The Role of the Hypothalamus-Pituitary-Gonadal Axis in Breast Cancer; a candidate gene approach

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The Role of the Hypothalamus-Pituitary-Gonadal Axis in Breast Cancer; a candidate gene approach

De rol van de hypothalamus-hypofyse-gonade as in borstkanker, een kandidaatgen benadering

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CHAPTER ONE

General Introduction

Parts of this introduction are based on:
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LH receptor gene mutations and polymorphisms: an overview.
Mol Cell Endocrinol 260-262:282-286

General introduction

This chapter provides a general overview of breast cancer, including the possible role of genetic and exogenous factors and an overview of the role of hormones in carcinogenesis of the breast. Variability in susceptibility to the disease, timing of development, as well as tumor characteristics upon presentation and outcome of breast cancer, are likely to be affected by differences in both genetic and exogenous factors. The role of polymorphic variation in genes involved in hormonal control of carcinogenesis will be introduced.

1.1 BREAST CANCER

Facts and figures

Breast cancer is the most frequently occurring malignancy in women, affecting women of all ages. More than 1 million new cases are diagnosed each year, accounting for almost one third of incident cases of cancer in women in Western industrialized countries (1, 2). The cumulative lifetime risk, documented between 2000 and 2002, for women in the USA is 13% (1:8) (2), while in The Netherlands the approximate lifetime risk is currently about 11% (1:9) (www.kankerregistratie.nl). In total over 400,000 women die each year worldwide of breast cancer (www.iarc.fr), making it the leading cause of death among women 35 to 55 years of age (3). Important aims of breast cancer research are prevention, early detection and reduction of mortality. Both basic molecular biological research and epidemiological investigations can help to achieve these goals by identification of women at risk, development of techniques for early diagnosis, prediction of outcome and response to therapy and finally optimization of targeted therapies resulting in tailored-treatment. In essence, breast cancer, like all cancers, is a genetic disease resulting from an accumulation of somatic mutations and/ or altered expression of genes. In addition, breast cancer is a complex, multifactorial disease in which environmental factors and individual genetic background, including germline mutations and polymorphisms, may influence susceptibility, prognosis and response to treatment (4, 5).

Risk factors and heritability

Among the most important risk factors are gender (male vs. female = 1:150), age, mutations in high susceptibility genes and family history (two-fold relative risk in first-degree relatives) (6). In addition, key factors affecting breast cancer development are those relating to reproductive history (parity and breast feeding, age at menarche, birth of first child and menopause), otherwise relating to exposure to endogenous (e.g., body mass index,

physical exercise) and exogenous estrogens (mainly oral contraceptives and hormone replacement therapy), several lifestyle factors (e.g., cigarette smoking, alcohol use and diet) and general factors such as immune status (7, 8).

Although most of the latter risk factors mentioned above are apparently environmental or exogenous, several are likely to reside in or interact with germline genetic variation. Any genetic site for which different structural DNA variants are present in more than 1% of the population is defined as a polymorphism (9). In contrast to high-risk DNA mutations, the consequences of polymorphic DNA variants on gene function or expression are relatively small. However, subtle changes in function of the encoded protein have been documented, with mild phenotypic expression in the individual. Over a lifetime period this may result in clinical consequences, such as susceptibility to disease, either or not via increased susceptibility to disease-related exogenous factors. Moreover, since polymorphisms are often widespread in the population, these relatively small phenotypic consequences may have significant impact on clinical parameters at the population-wide level.

Total breast cancer is comprised of 5-10% cases of familial breast cancer, the remaining 90-95% of cases are called "sporadic". Mutations in high susceptibility genes, such as Breast Cancer (BRCA) genes 1 (10) and 2 (11), TP53, PTEN and CHECK2 (12), are thought to account for only 20-25% of familial breast cancer (13-15). In addition, heritability, estimated from twin studies, has been shown to account for up to 25% of total susceptibility to breast cancer (16, 17). Shared exogenous factors such as lifestyle and environmental may explain part of heritability. In addition, so-far unknown low-penetrance or minor susceptibility genes are likely to be associated with a small to moderate increased risk for breast cancer (18), both in the remaining 75% of familial cases and in sporadic breast cancer (14). This is illustrated in Figure 1.

Candidate low-penetrance genes for breast cancer may include those involved in detoxification of chemicals, DNA repair, immune surveillance, angiogenesis and steroid hormone metabolism (18-20). Assuming a polygenic model, it has been estimated that individuals carrying several low-penetrance alleles with additive and/or multiplicative effects, might suffer an increased lifetime risk of breast cancer development of up to 50% (21).

Prognosis and treatment

Mammary carcinoma generally develops from the glandular epithelium of the terminal ducts and lobular units. At present roughly half of all women diagnosed with breast cancer will survive without recurrence of the disease. However, about one-third will die of metastasis within 10-15 years after diagnosis, whereas 15% shows recurrence after 15 years. Once breast cancer is diagnosed, patient characteristics such as age and menopausal status, in addition to several clinical and cell biological factors, are employed to assess

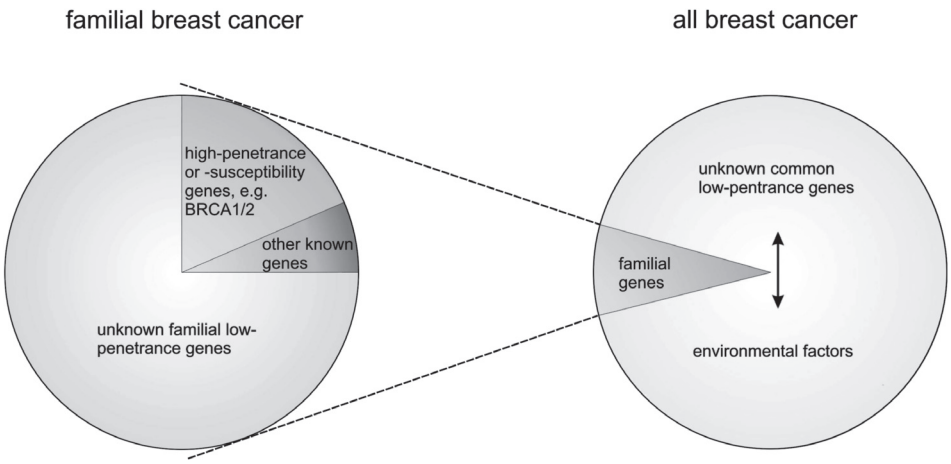

Figure 1. *Total breast cancer (right) constitutes of familial breast cancer (5-10%) and sporadic (90-95%). The known major susceptibility or high-penetrance genes (BRCA1/2, and TP53, PTEN and CHECK2) explain maximally 25% of familial breast cancer. So far unknown genetic variants, many of which may interact with environmental factors, are thought to contribute both to familial and sporadic breast cancer.*
Adapted from: Balmain A, Gray J, Ponder B 2003 The genetics and genomics of cancer. Nature Genetics 33:238-244

prognosis and treatment options. Clinical staging is based on three clinico-pathological characteristics: diameter of the tumor (T), involvement of loco-regional lymph nodes (N) and presence of distant metastases (M) at time of diagnosis. This TNM staging, applied to solid tumors in general, is the most widely used classification for assessment of prognosis, primary therapy selection and prediction of response to treatment as reviewed in (22). A second important prognostic and predictive characteristic of the tumor is histological grade according to Bloom and Richardson (23). The grading system comprises the following parameters: degree of glandular differentiation, degree of nuclear atypia and mitotic index. The combined score results in a classification of grading from well (grade I), to moderately (II), to poorly differentiated (III). In addition, expression of the estrogen and progesterone receptors has important prognostic value and is a strong predictor of response to endocrine treatment (24, 25). A relatively new etiological and prognostic factor is the oncogene HER-2/neu, a member of the epidermal growth factor family, first described in 1987 as a predictor for relapse in breast cancer patients (26). Amplification of HER-2/neu, resulting from amplification of the 17q12-21 region (27), is found in 20-30% of breast tumors (26) and is associated with increased proliferation, invasion and metastasis of tumor cells *in vivo* and *in vitro* (28, 29). Interestingly, HER-2/neu is not frequently overexpressed in BRCA1 tumors as opposed to sporadic and BRCA2 tumors (30). Most recent developments in the etiological, prognostic and therapeutic molecular biology field include molecular profiling, using micro-arrays to further characterize individual tumors (31-34).

Major breast cancer susceptibility genes, such as BRCA1 and 2, have been extensively studied both with respect to clinico-pathological characteristics and value for prognostic outcome (35, 36 and reviewed in 37, 38). In contrast, relatively little is known about the significance of more common germline polymorphic variation for risk, clinical presentation, response to treatment and outcome of breast cancer.
The following sections will discuss the importance of estrogens in breast cancer development and progression and the involvement of regulatory mechanisms, important in estrogen exposure, mainly assigned to the hypothalamus-pituitary-gonadal (HPG) axis. In addition, candidate genes in the HPG axis and candidate polymorphisms herein are introduced. This chapter ends with the aims of this thesis work.

1.2 ESTROGENS, ESTROGEN RECEPTOR AND BREAST CANCER

Etiological aspects

Factors relating to increased or prolonged cumulative estrogen exposure have been identified as major risk factors for breast cancer development and progression. Endogenous estrogens mainly originate from the ovaries in premenopausal women and from peripheral aromatization of adrenal androgens in postmenopausal women. For a more detailed discussion of regulation of estrogen production, the reader is referred to chapter 1.3 and Figure 3. Factors contributing to increased endogenous estrogen exposure are: early menarche, late age at first full term pregnancy or nulliparity, late menopause and postmenopausal obesity (7, 8). It is anticipated that a growing number of women will be diagnosed at younger age as a result of changes in lifestyle, partly expected to influence cumulative estrogen exposure (39). Breast cancer in postmenopausal life is primarily influenced by extraovarian sources of estrogens, for example postmenopausal hormonal replacement therapy (40, 41).
Estrogens, in cooperation with progesterone (42), prolactin (43) and growth hormone (44), have an important role in the regulation of proliferation and differentiation of the mammary gland. Especially the proliferative response of breast epithelial cells to estrogens may be involved in the pathological changes that ultimately lead to hyperplasia (45). Estrogens exert a proliferative effect on mammary gland epithelial cells either directly or via paracrine stimulation by neighbouring stromal cells, through binding to specific estrogen receptors (ER) (45). Although a small fraction of ER is localized on the plasma membrane, responsible for rapid non-genomic effects of estrogen signaling (46, 47), the major proliferative effects are thought to be mediated by the nuclear estrogen receptor. Upon ligand binding the ER interacts with specific DNA sequences (estrogen response elements, EREs) in the promoter region of estrogen-target genes, and with large complexes of transcription factors and co-activators or co-repressors resulting in regulation of the

transcription of genes involved in proliferation and survival or involved in maintaining tissue architecture (48-50).

Clinical phenotype

Despite the central role of estrogens in breast cancer development and progression not all newly diagnosed breast tumors show ER expression. Epidemiological data suggest that several factors related to increased estrogen exposure, such as listed above, are associated with development of breast tumors that express ER and progesterone receptor (PR) and less so with development of receptor-negative tumors. However, the results from several epidemiological studies are inconclusive, suggesting that several patient and tumor characteristics, interactively influence development of breast tumors and receptor expression of these tumors, as reviewed by Ursin et al (51). Furthermore, receptor expression also depends on tumor features such as histology, lobular tumors being more often receptor-positive than ductal tumors (51). Most newly diagnosed breast tumors are found in an early stage and at presentation the majority (75.1%) is ER-positive (Figure 2). ER-negative breast cancer is more often found in premenopausal patients (46% of premenopausal patients) and is associated with larger tumors. Despite the growth-stimulatory effect of estrogens, exerted by the ER, breast tumors without detectable ER expression tend to grow more rapidly and less differentiated, i.e., more aggressively. It is still unclear why premenopausal patients more often suffer ER-negative breast cancer.

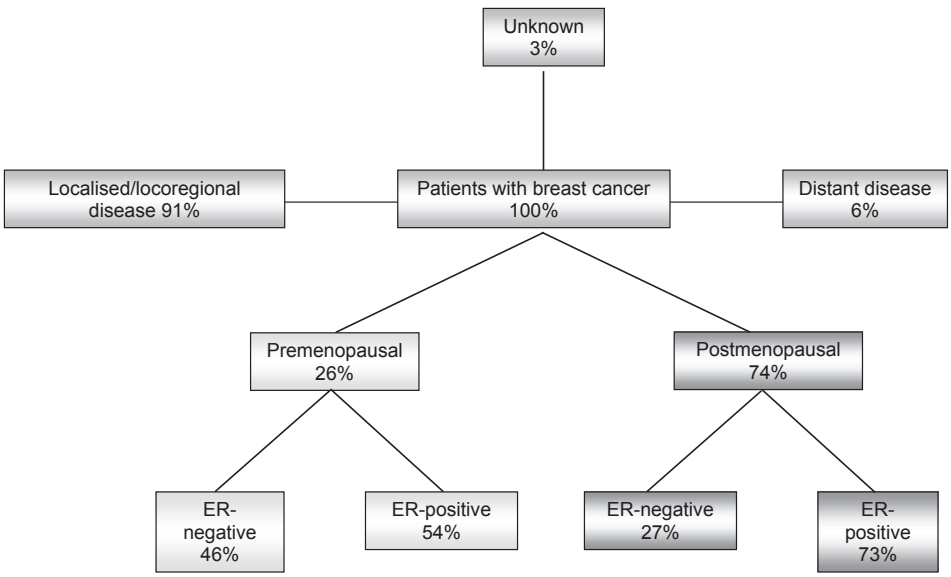

Figure 2. *Distribution of ER expression at diagnosis*

Adapted from: Jonat W, Pritchard KI, Sainsburry G, Klijn JGM 2006 Trends in endocrine therapy and chemotherapy for early breast cancer: a focus on the premenopausal patient. J Cancer Res Clin Oncol 132(5):275-86

Recent insight has suggested a model for mammary carcinogenesis resulting from the transformation of primitive stem or progenitor cells, as reviewed in (52, 53). In early fetal life an ER-negative stem cell population generates myoepithelial progenitor cells and primitive ductal epithelial progenitors. Later in fetal development ER-positive stem cells or early progenitor cells arise. The ER-positive cells proliferate in response to estrogens and produce paracrine factors that influence proliferation and/ or differentiation of adjacent cells, including ER-negative (stem) cells. Different subsets of mammary carcinoma may thus arise from different populations of stem or early progenitor cells that are either ER-negative or -positive and more or less primitive. This stem/ progenitor cell concept accounts for the heterogeneity of breast tumors, including their ER and PR expression. Importantly, since ER-negative tumors are proposed to arise from the most primitive cells, the model explains the poor histological differentiation and clinical aggressive behavior of ER-negative tumors. In addition, the stem cell model is in line with recent understanding from molecular profiling studies, showing that expression patterns of metastatic tumor cells are highly similar to those of primary tumor cells (54).

Treatment

Systemic treatment for breast cancer is indicated for patients with metastatic disease and for those with localized disease at risk to develop distant metastases (i.e., adjuvant treatment). The latter group, patients with high-risk localized disease, is identified by clinical parameters, e.g., presence of regional lymph node metastases, and by primary tumor features including tumor size and differentiation. Based on these prognostic factors, several guidelines for adjuvant treatment have been developed, for example the St Gallen criteria (55), while in The Netherlands such treatment guidelines have been set up by a task force, chaired by dr. Marijke Bontenbal, as part of the "kwaliteitsinstituut voor de Gezondheidszorg, CBO" (www.cbo.nl/product/richtlijnen/). Several distinct forms of systemic therapy can be distinguished that can be applied in the treatment of breast cancer patients. These include cytotoxic chemotherapy, hormonal therapy and immunotherapy (i.e., monoclonal antibodies). Of these, hormonal therapy plays a pivotal role in the treatment of women with hormone-sensitive breast cancer, which is determined by ER and/ or PR expression of the tumor. Considering the role of estrogen as driving factor in such tumors, hormonal therapy aims to inhibit estrogen-mediated tumor growth through blocking or degrading the ER, or reducing estrogen levels. One of the most frequently applied hormonal agents is tamoxifen. In breast cells, tamoxifen is a competitive inhibitor of estrogen binding to the ER. The subsequent inhibition of expression of estrogen-regulated genes leads to a block in G0/G1 phase of the cell cycle, resulting in attenuating of cell proliferation (56-58) and possibly induction of apoptosis (59). This drug was approved by the Food and Drug Administration (FDA) in 1977 for the treatment of women with advanced (metastatic) breast cancer and several years later

for adjuvant therapy of primary breast cancer. In metastatic (M+) disease, approximately half of the patients with ER-positive primary breast tumors respond to tamoxifen when given as first-line treatment. In the adjuvant setting, tamoxifen therapy results in an 11% improvement in 10-year survival in lymph node-positive patients, independent of menopausal status or age (60). The toxicity profile of tamoxifen is relatively mild. Tamoxifen is a so-called selective estrogen receptor modulator (SERM) and in some tissues it acts as a partial agonist, which is thought to mediate some beneficial side effects, such as prevention of bone loss (61). Also unwanted side effects can be observed, such as increased risk of endometrial cancer and thromboembolism. Another hormonal agent targeting the ER is fulvestrant, a selective ER degrader (SERD) or downregulator with no demonstrable agonistic effects (62). In second-line treatment of postmenopausal patients with tamoxifen-resistant disease, fulvestrant is equally effective as reducing local estrogen levels by use of aromatase inhibitors (63). However, the value of fulvestrant in the adjuvant setting remains to be established. In postmenopausal women, local estrogens produced after conversion of circulating androgens by the enzyme aromatase localized in adipose tissue, bone, breast and liver are responsible for the proliferative effects in breast cancer. As mentioned above, aromatase inhibitors (AI) are regarded as a class of agents that reduce estrogen levels. In advanced (M+) breast cancer, third-generation AIs have shown superiority to tamoxifen, and have replaced tamoxifen as first-line treatment for advanced postmenopausal breast cancer (64-66). The current view on adjuvant hormonal therapy for postmenopausal patients is that it should include an AI. In The Netherlands these patients are treated with 2-3 years of tamoxifen followed by 3-2 years with an AI, and this approach is supported by a recent meta-analysis (67). Although less severe than tamoxifen, aromatase inhibitors also exhibit side-effects, including bone loss and fractures, rheumatoid arthralgic symptoms and possibly negative effects on lipid metabolism and cognition (68).

In premenopausal women the ovaries are the main source of estrogen production, regulated by the integrated system of the hypothalamus-pituitary-ovarian axis. Inhibition of the ER by tamoxifen in premenopausal patients, results in a positive feedback signal to the central stimulus of ovarian estrogen production, i.e., hypothalamic Gonadotropin-Releasing Hormone (GnRH). This has been shown to result in an increase of estradiol levels, reducing the occupancy of ER by tamoxifen (69). Suppression of GnRH production can be achieved when pulsatility of endogenous GnRH secretion is overcome by regular administation of GnRH agonists, resulting in a downregulation of GnRH receptors and loss of stimulation of ovarian estrogen production. This may account for the observation that the combination of a GnRH agonist with tamoxifen, improves clinical outcome of premenopausal patients with advanced disease, compared with the use of GnRH agonists or tamoxifen alone (69, 70). Presently suppression of ovarian estrogen production in combination with tamoxifen is the standard endocrine therapy in metastatic disease

in premenopausal women (70), while it is increasingly applied in the adjuvant setting. Randomised trials, in the adjuvant and advanced setting, assessing whether the combination of a GnRH agonist with an aromatase inhibitor improves outcome over treatment consisting of a GnRH agonist and tamoxifen are ongoing in premenopausal women (71, 72). Ovarian suppression or ablation can be achieved not only by using GnRH agonists, but also by resection or radiation of the ovaries, or by chemotherapy (73). Important side effects of ovarian suppression and ablation include bone loss, hypertension, weight gain and diabetes (74).

Improved classification of patient groups can be valuable for fine-tuning of treatment modalities to increase benefit and decrease side-effects (22, 75). With regard to the identification of patients who are likely to respond to a certain type of hormonal treatment, numerous predictive factors have been revealed. These include gene-expression signatures for both adjuvant treatment (76) and metastatic disease (33), but it is clear that there is need for further improvement.

1.3 GENETIC ASSOCIATION STUDIES AND CANDIDATE GENE APPROACH

The contribution of genetic variants to the development and phenotypic presentation of complex diseases can be evaluated by genetic association studies (77). By comparing frequencies of candidate alleles in cases and controls, genetic association studies are a highly suitable method to search for minor susceptibility alleles (78). In addition, using a candidate-gene approach genetic association studies are an ideal tool to study specific traits of the complex disease by comparing phenotypic variables across the genotype under study (77). A hypothesis-driven selection of genes based on their biological role, is the first step using the candidate gene approach studying a complex trait. Subsequently, variants of the selected genes are identified that are likely to cause a change in function or expression, or are in linkage disequilibrium with functional genetic changes. This approach can reduce the number of subjects to be genotyped (79) and is likely to result in associations that can be replicated in independent studies (78). The next step involves characterization of the phenotype and selection of cases followed by genotyping of the variants in a population. The final step is the use of statistical methods to determine whether there is a correlation between those variants and the phenotype of interest (78).

In addition to cohort- or population based association studies, linkage studies can be used to study the genetic basis of hereditary disease. However, the individual effect of low-penetrance or minor susceptibility genes on complex diseases such as breast cancer, by definition is small. Therefore, even when acting as dominant alleles, minor susceptibility genes will not give rise to multiple-case families that can be used in linkage studies to search for regions with higher numbers of shared alleles than expected amongst cases

(21). A growing number of current genetic association studies explore the application of genome-wide approaches whereby the genome is typed for 500,000 SNPs to search for common genetic variation associated with complex diseases such as breast cancer (15). However, this hypothesis- free approach implies that the biological rationale no longer plays a role a priori, which can be considered a weakness of this kind of study (21).
Following the above-mentioned hypothesis-driven approach, candidate genes with a clear-cut role in the hormonal carcinogenesis of breast cancer, i.e., genes involved in the HPG axis, were selected. Biologically interesting polymorphisms were chosen based on their properties during protein biosynthesis (signal peptide polymorphisms in the LHR and GnRH genes), structural function of the protein (LH8Arg15Thr and LHR exon 10 SNPs) or solid literature reports (CYP19 and ESR1). Optimal selection and phenotyping of cases was performed by using patient samples from the tumor bank, collected and stored at the Erasmus University Medical Center. Most of the association analyses, were performed in a subset of tumor samples. The cohort is large, well-documented, has a long follow-up period and can be considered ethnically homogenous.

1.3.1 CANDIDATE GENES INVOLVED IN ESTROGEN PRODUCTION AND SIGNALING

In premenopausal women estrogens arise from the ovaries, where production is regulated by the hypothalamus-pituitary-gonadal (HPG) axis, the neuro-endocrine system consisting of hypothalamus, pituitary and gonads (as illustrated in figure 3). Internal and external stimuli are integrated in the brain, resulting in the pulsatile secretion of the hypothalamic neuropeptide Gonadotropin-Releasing Hormone (GnRH). GnRH reaches the gonadotroph cells in the anterior pituitary through the hypophysial portal circulation, where it stimulates *de novo* synthesis and secretion of the gonadotropins Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), which reach the ovaries in women through the circulation. In the ovary, FSH, acting via its receptor located on the granulosa cells, stimulates follicle maturation, including follicular LH receptor (LHR) expression and regulates aromatase activity. LH is responsible for the induction of ovulation through the mid-cycle LH-surge and the maintenance of corpus luteum function. In addition, LHR activation stimulates androgen production in theca cells surrounding antral follicles, as a substrate for the granulosa cell enzyme aromatase. In turn, ovarian sex steroid and peptide hormones (Inhibin A and B) provide feedback regulation, either on the gonadotroph cells in the pituitary or on GnRH secretion from the hypothalamus, regulating gene expression, synthesis and secretion of the gonadotropins (80).
The menopausal transition, resulting from the depletion of the primordial follicle pool (81), is characterized by disruption of this tightly balanced HPG axis system. The cessa-

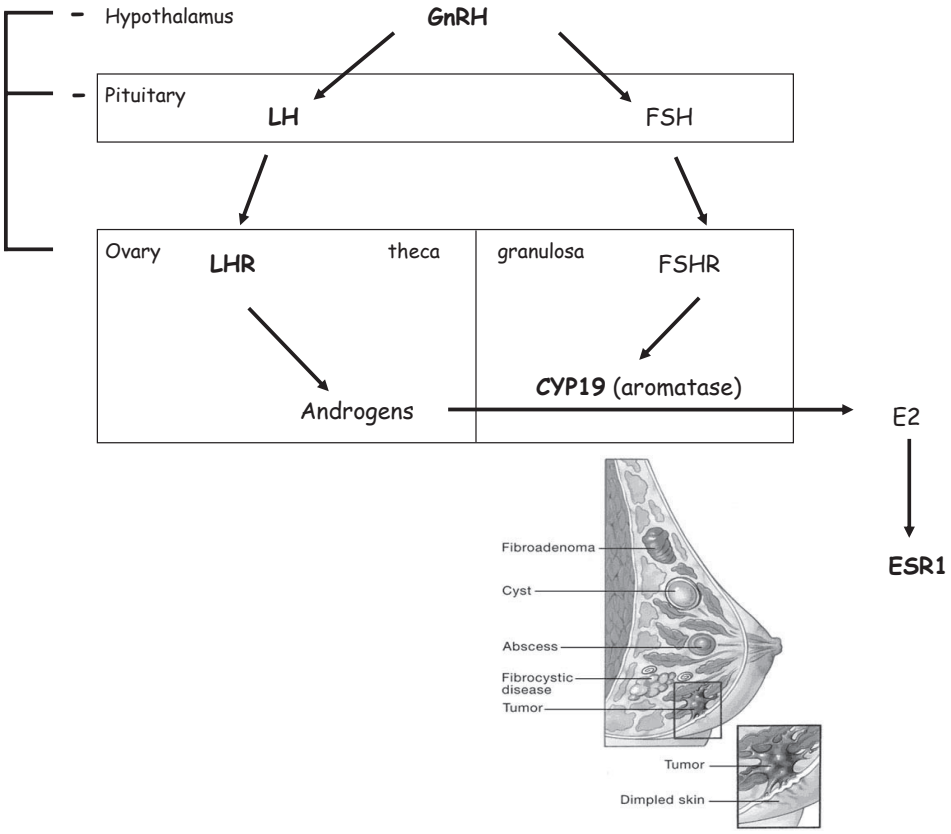

Figure 3. *Schematic overview of the Hypothalamus-Pituitary-Ovarian axis and candidate genes possibly involved in breast cancer development and progression in bold*

tion of ovarian function results in a loss of inhibitory feedback to the hypothalamus and pituitary. As a consequence menopause is characterized by continuously increased serum LH and FSH in combination with decreased levels of ovarian sex steroid hormones (82, 83). In postmenopausal life estrogens (estrone and estradiol) in women are mainly derived from local conversion of circulating androgens (androstenedione and testosterone, respectively) by peripheral aromatase, predominantly in the adipose tissue. The adrenal glands are the main producers of the circulating androgens, but approximately 10-25% is produced by the ovary, partially under the control of LH (84-88).

The research described in this thesis focuses on HPG genes (further described in section 1.3.2 and outlined in figure 3), that may influence timing and duration of estrogen production and signaling and may play a role in the development and progression of breast cancer.

1.3.2 CANDIDATE GENE POLYMORPHIC VARIANTS AND THEIR BIOLOGICAL RELEVANCE

The candidate polymorphisms described in this thesis will be introduced based on relevant aspects of the structure, function and expression. Their possible role in breast cancer will shortly be discussed as well as an overview of hypothesized consequences of the genetic variation.

Gonadotropin-Releasing Hormone

GnRH, as the name implies, is responsible for secretion of the gonadotropins FSH and LH from the pituitary. GnRH is a decapeptide synthesized in specific neurons of the hypothalamus. It is produced from a precursor polypeptide, consisting of GnRH, preceded by a so-called signal peptide and followed by a 56-amino acid GnRH-associated protein (GAP). After enzymatic cleavage GnRH is stored as the processed peptide, awaiting release from storage granules (89). Release of GnRH into the hypophyseal portal system occurs every 30-120 minutes to stimulate gonadotropin production from the pituitary gonadotrope cells. At the onset of puberty pulsatile secretion increases in diurnal fashion, under control of a hypothalamic GnRH pulse generator (90). Secretion of LH from intracellular storage is highly dependent on pulsatile GnRH signals, whereas FSH secretion is predominantly subject to FSH biosynthesis. High doses or frequent administration of GnRH analogous that abolish GnRH pulsatility desensitizes the gonadotroph cells resulting in downregulation of GnRH receptor expression and a decline of LH and FSH production, as reviewed in (91). As explained in section 1.2 this principle is used in the application of GnRH agonists in the endocrine treatment of breast cancer.

In general, deficiency of GnRH signaling, the most well-known example being Kallmann's syndrome, results in a heterogeneous phenotype of hypogonadotropic hypogonadism (92). The GnRH receptor has been shown to be expressed in breast cancer tissue (93-98). Therefore, besides indirect effects of GnRH agonists treatment through down-regulation of pituitary GnRH receptors and subsequent shutdown of the HPG axis (69), direct effects of GnRH on breast cancer cannot be excluded.

In search for causes of idiopathic hypogonadotropic hypogonadism several naturally occurring GnRHR mutations have been identified (99), but so far none in the GnRH gene itself (100). Polymorphic variation in the genes encoding for GnRH and its receptor have been studied for a possible role in the physiological variation of pubertal timing, but no clear associations were observed (99). However, a potentially functional polymorphic variant can be identified in the GnRH gene, located on chromosome 8p11.2-p21. A Trp to Ser change at position 16 in the signal peptide (rs6185), first described by Nakayama et al (101). Genetic variation in signal peptides is potentially interesting because alterations

Box 1: *signal peptide biology*

Signal peptides are responsible for guiding nascent or completed proteins to a cytoplasmic residence, membrane insertion or secretion from the cell. Usually they are N-terminal extensions, however they can also be located within the protein or at its C-terminal end. Signal peptides can be subdivided into 3 regions. The most essential region for targeting and membrane insertion is the hydrophobic core (h-) region, generally thought to be comprised of 6-15 amino acids. For non-cleavable sequences, so-called signal anchor sequences, this number can be higher. At the N-terminal side is the, rather polar, n-region, which is usually positively charged. This region is most variable in size, contributing to size variations of the entire signal peptide. The polar C-terminal flanking c-region often contains helix-breaking proline and glycine residues. Furthermore it usually contains small, uncharged residues at positions -3 and -1, to determine the site of signal peptide cleavage. As mentioned above, signal peptides enable nascent secretory and transmembrane proteins to be targeted to the endoplasmic reticulum for translocation. This process can either be dependent on the signal recognition particle (SRP) and its receptor (docking protein/ SRP receptor) or be SRP independent. Largely, features of the signal peptide determine the discrimination between an SRP-dependent or SRP-independent pathway. In signal peptides that direct proteins to the SRP-dependent pathway the h-regions have been shown to be significantly more hydrophobic (102, 103). The current understanding of SRP-dependent protein translocation is that as the signal peptide emerges from the translating ribosome, it is recognised by SRP in the cytosol (104, 105). The association between the signal peptide and the SRP, results in the so-called elongation arrest of the nascent chain, a pause in translation. SRP subsequently mediates the targeting of the nascent polypeptide-ribosome complex to the endoplasmic reticulum membrane via interaction with the SRP/ docking receptor. The process of docking to the endoplasmic reticulum membrane releases the pause in translation. Once targeted to the endoplasmic reticulum membrane the complex of ribosome and nascent chain is transferred from SRP to the protein-conducting translocation channel. For many proteins, translocation is coupled to various maturation processes, such as signal peptide cleavage, N-linked glycosylation and association with chaperones to facilitate intramolecular folding and refolding. These processes start immediately after initiation of translocation in the endoplasmic reticulum lumen. Since signal peptide recognition is the first step in translocation, resulting in maturation of the nascent protein, signal peptides can influence the timing and efficiency of maturational events (106). A schematic overview of signal peptide-mediated translocation is shown in Figure 4.

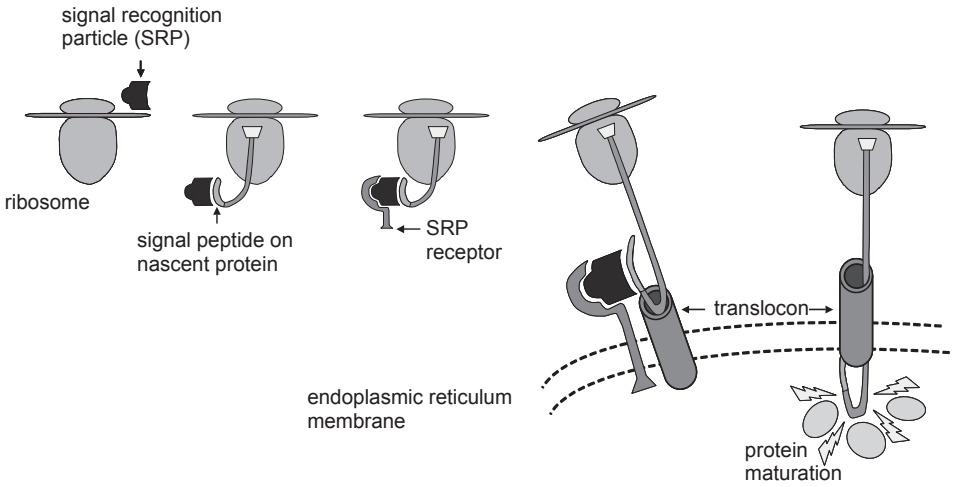

Figure 4. *Cartoon of signal peptide function*

Adapted from: Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD 1994 Molecular Biology of The Cell, third edition

in signal peptide function may influence cotranslational processing as well as protein expression, as described in the box.

Alterations in signal peptides that affect the h-region may influence kinetics of cotranslational folding (105) or exit from the endoplasmic reticulum (103) to the Golgi and subsequently the posttranslational maturation and modification in the Golgi apparatus. Mutations in the alkaline extracellular protease (AEP) signal peptide that significantly increase its hydrophobicity have been shown to increase the rate of translocation in an *in vitro* testing system (102). Other *in vitro* data indicate that increasing the hydrophobicity of the h-region results in more efficient processing and translocation of newly formed protein (104, 107). In addition, disruption of the hydrophobic core in signal peptides has been shown to impair co-translational translocation and posttranslational signal peptide cleavage, resulting in clinical disease. Thus, familial hypoparathyroidism resulting from impaired processing and secretion of parathyroid hormone associated with a signal peptide mutation has been described (108). In addition, impaired processing resulting in degradation of the B-UDP-glucoronyltransferase protein was shown to be associated with Crigler-Najjar type I disease in two patients with a homozygous mutation in the signal peptide (109). On the other hand, an increase of the hydrophobicity above a certain threshold is thought to impair co-translational processing. The polymorphic alteration of Thr to Ala in the signal peptide of the cytotoxic T-lymphocyte antigen, resulting in inefficient glycosylation, is suggested to result in a higher frequency of type I diabetes and other autoimmune disease (110).

Interestingly, the substitution of Trp by the less hydrophobic Ser amino acid at position 16 may change the efficiency of the GnRH signal peptide. Considering the clear role of GnRH in ovarian estrogen production and potential functional effect of this SNP, previously associated with another estrogen-dependent characteristic bone mineral density (111), the GnRH 16Trp/Ser is an interesting candidate breast cancer gene.

Luteinizing Hormone Receptor

Adapted from (112)

LHR activation results in production of androgens by the ovary as a substrate for estrogen production. The LH receptor protein is a member of the large receptor family of GTP-binding protein coupled receptors, GPCRs (113-115). GPCRs are characterized by the presence of a transmembrane domain consisting of seven transmembrane helices connected by three extracellular and three intracellular loops (Figure 5). Most of the GPCR family members are encoded by a relatively small gene consisting of one exon. In contrast, the LH receptor, just as the closely related FSH and TSH receptors, has a large ectodomain that binds the large glycoprotein hormone. The ectodomain, encoded by exons 1-10 in the case of the LH receptor, has a characteristic structure with as a main

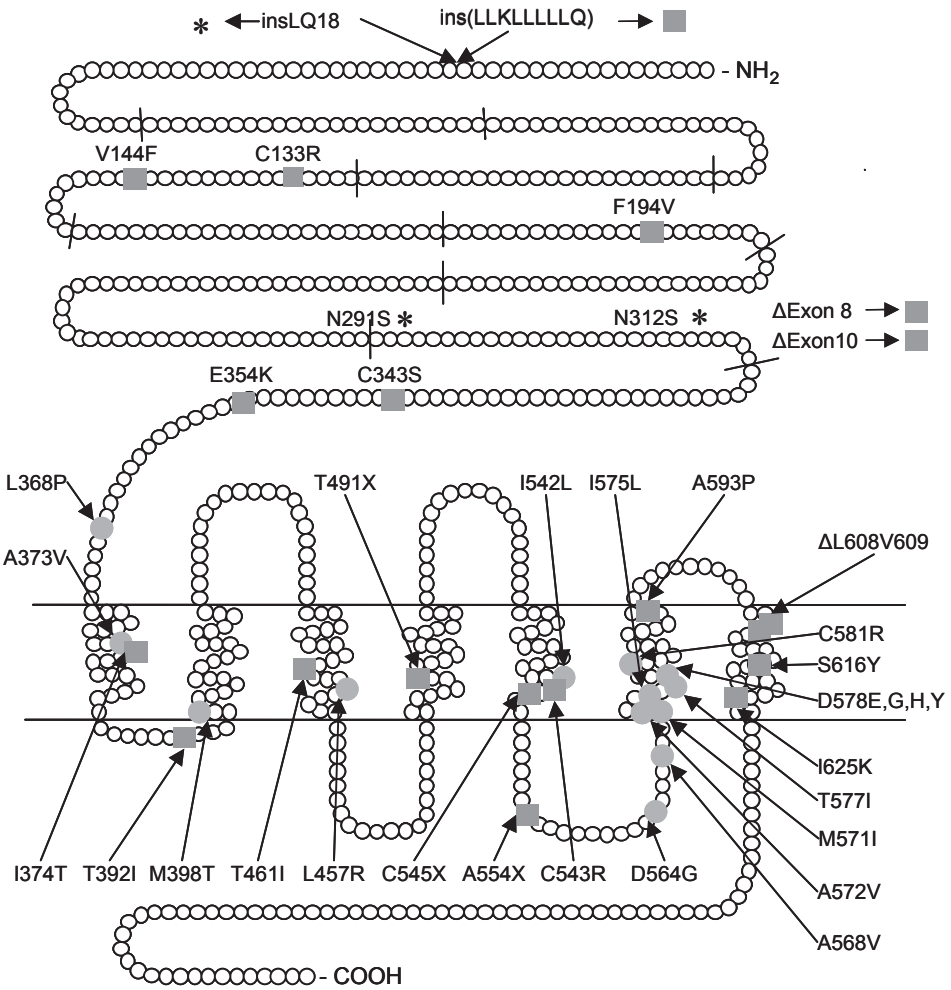

Figure 5. *Schematic representation of activating (circles) and inactivating (squares) amino acid changes in the LH receptor protein. Polymorphic amino acid residues have been indicated by an asterisk (see text). The information has been taken from the published literature (121, 177, 178).*

feature a tandem repeat of leucine rich repeat structures that forms a concave hormone binding surface consisting of short beta turns, bordered at each side by a cysteine-rich domain (116, 117).

Since the cloning of the LH receptor in 1989 (113, 114), it did not take long for the first clinical data to appear that were a result of mutations in the LH receptor gene located on chromosome 2p16.3 (118, 119). The first mutations that were identified were missense amino acid changes that cause activation of the LH receptor protein in the absence of hormone. These mutations are found in heterozygous boys with familial male-limited precocious puberty (FMPP). FMPP is characterized by an early puberty (before the age

of four years), high serum testosterone levels and low serum LH (120). Most mutations are found in the sixth transmembrane helix and its flanking third intracellular loop; a domain in the family of GPCRs that has been shown to be intimately involved in GTP-binding protein interaction and activation (Figure 5).

At the other end of the clinical spectrum lies the phenotype of the inactivating LH receptor mutations (121). Such mutations render the LH receptor protein completely inactive, and upon transfection in cultured cells, no hCG response can be observed. Inactivating LH receptor mutations occur much more rarely and show a recessive inheritance pattern, thus only homozygote or compound heterozygote carrier individuals are affected. Inactivating LH receptor mutations were first identified in 46XY pseudohermaphrodites and the disease has been named Leydig cell hypoplasia (LCH) referring to the absence of recognizable adult-type Leydig cells in testicular sections from the affected patients (122, 123). In LCH an excellent correspondence has been found between strength of the phenotype and residual LH receptor activity *in vitro*. Thus, LCH patients who are homozygous carriers of a mutation that renders the LH receptor protein completely inactive are 46XY pseudohermaphrodites with a complete female habitus. Less severe phenotypes also correlate with activity. In a patient with severe hypospadia, a combination of a partial inactive LH receptor mutant (S616Y) with an exon deletion on the other allele was found (124), whereas a homozygous partial LH receptor mutation was identified in a patient with a micropenis (125). In contrast to FMPP, inactivating LH receptor mutations also cause a phenotype in women. Sisters of complete 46XY LCH patients are infertile and show signs of low estrogen production such as a small uterus and diminished bone mass (126).

As described above, LH receptor gene mutations have profound effects on sex steroid hormone production and therefore on physiology of patients. In addition, the strength of the phenotype follows the *in vitro* activity of the various LH receptor mutant proteins. Thus, the question arises whether slight changes in LH receptor gene activity may have subtle effects, which may contribute to susceptibility to, or progression of disease. LH receptor genes that may be slightly more or less active may exist in the form of polymorphisms. In the LH receptor approximately 285 single nucleotide polymorphisms (SNPs) have been identified (http://SNPper.chip.org), at an average distance of about one SNP per 300 base pairs. Most of the SNPs are located in the large introns, which account for more than 95% of the LH receptor gene. Considering the role of LH in estrogen production and potential carcinogenic effect of estrogens on mammary cells, LH and LHR gene variants may have functional consequences in breast cancer development and/or progression. The most frequent LH receptor polymorphisms that involve an amino acid change are the absence or presence of a two amino acid insertion at position 18 in exon 1 (rs4539842; low frequency allele LHR insLQ: frequency 29%), and two variable amino acids at position 291 and 312 respectively: 291Asn/Ser (rs12470652; LHR 291Ser: 5%)

and 312Ser/Asn (rs2293275; LHR 312Asn: 45%) (127) (Figure 5). In addition a 124Arg/Gln has been described as a SNP but with low frequency (<2%) (127).

The 291Asn/Ser and 312Ser/Asn polymorphisms are of interest because of their location in exon 10. A deletion of exon 10 has been described in a patient with normal male sex differentiation, but delayed puberty, apparently because the del(exon10) LHR did not respond to LH, while it was sensitive to hCG (128). Indeed *in vitro* expression of the del(exon10) LHR showed a slightly decreased response to hCG whereas LH-induced receptor activation was severely impeded (129). These results indicate the possibility that polymorphic changes in exon 10 may have subtle effects on LHR sensitivity for LH and hCG. In addition, the 291Asn/Ser and 312Ser/Asn SNPs are located at or near glycosylation sites, respectively. Glycosylation plays an important role in the G protein-coupled receptor superfamily for stability, trafficking and cell surface expression (130). Three of the total of six potential glycosylation sites in the LHR protein can be identified in exon 10: N^{291}FS, N^{299}FS and N^{313}KT. For the pig LH receptor it has been shown that 36% of all glycan residues in the mature receptor can be found at the homologous 291 position (131).

The polymorphic insertion of two amino acids insLQ, is located at a position in the LH receptor that appears to be prone to changes. In two independent patients (127, 132) an insertion of 33 base pairs was found at position 18. As a result of this insertion, the LH receptor protein product of this allele contains eleven additional amino acids [LLKLLLLLQLQ] compared to the nonLQ-LH receptor variant (the last LQ pair is the polymorphic insLQ, suggesting that the insertion has occurred in the insLQ LH receptor allele). The 33 base pair sequence is a direct repeat of the sequence already present between codons 8 and 18, suggesting that aberrant duplication in the gene has caused the insertion (127). Position 18 is in the hydrophobic region of the signal peptide of the LH receptor protein, and the eleven amino acid extension of the hydrophobic region probably renders the signal peptide inactive (see Box 1). Indeed, *in vitro* expression of the mutant LH receptor shows complete absence of response to hCG or LH (132). The observation of the insLQ-LH receptor as an allelic variant was first described by Rodien and co-workers (133), who also reported no large functional difference between the two LH receptor variants after *in vitro* expression. However, it may be argued that the differences between the two receptor forms may be subtle, because strong changes in LH receptor activity may well have a clearly discernable clinical effect which has not been observed in LHR insLQ carriers as yet.

In conclusion, considering the role of LH in estrogen production and potential carcinogenic effect of estrogens on mammary cells, common genetic variation in the LHR may have functional consequences in breast cancer development and/or progression.

Luteinizing Hormone

Besides a role in ovarian estrogen production, a major reproductive function of LH is the induction of ovulation and the maintenance of progesterone production by the corpus luteum. In case of pregnancy in humans chorionic gonadotropin (hCG), produced by the trophoblast takes over the role of maintaining progesterone synthesis, by activation of the same receptor (LHR). Furthermore, hCG triggers male sexual differentiation during prenatal development. Postnatal pituitary-derived LH is essential for virilisation and normal gametogenesis.

LH is a member of the glycoprotein hormone family, together with the other gonadotropins FSH and hCG as well as Thyroid Stimulating Hormone (TSH) and the recently discovered human thyreostimulin (134). All members are relatively large glycosylated, non-covalently associated heterodimers consisting of a species-specific common α-subunit and a hormone-specific β-subunit. The common α-subunit consists of 92 amino acids encoded by a single gene. LH and hCG both act on the LHR, hence high sequence similarity (83%) can be observed between LH and hCG. The major divergence between the two gonadotropins is ascribed to the 29-amino acid C-terminal extension of hCG. This so-called C-terminal peptide (CTP) contains four additional O-linked glycosylation sites, which are absent in LHβ, accounting largely for the longer circulating half-life and higher biopotency of hCG over LH (121).

In general, data from epidemiological studies indicate that pregnancy, especially early pregnancy, protects against breast cancer development. The hypothesis has arisen from this observation that the high levels of the pregnancy hormone hCG, acting via the LHR, may protect the mammary gland from malignant transformation. Expression of the LHR has been detected in different breast cancer cell lines (135) and LHR mRNA and protein expression has been shown in normal and malignant breast tissue (135). In addition, hCG treatment has been shown to induce mammary gland differentiation and protect against malignant transformation induced by the carcinogenic chemical 7,12-dimethylb enz[*a*]anthracene (DMBA) in a rat model. In summary, direct effects of LHR activity in breast cancer, induced by hCG, and possibly by LH as well, cannot be ruled out.

In the common α-subunit of glycoprotein hormones, so far, no germline mutations or non-synomous polymorphisms have been identified. The β-subunit of LH, encoded by chromosome 19q13.33, carries an inactivating mutation resulting in male infertility and hypogonadism (121). Petterson et al (136) described a polymorphic variant of LH (LH 8Arg15Thr, also referred to in literature as variant (V)-LH) in healthy people in the early 1990's. The genetic origin of this change was clarified in 1994 by the existence of two single nucleotide polymorphisms (SNPs) in exon two of the gene encoding LHβ, consisting of two T/C substitutions resulting in a Trp to Arg alteration at codon 8 (rs1800447), and an Ile to Thr at codon 15 (137-139). The carrier frequency of LH8Arg15Thr worldwide has been extensively studied and ranges from 0 to 53%

in various populations. Detailed genotyping has shown complete linkage of the two SNPs in all individuals investigated so far (140). Both amino acid changes introduce hydrophilic residues identical to the corresponding positions in hCG. Moreover the Ile/Thr substitution at position 15 results in an extra potential N-linked glycosylation site, $N^{13}XT/S$, in the LHß chain. In hCGß the same triplet structure is present were 13Asn is glycosylated. The *in vitro* activity of LH8Arg15Thr at the receptor site was shown to be higher compared to that of wild-type LH (141, 142). However, the half-life in circulation of LH8Arg15Thr is almost half that of WT-LH (141). There seem to be no compensatory changes in the pulsatile secretion pattern of LH8Arg15Thr or for the response to gonadotrophin-releasing hormone (GnRH) stimulation (141). Sequencing of the LH8Arg15Thr promoter region has resulted in a total of eight SNPs within the first 650 nucleotides of its 5'-flanking sequence. In transfection studies the activity of this mutant promoter was 40% higher and some qualitative differences in response to hormonal stimulation were observed in an immortalized mouse pituitary cell line $L\beta T_2$ and in a human embryonic kidney cell line HEK 293 (143).
From clinical experience the net result of this higher but shorter LHR-activation appears to be weaker *in vivo* functional activity, however carriers of the LH8Arg15Thr gene do maintain roughly normal gonadal function and fertility (141, 144). LH8Arg15Thr has been associated with changes in ovarian steroidogenesis (145), delayed progression of puberty and gain of height in boys (146) and with features of PCOS, although not consistently (144, 147). In summary, LH8Arg15Thr is an interesting candidate polymorphism and some data already suggest that the variant is associated with pathologies of LH action. Hence, breast cancer is an interesting clinical endpoint to include in association analyses involving LH8Arg15Thr.

CYP19

Aromatase, responsible for the enzymatic aromatization of androgen precursors, is encoded by the CYP19 gene on chromosome 15q21.2. In contrast to other mammals, expression of aromatase can also be found outside the gonads and brain. In women these extragonadal sites of aromatase expression, mainly adipose, but also (smooth) muscle, bone and endothelium have an essential role in local estrogen production in postmenopausal life (87). Inactivating mutations of aromatase have an effect in fetal life. Absence of aromatase activity results in an accumulation of androgens produced by the fetal adrenal gland, which cannot by converted to estrogens. These androgens accumulate in both the fetal and maternal circulation. In the fetus this leads to ambiguous genitalia and masculinization of female fetuses and in the heterozygous pregnant mother to progressive virilization (148, 149). Postnatally, females with aromatase deficiency fail to develop secondary sexual characteristics at puberty and exhibit hypergonadotropic hypogonadism and progressive virilization. Non-reproductive consequences of aromatase deficiency are

similar in males and females, including osteoporosis and continued linear growth due to non-closure of the epiphyses (150).
As reviewed by Simpson and Davis (151), at least 10 different first exons can be identified in the CYP19 gene (152). Each is spliced into different 5'-untranslated regions and associated with specific 5' promoter regions that are tissue-specifically regulated by distinct endocrine and paracrine signals. In the ovary aromatase expression is regulated via promoter II by FSH acting through cyclic AMP (cAMP). In normal breast tissue, aromatase is expressed at low levels under the control of the adipose promoter I.4, regulated by glucocorticoids and various cytokines (153). In contrast to the ovarian promoter II, promoter I.3 and more recently the endothelial promoter I.7 have been identified as the major promoters in breast tumors (154-157).
Polymorphic variation in the CYP19 gene might alter aromatase expression in a tissue specific manner (158). If androgen precursors are not limiting, then increased aromatase expression will result in increased estrogen production, possibly affecting estrogen-related clinical endpoints. It can be hypothesized that CYP19 polymorphisms affecting ovarian aromatase activity may exert an effect in premenopausal life, as recently suggested by an association between CYP19 genetic variation and age at menarche (159). In addition, a T/C SNP 1531 basepairs downstream of the translation initiation site in the 3'-UTR region of the CYP19 gene (rs10046) has been shown to result in increased aromatase mRNA levels and increased use of a breast cancer specific promoter (158). Hence, the CYP19 1531T/C may affect clinical phenotype and outcome of breast cancer, either in pre- or postmenopausal patients or both.

ESR1

As outlined above, the estrogen receptor is crucial for the proliferative effect of estrogens and is a major target for therapy. The final step in estrogen signaling is exerted by two different estrogen receptors, i.e., ESR1 (ER-α) and ESR2 (ER-β). The role of estrogen-signaling through the latter in growth and development of normal and malignant breast tissue still needs to be established, whereas, animal studies indicate a clear-cut role for ESR1 in proliferation and differentiation of the mammary gland (160). Of note, ER-α knock-out mice (αERKO) are insensitive to estrogen-induced mammary gland development during puberty and adulthood. In addition, although the mammary gland of αERKO mice can be transformed to hyperplasia and tumorigenesis, tumors exhibit delayed growth rate compared to those in wild-type mice (161).
To date, one case has been described with complete estradiol resistance resulting from a recessive mutation in the ESR1 gene, located on chromosome 6q25.1(150), whereas reports of activating germline ESR1 mutations cannot be found in the literature. In RNA samples from breast tumors several mutations and splice variants of ESR1 have been described. The exact mechanisms by which acquired ESR1 mutations affect breast cancer

are not yet completely understood (162). More common polymorphic genetic variation in the ESR1 gene has been associated with several estrogen-dependent clinical endpoints (163-170), including breast cancer (171). Many association studies involving ESR1 have investigated a T/C SNP 397 base pairs upstream of exon 2, i.e., the –397T/C SNP in intron 1 (rs2234693, also referred to as as the PvuII restriction site) and/or linked polymorphisms, mainly the –354A/G SNP (rs9340799, also named after the XbaI restriction site) in intron 1. Although not in a coding region, polymorphisms in intronic regions may affect gene function by altering stability, splicing or localization of mRNA. In addition, functional assays suggest that the ESR1 –397T/C SNP affects a binding site for the myb family of transcription factors (172, 173). Considering the *in vitro* effects of this ESR1 –397T/C SNP and associations with estrogen-dependent outcome in a Dutch Caucasian female population, the ESR1 –397T/C SNP is an interesting candidate SNP in breast cancer association studies (174-176).

1.4 AIMS AND OUTLINE OF THE THESIS

Breast cancer is a major life-threatening disease in women worldwide. About one half of women diagnosed with breast cancer will eventually die, mainly caused by metastatic progression or recurrence of the disease. The main factor involved in progression and therefore a major treatment target is estrogen action. Genetic variability, i.e., germline polymorphic variation, contributes to inter-individual differences in breast cancer susceptibility, clinical phenotype, prognosis and response to treatment of the disease.
The aim of the thesis is to study the role of genetic variation of the HPG axis in breast cancer. For this study a candidate gene approach was used. In Chapter 2 the associations of two common, biologically interesting polymorphic variants of the genes encoding LH and its receptor, with breast cancer is described. Chapter 3 extends on the positive associations reported in Chapter 2 by studying the functional effects of a polymorphism shown to be associated with breast cancer. In addition the association analyses are repeated and extended in an independent cohort. In Chapter 4 the HPG axis and possible ovarian contribution to the associations are further investigated and a second polymorphic HPG gene variant (GnRH), is included in the association analyses with breast cancer. Chapter 5 focuses on two other polymorphisms in a specific biologically interesting domain of the LHR gene and describes associations of these SNPs with breast cancer. Chapter 6 studies associations with breast cancer of two SNPs in two further downstream determinants in the production and action of estrogens, i.e., the aromatase gene (CYP19) and the estrogen receptor gene (ESR1). Chapter 7 reviews and discusses the major findings and concludes with suggestions for future research.

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CHAPTER TWO

Luteinizing Hormone signaling and breast cancer: Polymorphisms and age of onset

Powell BL, Piersma D, Kevenaar ME, van Staveren IL, Themmen APN, Iacopetta BJ, Berns EMJJ 2003 J Clin Endocrinol Metab 88:1653-1657

ABSTRACT

Estrogen exposure has repeatedly been shown to associate with the risk of developing breast cancer. Estrogen synthesis is under the control of LH and FSH, where LH, through its receptor (LHR), stimulates production of ovarian androgens and FSH their aromatization to estrogens. Here, we investigated whether functional polymorphic variants in the LH signaling pathway are associated with the risk of breast cancer or its clinical phenotype.

A PCR-restriction fragment length polymorphism genotyping approach was used to investigate this in 266 breast cancers. The LHR insLQ allele does not seem to influence breast cancer risk. However, women who were homozygous for the LHR insLQ allele were, on average, 8.3 years younger at diagnosis, compared with those homozygous for the wild-type LHR allele (mean age 51.9 years *vs.* 60.2 years, P=0.03). Trends were observed for associations between the LHR insLQ carriers and nodal involvement or larger tumor size. Patients who were LHR insLQ carriers revealed a significantly worse overall survival, compared with those who were homozygous for LHR (HR=2.4; 95% CI 1.3-4.3; P=0.006). In contrast, no associations between the LH genotype and any of the clinical parameter were observed. Our findings suggest that the LHR insLQ gene polymorphism determines an earlier age of disease onset is prognostic for poor outcome of breast cancer.

INTRODUCTION

Breast cancer is the most common form of cancer among women in industrialized countries and is the leading cause of death between the ages of 40 and 55 yr (1). In addition to age and family history of the disease, exposure to endogenous and exogenous estrogens is a well-known risk factor. Elevated serum estrogen levels and increased urinary excretion rates of E1, E2 and E3 have been found in breast cancer cases compared with controls. Because exposure to estrogens is known to influence breast cancer risk, genetic variants within the hormone metabolic pathway, including enzymes involved in the biosynthesis and metabolism of estradiol (i.e., CYP17, CYP19, CYP2D6, CYP1A1, COMT), are currently being considered as candidate low-risk factors for breast cancer. The data from most of these studies, however, is still inconclusive (reviewed in 2, 3). In addition to increasing the risk of cancer, polymorphisms in genes involved in hormone synthesis and metabolism are also associated with various clinicopathological features of tumors and with patient prognosis. For example, variants of the human steroid 5-α reductase type II gene are associated with an earlier age of disease onset, poor tumor grade, and breast cancer patient (disease free) survival (4), whereas variants of the CYP19 and CYP1B1 genes have been associated with positive estrogen receptor and progesterone receptor tumor status (3).

Estrogen production in the ovary is under the control of the pituitary hormones FSH and LH (reviewed in 5). FSH regulates aromatase (CYP19) activity, whereas LH is responsible for the actual production of androgens in the ovarian theca cells, thus providing the substrate for aromatization to estrogens in the granulosa cells (reviewed in 5). LH and its placental homologue human chorionic gonadotropin (hCG) act through the LH receptor (LHR), a member of the heptahelical, G-protein-coupled receptor family. LHR is expressed in the gonads as well as various other tissues, including normal breast tissue, primary breast tumors and breast cancer cell lines. Inactivating mutations in LHR are associated with strong phenotypic effects, such as 46,XY pseudohermaphroditism primary amenorrhea and anovulation in women. However, linkage of polymorphic variants of *LHR* to disease phenotypes has not been reported. Since LH and the LHR are both involved in estradiol synthesis, functionally important polymorphisms in these genes could alter the level of estrogen exposure and thereby contribute to breast cancer risk determination.

A genetic variant of LHβ has recently been described that is characterized by a single nucleotide polymorphisms resulting in amino acid change Trp8Arg at codon 8 (LH8Arg allele). This polymorphism is linked to another SNP, resulting in amino acid change Ile to Thr at codon 15, introducing an extra glycosylation site at codon 13 (6). Compared to wild-type LH (LH8Trp allele) this variant has higher *in vitro* bioactivity and is associated with higher levels of circulating estradiol and testosterone but has a shorter circulatory

half-life (7). The variant LH is thought to be functionally weaker than the wild-type form.

Seven polymorphisms have been described in the LHR gene (reviewed in 8). Six are located in the extracellular ligand-binding domain and three of these may have functional relevance. They include two single nucleotide polymorphisms, resulting in amino acid changes Asn to Ser at codon 291 and Ser to Asn at codon 312 in exon 10, both of which are potential N-linked glycosylation sites that may be involved in hormone binding. The remaining polymorphism is a palindromic insertion of 6 bp (CTGCAG, Leu-Gln at codon 18) in exon 1, position 55-60, near the N-terminus of the mature protein (LHR insLQ) (9, 10). It is located immediately upstream of the proposed signal peptide cleavage site and may therefore interfere with protein synthesis, posttranslational modification and targetting to the plasma membrane. Interference with the LHR protein at this location has a high probability of changing receptor function, because an insertion of 33 amino acids at the same position causes complete female phenotype in a 46,XY patient (11).

Interestingly, the frequency of LH8Arg allele seems to be generally higher in populations from Northern Europe, compared to Asian populations (6, 12), while LHR insLQ has been reported to be virtually absent from the Japanese population (10). Because the incidence of breast cancer in Northern Europe is two-fold higher than in Asian or Japanese populations (13), we hypothesized that LH8Arg allele and LHR insLQ could be related to breast cancer occurrence, clinicopathological features, and patients prognosis.

The aim of the present study was to assess whether polymorphisms in the LH and LHR genes are possible susceptibility alleles and determinants of clinical phenotype in a cohort of breast cancer patients of Caucasian descent.

MATERIALS AND METHODS

Subjects and sample preparation

Eligible cases were women with primary breast cancer (n=266) who were treated by mastectomy or breast conserving surgery between 1990 and 1993 at two main hospitals, the Sir Charles Gairdner and the Royal Perth Hospitals in Perth, Western Australia. Genomic DNA was extracted from surgically resected tumor samples, using standard procedures. The LH and LHR genes are located on chromosomal arms 19q and 2p, respectively, regions that are infrequently lost in breast cancer. However, because the primary breast tumor specimens form which the DNA was obtained for genotyping always contains a relative high proportion (>50%) of nontumor tissue (stroma, lymphoid, nontumor areas), this contribution of germ line DNA ensures accurate genotyping of the LH and LHR genes.

The median age at surgery was 58 years (range, 18-92 years) and the median follow-up time was 87 months (range, 2-116 months). Additional patient and tumor characteristics for this tumor series were described by Soong et al (14) and are summarized in Table 1. Information on family history of breast cancer was not available. The majority of subjects (>95%) were Caucasian of North-Western European descent. Ethics approval was obtained from the local ethics committee. To test for control frequencies, a comparable local healthy population was chosen. This control series comprised DNA samples from 110 healthy women from the same population that were age-matched to the breast cancer patients.

PCR-restriction fragment length polymorphism (RFLP) assay and sequencing for LHß genotype analysis

High molecular weight genomic DNA was used as template for PCR amplification of exon 2, intron 2 and exon 3 of LH-β, essentially as described by Furui et al (15). For the RFLP-assay, the restriction enzymes *Nco*1 and *Fok*1 (Amersham Pharmacia Biotech, Little Chalfort, Buckinghamshire, UK) were used to detect the linked polymorphisms at codons 8 and 15 in LHß, respectively, as described (15). Suspected LH variants were confirmed by sequence analysis using the Cycle Reader DNA Sequencing kit (Fermentas, St Leon-Rot, Germany) and 5'-^{33}P end-labeled primers. The terminated PCR products were separated on a 6% denaturing polyacrylamide gel containing 8 M Urea. Gels were dried and exposed overnight with Biomax MR films (Eastman Kodak Co., Rochester, NY). The presence of the LH variant was confirmed by comparison with the published wild-type sequence (Hs.: 154704; OMIM: 152780).

PCR-RFLP assay for LHR genotype analysis

High molecular weight genomic DNA was used as template for PCR amplification of exons 1 and 10 of LHR (Hs.: 1796; OMIM: 152790) as described by Atger et al, (9). The RFLP-assay was performed as described by Rodien et al, (10). To detect the 6-bp insertion at position 55 in exon 1 of LHR (LHR insLQ), the restriction enzyme PvuII (Amersham Pharmacia Biotech, Little Chalfort, Buckinghamshire, UK) was used. To confirm the results, PCR-RFLP analysis was repeated on samples that tested heterozygote or homozygote LHR insLQ, as well as 10% of LHR wild-type samples.

Statistical analysis

Pearson's χ^2 test was used to test for independence of the alleles (Hardy Weinberg Equilibrium). The χ^2 test was used to determine associations between the various patient and tumor characteristics and the LH or LHR genetic variants. Information on patient survival was obtained from the Western Australian Health Department death registry. At the end of the study 45 patients (17%) had died of their disease. Univariate and multivariate survival analysis was carried out using Cox regression. All tests were two-tailed and

statistical significance was assumed at $P \leq 0.05$. Statistical analyses were carried out using the SPSS software package (Chicago, IL, USA).

RESULTS

Variant-LH and LHR insLQ genotype frequencies

PCR-RFLP was used for the detection of polymorphisms in LH and its receptor, LHR, in a total of 266 breast cancer cases and 108 control samples. Samples were classified as "carrier" if they were confirmed as being either heterozygote or homozygote for the polymorphisms. The combined homo- and heterozygote frequency of LH8Arg allele in this breast cancer series was 16.5% (Table 1). The observed *V-LH* frequency is similar to that reported for healthy control subjects from Western and Southern European Caucasian populations, i.e., 14-15% in 569 subjects. The combined homo- and heterozygote frequency of the LHR insLQ allele was 44.4% (Table 1).

This LHR insLQ carrier frequency is not significantly different from that of the control group of 108 healthy women from the same hospital (54.6%) or from the 44.1% as reported for 102 subjects (6, 10, 12). The genotype distribution for both the LH and

Table 1. *Associations between LH8Arg or LHR insLQ polymorphisms[a] and clinicopathological features of breast cancer patients*

Feature (n)	LH8Arg (%)	LH (%)	P	LHR insLQ (%)	LHR (%)	P
Breast cancer cases (266)	44 (16.5)	222 (83.5)		118 (44.4)	148 (55.6)	
Age (266)						
≤ 57.8 yr (133)	24 (18.0)	109 (82.0)		67 (50.4)	66 (49.6)	
> 57.8 yr (133)	20 (15.0)	113 (85.0)	0.51	51 (38.3)	82 (61.7)	0.05
Lymph node involvement (211[b])						
Negative: 0 nodes (112)	20 (17.9)	92 (82.1)		46 (41.1)	66 (58.9)	
Positive: 1-4 nodes (62)	10 (16.1)	52 (83.9)		31 (50.0)	31 (50.0)	
Positive: ≥4 nodes (37)	4 (10.8)	33 (89.2)	0.60	22 (59.5)	15 (40.5)	0.13
Histological grade (208[b])						
Well/moderate (130)	20 (15.4)	110 (84.6)		54 (41.5)	76 (58.5)	
Poor (78)	16 (20.5)	62 (79.5)	0.34	38 (48.7)	40 (51.3)	0.31
Tumor size (236[b])						
≤2 cm (134)	22 (16.4)	112 (83.6)		52 (38.8)	82 (61.2)	
>2 cm (102)	17 (16.7)	85 (83.3)	0.96	52 (51.0)	50 (49.0)	0.06
Estrogen receptor status[c] (260[b])						
Negative (81)	14 (17.3)	67 (82.7)		37 (45.7)	44 (54.3)	
Positive (179)	29 (16.2)	150 (83.8)	0.83	78 (43.6)	101 (56.4)	0.75

[a] Homozygote and heterozygote cases are combined.

[b] Numbers are missing since data are not available on full cohort of 266

[c] Threshold for positivity: 10 fmol/mg protein

LHR genes was in Hardy-Weinberg equilibrium (P= 0.26 and P=0.66, respectively). The LHR 291Asn/Ser (A*A*T→ A*G*T) allele frequency was examined but too low (6%) to be informative and therefore was not further analyzed (results not shown). No association between the carriers was apparent: 44% of patients who were wild-type for LH had the LHR insLQ allele, compared to 48% for patients with the LH8Arg allele (P=0.62).

Variant-LH and LHR insLQ genotype and clinicopathological features

Associations between LH8Arg, LHR insLQ and clinicopathological features of breast cancer are shown in Table 1. The LHR insLQ variant does not appear to predispose to breast cancer but does seem to influence the phenotype of the tumor that develops. There was a significant correlation between median age at diagnosis of breast cancer and *LHR* genotype (P=0.05). Trends were observed for associations between LHR insLQ and nodal involvement (P=0.13) and larger tumor size (P=0.06). In contrast, no evidence was found for associations between LH8Arg genotype and any of the established clinical or pathological parameters. Moreover, no difference in overall survival was seen between LH8Arg and wild-type LH breast cancer patients (HR=0.7; 95% CI 0.3-1.8; P=0.49).
We analyzed, in further detail, the relationship between LHR insLQ genotype and the mean ages of diagnosis in affected patients (Table 2). Breast cancer cases who were ho-

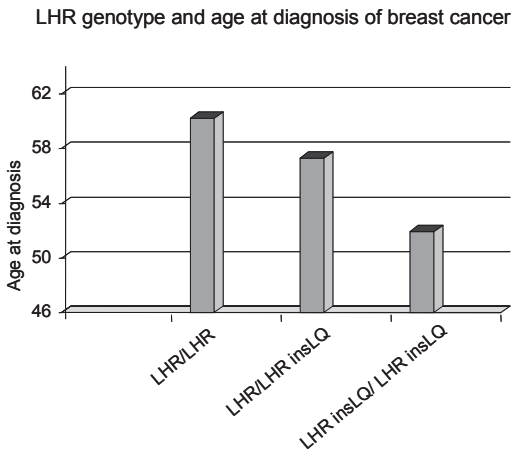

Figure 1. *LHR genotype and mean age at diagnosis of breast cancer.*

Table 2. *LHR genotype and mean age at diagnosis of breast cancer*

	LHR/ LHR	LHR/ LHR insLQ	LHR insLQ/ LHR insLQ	P-value
Mean age (yrs)	60.2	57.3	51.9	0.03[a]
No. of patients	148	99	19	
Percentage	55.6	37.2	7.2	

[a] Linear regression analysis comparing means of homozygotes

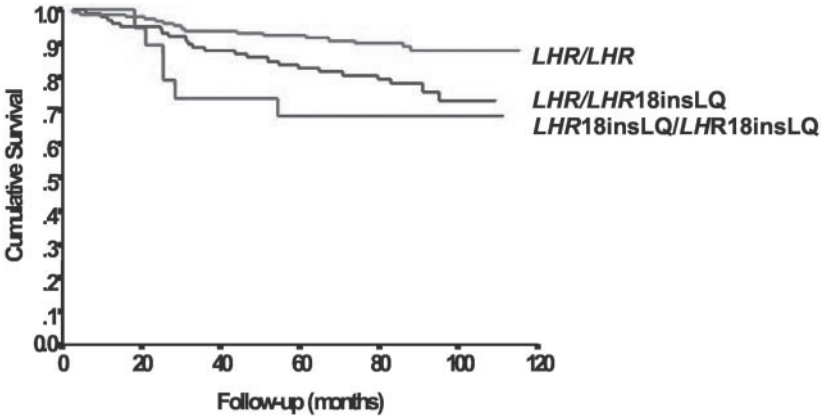

Figure 2. *Kaplan-Meier survival analysis, comparing the overall survival of patients homozygous for wild-type LHR (n=148) with those heterozygous (n=99) and homozygous (n=19) variant, LHR insLQ. Curves were compared using Cox regression analysis.*

mozygous for LHR insLQ were significantly younger than those who were homozygous for wild-type LHR (mean 51.9 years *vs.* 60.2 years, P=0.03; Fig. 1), with a calculated 3.5-yr-earlier age of diagnosis per allele copy. Cox regression univariate analysis revealed significantly worse overall survival for patients who were carriers of the LHR insLQ allele, compared with those who were homozygous for wild-type LHR (HR=2.4; 95%CI 1.3-4.3; P=0.006). Significantly worse overall survival was also seen when patients who were homozygous for wild-type LHR were compared with those who were homozygous for the LHR insLQ allele (HR=3.2; 95% CI 1.2-8.1; P=0.01) or patients heterozygous for the LHR insLQ allele (HR=2.2; 95% CI 1.2-4.2; P=0.01; Fig. 2). The LHR insLQ was not an independent factor for survival in multivariate analysis (results not shown).

DISCUSSION

The current study was undertaken to determine whether genetic variants in the LH signaling pathway were associated with the risk of breast cancer or its clinical phenotype. To our knowledge, this is the first report that a genetic variant in the LHR can modify the age of diagnosis or prognosis of breast cancer. The LHR insLQ allele showed significance for association with younger age of onset of breast cancer in this series, compared to patients with wild-type LHR. Carriers who were homozygous or heterozygous for the LHR insLQ allele were diagnosed, on average, 8 or 3 years earlier, respectively. In addition, patients carrying the LHR insLQ allele had significantly poorer outcome. This finding may be linked to the trends observed for association of the variant with lymph node involvement and larger tumor size (Table 1). These features are all characteristic of a more aggressive

breast cancer phenotype often seen in young women (16). Early-onset breast cancer is likely to differ genetically and biologically from late-onset disease (17, 18). Because no data on family history or on BRCA1 or BRCA2 status are available, the evaluation of a possibility of linkage of LHR with BRCA1 or BRCA2 could not be done for this study.

The frequencies of LH8Arg and LHR insLQ alleles were determined in a series of breast cancer cases that were previously characterized for clinicopathological features and patient outcome (14). The present data suggests that the breast cancer patients investigated in this series do not have increased LH8Arg or LHR insLQ allele frequencies, when compared with a healthy control population from the same area or with other healthy control groups as reported in the literature. Moreover, the genotype distribution for the LH and LHR genes was in Hardy-Weinberg equilibrium. Based on these data, it seems unlikely that the detected polymorphisms reflect somatic changes in the DNA, which was obtained from the breast tumor studies. Furthermore, our results confirm two previous studies for variant LH that also found no difference in the frequency of LH8Arg between breast cancer cases and controls (19, 20). These studies also suggest that the LH8Arg is not associated with increased risk of breast cancer. Although LH and the LHR are involved in estradiol synthesis, there is no relation to the ER status of the breast tumors. Furthermore, there were no significant associations between the LH8Arg allele and clinicopathological features of breast cancer, nor were there differences in overall survival between women who carried the wild-type LH allele or the LH8Arg allele. Information on recurrence-free survival was incomplete and therefore not included in the analyses (as described by us earlier, ref. 14). Interestingly, the LHR insLQ allele showed a significant association with age of diagnosis and a worse overall survival of breast cancer patients.

Even though the breast is not typically considered an LH-responsive tissue, receptors for LH/hCG have been found in human breast tumors and breast cancer cell lines. HCG can inhibit proliferation of some breast cancer cell lines *in vitro* (21). Furthermore, the protective effect of pregnancy on breast cancer has been proposed to be caused by binding of hCG to the LH/hCG receptor on breast epithelial cells, thereby causing differentiation, which, in turn, renders the cells less susceptible to neoplastic transformation (21; reviewed in (13)). Finally, Tanaka et al (22) have proposed a gonadotropin-stimulated, intramural estrogen synthesis in which elevated gonadotropin levels, during peri- and postmenopausal period, stimulate breast tissue to synthesize estrogens. Because this involves both LH and LH/hCG receptor, it is conceivable that polymorphisms in these genes also influence the level of local estrogen synthesis.

Although these data are intriguing, studies on ovariectomy in mouse models have shown that the effect of LH on regression of the mammary gland in indirect, through stimulation of estrogen production by the ovary. This is supported by recent finding on transgenic mice that overexpress LH. Milliken et al (23) showed that persistent overexpression of

LH from the pituitary of transgenic LHbetaCTP mice leads to precocious mammary gland development and ovary-dependent mammary hyperplasia. These mice develop spontaneous mammary tumors, compared with nontransgenic controls, where the ovary was the obligatory intermediate of LH action. In contrast, complete absence of LH signaling has a more subtle effect, as shown in one patient: a normal female phenotype with infertility caused by anovulation. In addition, signs of severe hypoestrogenization are present: small uterus, hyposecretory vagina, decreased bone mass, again illustrating the clear link between LH signal transduction and estrogen exposure (24).

Although the functional implications of the LQ insertion for LHR activity are still not clear, a possible role in breast carcinogenesis might be explained by differences in estrogen exposure or by an altered LH responsiveness. Whether the variant form of the receptor alters estrogen production by the ovary, and in this way indirectly affects breast carcinogenesis, needs further study.

In conclusion, our data suggest that neither the LH8Arg nor LHR insLQ genotypes are associated with an increased risk of breast cancer. The LHR insLQ variant is, however, implicated in an earlier age of onset of this disease and with a more aggressive phenotype. To shed further light on these associations, the role of the LHR insLQ allele in (early-onset) breast cancer needs to be investigated further, preferably in high-power longitudinal studies recording the incidence as well as prevalence in large populations and with documentation of estrogen serum levels.

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CHAPTER THREE

A common polymorphism renders the Luteinizing Hormone receptor protein more active by improving signal peptide function and predicts adverse outcome in breast cancer patients

Piersma D, Berns EMJJ, Verhoef-Post M, Uitterlinden AG, Braakman I, Pols HAP, Themmen APN 2006 J Clin Endocrinol Metab 91:1470-1476

ABSTRACT

Epidemiological and animal studies indicate a carcinogenic role of estrogens in breast tissue. The pituitary gonadotropin Luteinizing Hormone (LH) is an important regulator of estrogen production in premenopausal women, whereas even in women after menopause 10 to 25% of ovarian steroid hormone production is LH-dependent.

We hypothesized that an LH Receptor (LHR) gene variant may affect LH receptor function and thereby influence disease outcome in breast cancer patients.

Design: The association of a polymorphic CTCCAG (Leu-Gln) insertion (insLQ), in the signal peptide encoded by exon 1 of the LHR gene with breast cancer risk, (disease-free) survival and clinico-pathological features was studied in a large cohort of 751 breast cancer patients with complete follow-up. Functional analysis of the insLQ-LHR and nonLQ-LHR (no LQ insertion) was carried out using transfection studies.

We found a significant association between the LHR insLQ and a shorter DFS (HR 1.34; CI 1.11-1.63; P=0.003). The mechanism of the effect of insLQ on LHR function involves increased receptor sensitivity (insLQ-LHR has a 1.9 lower EC50 than nonLQ-LHR; P=0.02) and plasma membrane expression (insLQ-LHR has 1.4 higher Bmax; P=0.0006) rendering the LHR insLQ allele more active.

The insLQ polymorphism increases LHR activity, thereby shortening breast cancer DFS, probably by increasing estrogen exposure in female carriers.

INTRODUCTION

Worldwide the diagnosis of breast cancer is made one million times per year. Factors contributing to an increased cumulative life long estrogen exposure have clearly been shown to increase the risk of breast cancer development (1, 2). In addition to indirect epidemiological evidence, multiple studies in animals indicate a carcinogenic role of estrogens in breast tissue (3). In premenopausal women the major source of estrogen production are the ovaries, which are under control of the pituitary gonadotropins Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH) (4). LH stimulates production of androgens in theca cells that surround growing follicles. FSH subsequently regulates conversion of androgens to estrogens by the granulosa cell enzyme aromatase. Estrogens (estrone and estradiol) in postmenopausal women are mainly derived from local conversion of circulating androgens (androstenedione and testosterone, respectively) by peripheral aromatase, predominantly in the adipose tissue. The adrenals are the main producers of the circulating androgens, but approximately 10-25% is produced by the ovary, partially under the control of LH (5-8).

Considering the role of LH in estrogen production and potential carcinogenic effect of estrogens on mammary cells, LH and LH Receptor (LHR) gene variants may have functional consequences in breast cancer development and/or progression. Indeed, in our previously reported pilot study of 266 breast cancer patients, we found an association between a polymorphic CTCCAG insertion (resulting in the insertion of two amino acids, Leu-Gln (LQ)), in exon 1 of the LHR gene and younger age of onset and adverse overall survival (9). This LQ insertion, with allele frequency in Caucasian and African populations of approximately 0.3, is located in the hydrophobic (h-) region of the signal peptide (10, 11). Signal peptides enable nascent proteins to be targeted to the endoplasmic reticulum membrane for translocation, which is coupled to protein maturation processes such as glycosylation, folding and disulfide bond formation (12). However, the effect of the LQ insertion on the LHR signal peptide is not known.

The present study focuses both on the functional and on the clinical impact of insLQ. Our *in vitro* studies show that the insLQ-LHR protein is translocated to the endoplasmic reticulum more efficiently, resulting in a more sensitive mature LHR protein that is expressed at a higher level than its nonLQ counterpart. Moreover, we establish that the insLQ polymorphism is associated with a shorter disease-free survival.

MATERIALS AND METHODS

Study populations and subjects

Breast tumor samples

DNA samples from two series of breast tumor specimens were available for analysis. Study design was approved by the Medical Ethical Committee of the Erasmus MC Rotterdam, The Netherlands (MEC 02.953). The first series were drawn from studies on prognostic and predictive markers between 1978 and 1986. The second series were drawn from consecutive unselected cases diagnosed in the year 1990 (13). Patients underwent surgery for primary breast cancer at the median age of 60.3 (range: 28 to 94). Analyses of survival and clinico-pathological features were performed in 751 cases of which complete follow-up is currently available. The median follow-up period of patients alive is 130 months from primary surgery (range: 13 to 255 months).

Eindhoven Perimenopausal Osteoporosis Study (EPOS)

The EPOS study is a population-based cohort study of pre-, peri- and postmenopausal women born between 1941 and 1947, living in the city of Eindhoven, The Netherlands. Rationale and design have been described previously (14). For the present study we included 1759 control subjects from this cohort, after excluding women with a history of breast carcinoma. All subjects (median age 49.5 years) are of Caucasian Dutch descent (15). All participants gave their written informed consent, the study was approved by local medical ethical committees.

Rotterdam Study

The Rotterdam Study is a prospective population based cohort study of individuals aged 55 years and over. 3640 women, median age 70.2 years, were included from this cohort to determine the LHR insLQ genotype frequency. The cohort is ethnically homogeneous (Caucasian) and is relatively stable with respect to migration of study participants. The main focus of this study is on cardiovascular, neurogeriatric, ophthalmologic and endocrine disease. Rationale and design have been described previously (16). Baseline examinations took place between 1989 and 1993, including a home interview and an extensive physical examination. The Rotterdam Study was approved by the medical ethics committee of the Erasmus MC.

Genotyping

High molecular weight genomic DNA was used as template for PCR amplification of exon 1 of the LHR gene for detection of the LQ insertion by fragment size analysis. The LHR gene is located on chromosome 2p21, a region that is not frequently lost in breast

cancer. Exon 1 of the LHR gene was amplified as described in (17), using a 5'-hexachlorofluorescein labeled forward primer. Separation and sizing of the PCR fragments and assignment of LQ status was performed using the ABI Prism 3100 automated capillary DNA sequencer and Genescan and Genotyper software packages (Applied Biosystems, Perkin Elmer, Nieuwerkerk aan den IJssel, The Netherlands).

Construction of hLHR cDNA expression vectors

The pSG5-hLHR-EGFP plasmid expressing the coding region of the hLHR extended with a hemagglutinin (HA1) immunotag and EGFP (enhanced green fluorescent protein) was used to construct the insLQ and nonLQ full-length LH receptor expression plasmids (18). As described before, neither the HA1 immunotag nor the EGFP extension affect expression and signal transduction of the LH receptor (18). The LH receptor cDNA in pSG5 carrying the insLQ insertion (pSG5insLQ-LHR) as originally described in (19) was used to construct the ectodomain (ECD) expression plasmids. The 3' half of the cDNA in the plasmid was removed by digestion of pSG5insLQ-LHR with restriction enzymes Bsu36II and BglII, followed by blunting and religation. This resulted in a truncation of the hLHR protein after R365 (all LHR amino acid numbering is according to the LHR protein that contains the LQ insertion, taking the methionine start as #1). This procedure rescues the BglII site which was used to insert a synthetic double-stranded oligonucleotide encoding a combined FLAG and HA1 immunotag: 5'-T GAT TAC AAG GAC GAC GAT GAC AAG TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC T-3', resulting in an extension of the LHR protein: ^{365}R-SDYKDDDDK-YPYDVPDYAS. This extension did not change expression, affinity or activity of the LHR protein (results not shown).

The insLQ from both full-length and ECD LHR expression plasmids was removed using a PCR-based exchange method (20) using a central primer that did not carry the CTGCAG insertion that encodes the LQ. The various signal peptide expression plasmids were constructed on the basis of the pSG5-insLQ-hLHR-ECD. The signal peptide was removed by digestion with EcoRI (in the vector at the 5'-side of the insert) and Eco47III followed by re-ligation. A new translation start site was constructed using site-directed mutagenesis with the primer: 5'-G GGC GAA ATG CTG CGC GAG-3'. The hemagglutinin signal peptide was constructed by insertion of a synthetic double-stranded oligonucleotide: 5'-AA TTC ATG AAG ACC ATC ATT GCT TTG AGC TAC ATT TTC TGT CTG GCT CTC GG-3' in the EcoRI/Eco47III digested pSG5-insLQ-hLHR-ECD plasmid. The plasmid containing the signal peptide with the deleted hydrophobic region was constructed by digestion with PstI and re-ligation, removing amino acid L_{10}-Q_{20}. All mutations were confirmed by DNA sequence analysis of both strands as well as by digestion with appropriate restriction enzymes.

Analysis of LH receptor function

Transfection experiments concerning signal transduction and binding in HEK293 cells used a calciumphosphate transfection method as previously described (20). The extracellular domain-containing expression plasmids were transfected in HEK293 cells using the PolyFect transfection kit according to the manufacturer's instructions (Qiagen, Westburg, Leusden, The Netherlands).

Analysis of signal transduction and cell surface expression

In order to obtain dose response curves for human chorionic gonadotropin (hCG)-dependent induction of cAMP subconfluent HEK293 cells were transiently transfected with 2 μg of the cAMP-reporter plasmid $pCRE_6$ Lux (21), 2 μg SV40Renilla (transfection efficiency), and 10 μg of either pSG5-insLQhLHR-GFP or pSG5-nonLQhLHR-GFP and 6 μg carrier DNA per 75 cm^2. Two days after transfection the cells were trypsinized and plated in 24-well tissue culture plates (Nunc, Roskilde, Denmark) for 6 hours in medium containing 0.1% BSA and increasing concentrations of hCG. Cells were lysed and cAMP response element-dependent luciferase activity was measured as well as Renilla luciferase activity as a control for transfection efficiency. Bmax (single point determinations) and Kd (Scatchard analysis) were carried out as described previously (20, 22)

LH receptor protein analysis

In most proteins signal peptide function is dependent on features of the signal peptide itself, not on the protein that follows. Therefore we decided to use signal peptide-LHR-ectodomain (LHR-ECD) constructs for western blot analysis, since the high hydrophobicity of the transmembrane domain of the LHR is expected to complicate western blot protein analyses. Seventy-two hours after transfection, the HEK293 cells were washed twice in Phosphate Buffered Saline (PBS) and collected in PBS, 20 mM N-ethylmaleimide, with 1x protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, The Netherlands) and centrifuged for 1 min at 2000 rpm. Immediately after pelleting, cells were snap frozen in liquid nitrogen and stored at -80 C before analysis. Cell pellets were resuspended in Laemmli sample buffer to a final concentration of 1 μg/μl and disrupted by 10 passages through a 23-gauge needle. Samples were denatured at 95 C for 5 min and some samples were reduced by adding 20 mM dithiothreitol (DTT) prior to denaturing.

For deglycosylation reactions the manufacturer's instructions were followed (New England Biolabs, Westburg, Leusden, The Netherlands). Briefly, cell pellets were resuspended in denaturing buffer (0.5% sodium dodecyl sulphate (SDS); 1% β-mercaptoethanol) of pH 5.5 for Endo H treatment and of pH 7 for PNGase F treatment. PNGase F reactions were carried out in a final volume of 20 μl, containing 1x G7 buffer and 1% NP-40 and 1.0 μl of 500 U/μl PNGase F. To carry out Endo H deglycosylation 1 μl Endo H was added to a volume of 20 μl, containing 1xG5 buffer. The reaction mixtures were incubated at 37

C for 1h and reactions were stopped by addition of 5 µl 5X Laemmli sample buffer. Prior to electrophoresis, protein concentrations were determined using Protein assay reagent (Cytoskeleton Inc., Denver CO, USA). Four µg of deglycosylated protein was separated on a 15% polyacrylamide separating gel with a 4% stacking gel using the Mini-Protean System 3 (Biorad Laboratories BV, Veenendaal, The Netherlands). Proteins were then transferred to nitrocellulose membranes (Schleicher & Schuell BioScience GmbH, Dassel, Germany) of 45 µm pore size, and blocked for 1 h in blocking solution (5% nonfat milk powder, 0.1% Tween in PBS). LHR protein was detected using a conjugated HA1-horse radish peroxidase rat monoclonal antibody (Roche Diagnostics Nederland BV, Almere, The Netherlands), by incubating for 1 h with 1:5000 dilution of antibody in 5x diluted blocking solution. The membranes were washed 4 times with 0.1% Tween in PBS and developed using the ECL plus Western Blotting Detection System kit (Amersham Biosciences UK Limited, Buckinghamshire, UK).

Statistical analysis

Pearson's χ^2 analysis was used to test for independence of the alleles (Hardy-Weinberg equilibrium, HWE), for the allelic distribution in breast cancer cases and the control populations (EPOS, Rotterdam Study) and to compare genotype frequencies between the three groups. Associations between the various patient and tumor characteristics and the insLQ polymorphism were investigated using Pearson's χ^2 test. Uni- and multivariate overall (endpoint: death) and disease free (endpoint: recurrence including second primary breast tumor) survival analyses were carried out using proportional hazards regression analysis. The assumption of proportional hazards was investigated using a test based on the Schoenfeld residuals (23) and was not violated for insLQ genotype. Hazard ratios (HR) for the insLQ allele are presented with their 95% confidence interval (CI). Differences between HRs for insLQ carriers vs. non-carriers were tested using the likelihood ratio test associated with the Cox regression analysis. In the multivariate model adjustment for classical prognostic factors (age, menopausal status, nodal status, tumor size, differentiation grade and receptor status) was included. Disease-free and overall survival probabilities were calculated using the actuarial method of Kaplan-Meier (24). Survival probabilities were compared between insLQ carriers and non-carriers using the log-rank tests for equality of survival functions. All computations were carried out using the STATA statistical package, version 8.2 (Stata Corp., College Station, TX, USA). Statistical significance was assumed at $P \leq 0.05$, P-values are two-tailed and relate to data during the total period of follow-up.

RESULTS

LHR insLQ genotype, breast cancer risk and clinico-pathological features

Genotype determination was successful in 1240 breast cancer samples: nonLQ/nonLQ: 631; nonLQ/insLQ: 516; insLQ/insLQ: 93. Genotypes were found to be in Hardy Weinberg equilibrium (HWE: P=0.37). The genotype distribution in control population cohorts EPOS (nonLQ/nonLQ: 903; nonLQ/insLQ: 708; insLQ/insLQ: 148; HWE: P=0.58) and ERGO (nonLQ/nonLQ: 1887; nonLQ/insLQ: 1443; insLQ/insLQ: 310; HWE: P=0.15) did not differ significantly from case frequencies (P=0.75). The observed insLQ allele frequency in the breast cancer group was 0.28, which was not significantly different from the frequencies in the control population cohorts (EPOS: 0.29; Rotterdam Study: 0.28; P=0.98). Comparison of the genotype frequencies between cases and controls showed that carriership of the insLQ allele does not affect the chance to be diagnosed with breast cancer.

Based on our previously published results (9), the insLQ allele was designated as a risk allele for worse prognostic outcome and patients were divided into cohorts on the basis of carriership of the LHR insLQ allele. For 751 patients detailed clinical follow-up is available and patient and tumor characteristics were compared in non-carriers versus hetero- and homozygous carriers of the LQ insertion. The LQ insertion was not significantly associated with age, menopausal status, lymph node status, histological grade and estrogen or progesterone receptor status. However, the insLQ polymorphism is significantly associated with larger tumor size (P=0.03) (Table 1).

LHR insLQ and survival analysis

The Kaplan-Meier curves as a function of overall and disease free survival for insLQ carriers versus non-carriers are shown in Figure 1. InsLQ carriers show shorter overall survival as compared to non-carriers (HR=1.25; 95% CI (1.03-1.51); P=0.03; Figure 1A).

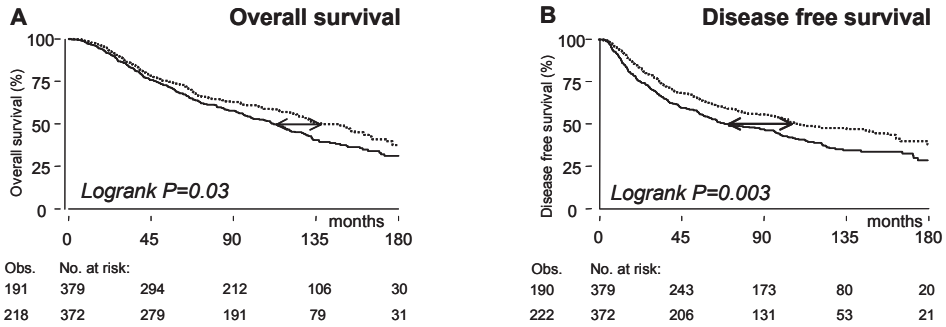

Figure 1. *Kaplan-Meier survival curves for patients homozygous for the nonLQ allele (non-carriers; dotted line) versus those heterozygous or homozygous for the insLQ allele (insLQ carriers; solid line). Difference in median survival is indicated by a two-headed arrow.*

Overall and disease-free survival of non-carriers vs. insLQ carriers are shown in A and B, respectively.

Table 1. *Characteristics of 751 breast cancer patients stratified by LHR insLQgenotype*

		LHR insLQ genotype		
Feature	(%)	non-carriers (%)	carriers (%)[a]	P-value[b]
Breast cancer cases	100	379 (50.5)	372 (49.5)	
Age in years				
<40	9	32 (45.1)	39 (54.9)	
40-50	25	102 (54.0)	87 (46.0)	
50-60	23	92 (54.4)	77 (45.6)	
60-70	24	77 (43.3)	101 (56.7)	
> 70	19	76 (52.8)	68 (47.2)	0.15
Menopausal status				
Pre	39	154 (52.6)	139 (47.4)	
Post	61	225 (49.1)	233 (50.9)	0.36
Nodal status				
Negative	42	168 (52.8)	150 (47.2)	
Positive	58	211 (48.7)	222 (51.3)	0.27
Histological grade[c]				
Well/moderate	21	84 (54.2)	71 (45.8)	
Poor	56	220 (52.1)	202 (47.9)	0.66
Tumor size[d]				
≤ 2cm	35	146 (56.2)	114 (43.9)	
> 2cm	65	233 (47.5)	258 (52.6)	0.03
Estrogen receptor status[c,e]				
Negative	24	82 (45.1)	100 (55.0)	
Positive	75	295 (52.0)	272 (48.0)	0.10
Progesterone receptor status[c,e]				
Negative	28	100 (47.0)	113 (53.1)	
Positive	67	259 (51.3)	246 (48.7)	0.29
Adjuvant therapy				
No	75	288 (51.0)	277 (49.0)	
Hormonal	6	19 (40.4)	28 (59.6)	
Chemotherapy	19	72 (51.8)	67 (48.2)	0.36

[a] Homozygous and heterozygous carriers have been combined

[b] Associations between patient/tumor characteristics and insLQ status were tested with Pearson's χ^2

[c] Numbers in cells may not add up due to incomplete information on histological grade, and/ or receptor status

[d] Tumors of unknown size (n=10) are included as tumor size > 2cm

[e] Cutoff value used: 10 fmol/mg protein

Median OS is 112 months versus 138 months for insLQ carriers vs. the non-carriers (26 months difference). In multivariate analysis, however, the insLQ allele was not an independent factor for overall survival (results not shown). InsLQ carriers show significantly shorter disease free survival (DFS) as compared to non-carriers by univariate analysis (HR 1.34; 95% CI 1.10-1.63; P=0.003; Figure 1B). Next, the prognostic value of the insLQ

allele was tested in the multivariate model for DFS, including classical prognostic factors: age, menopausal status, nodal status, tumor size, differentiation grade and receptor status. The presence of the insLQ allele is an independent negative prognostic factor for DFS (associated likelihood ratio-test), since the addition of insLQ to the model contributed to an increase of χ^2 from 142.25 to 150.81 ($\Delta\chi^2$=8.56 (df=1); P=0.003). It is anticipated that adjuvant therapy improves DFS, therefore we have excluded patients treated with adjuvant chemo or hormonal therapy, which was given to 25% of these patients. Adjusting for adjuvant therapy did not change the association (HR=1.35; 95% CI 1.11-1.64; P=0.003).

The LQ insertion affects LHR protein function

The possible effects of the insLQ polymorphism on hCG-dependent LHR signal transduction *in vitro* were investigated using a cAMP-responsive reporter system in insLQ-LHR or nonLQ-LHR transfected HEK293 cells. Cells expressing the insLQ-LHR construct

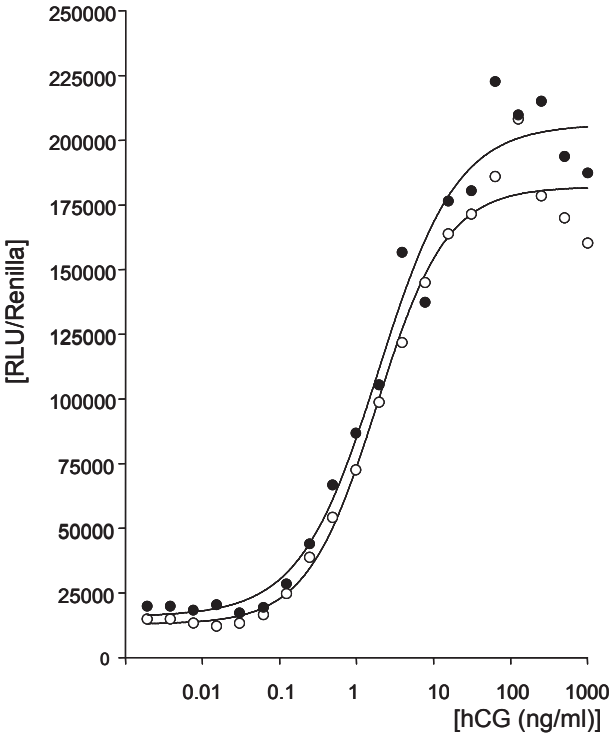

Figure 2. *hCG-induced cAMP response element activation by the insLQ- and nonLQ-LHR variants. HEK293 cells were transfected with pSG5-hLHR-HA1 with the LQ (closed circles) or without the LQ insertion (open circles), in the presence of a cAMP-responsive luciferase reporter plasmid (pCRE$_6$luc). Subsequently, the cells were incubated for 6 h with different concentrations of hCG and luciferase activity was determined in the cell lysates. Cells expressing the insLQ LHR protein are more sensitive to hCG (lower EC50) and show a higher maximal response (see also Table 2). This graph is a representative of 6 independent experiments.*

Table 2. *EC50 and Bmax values for LHR insLQ and nonLQ variant*

	LHR insLQ	LHR nonLQ	n	P-value[a]
logEC50 (95%CI) [ng/ml]	0.19 (0.12-0.26)	0.32 (0.18-0.45)	6	0.02
Bmax (95%CI) [fmol/Renilla]	3.38 (2.64-4.12)	2.41 (1.89-2.94)	40	0.0006

[a] Difference between mean values was tested using Student's t-test

responded to hCG with a slightly higher maximal increase in luciferase activity, and a 1.7-fold lower EC_{50} (P=0.02) than nonLQ-LHR expressing cells (Figure 2 and Table 2). Using LH as ligand, similar results were obtained (results not shown).
Subsequently we determined whether the insLQ-LHR was expressed more efficiently at the cell surface. Single point [^{125}I]-labeled hCG Bmax values for insLQ-LHR-expressing HEK293 cells were highly significantly increased (P=0.0006) compared to nonLQ-LHR-expressing cells (Table 2). Kd values for insLQ- and nonLQ-LHRs from Scatchard analyses were not different (results not shown).

The LQ insertion enhances signal peptide efficiency

Signal peptides contain 3 domains. The N-terminal n-region is usually positively charged, whereas the more polar C-terminal c-region often contains helix-breaking proline and glycine residues. The most essential region for targeting and membrane insertion is the central hydrophobic core (h-) region (25). Since the LQ insertion is located in the hydrophobic region of the signal peptide, the increased receptor sensitivity and cell surface

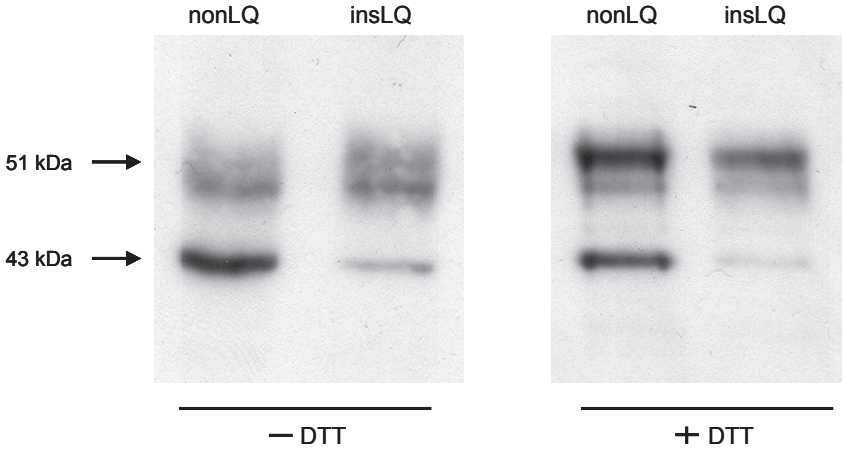

Figure 3. *The nonLQ-LHR-ECD transfected cells express more of a non-folded 43 kDa LHR-ECD isoform. HEK293 cells were transfected with pSG5-hLHR-ECD-HA1 with (insLQ) or without (nonLQ) insertion. After protein extraction, denatured non-reduced (-DTT) or reduced (+DTT) extracts were run on separate 12% polyacrylamide gels. Proteins were visualized using western blotting with an HA1 antibody. Reduction in electrophoretic mobility after reducing treatment suggests that the protein forms in the 51-kDa band group contain internal disulfide bands. Resistance of the lower 43-kDa fragment to reducing treatment, suggests a native, unfolded condition of this protein form.*

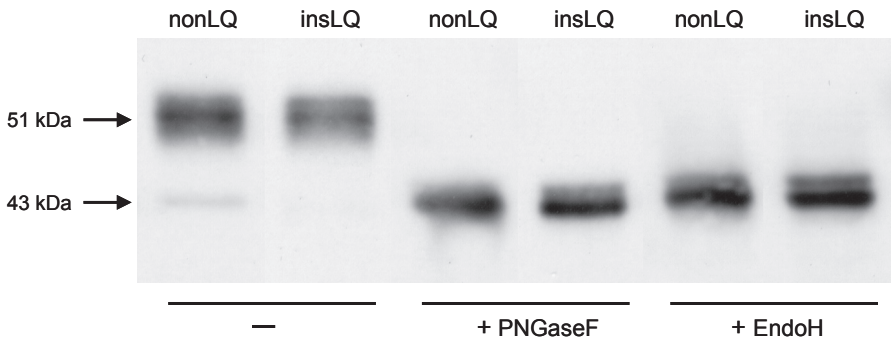

Figure 4. *The 43-kDa LHR-ECD species is not glycosylated, indicating a cytosolic location.*
HEK293 cells were transfected with pSG5-hLHR-ECD-HA1 with (insLQ) or without (nonLQ) insertion. Protein was extracted, left untreated (-), treated with N-Glycosidase F (+PNGaseF) or with Endoglycosidase H (+Endo H), and subsequently separated using 8.5% polyacrylamide gels and analyzed using western blotting with an HA1 antibody. Under both treatments the glycosylated 51-kDa band changes mobility to an apparent molecular mass slightly smaller than 43 kDa, whereas the 43-kDa band does not change mobility.

expression may result from altered function of the signal peptide. This was tested using signal peptide-LHR-ectodomain (LHR-ECD) constructs for western blot analysis (see also the Materials & Methods section).

Expression of insLQ-LHR-ECD and nonLQ-LHR-ECD in HEK293 cells yielded two major bands at 51 and 43 kDa (Figure 3). However, the intensity of the 43-kDa band was much lower in the insLQ-LHR ectodomain-expressing cells. To investigate the identity of this fragment, PAGE separation was carried out after reduction of possible disulfide bonds with DTT or in the presence of N-ethylmaleimide, an agent that prevents further oxidation of cysteines and rearrangement of disulfide bonds (results not shown). Both treatments affected electrophoretic mobility of the 51-kDa, but not the 43-kDa band, indicating that the lower LHR protein band has not undergone co-translational protein folding and disulfide bond formation at the luminal side of the endoplasmic reticulum membrane. Thus, the 43-kDa fragment may not have been translocated to the endoplasmic reticulum lumen, but rather be still located in the cytosol, in which case it should not be glycosylated. Treatment of lysates of transfected cells with PNGase F, a glycosidase that removes all types of N-linked glycans, revealed that the 51-kDa LHR band shifted to higher mobility, whereas the 43-kDa band was totally resistant to PNGaseF (Figure 4). The same effect was obtained using EndoH, a glycosidase for high-mannose immature carbohydrate chains, confirming the localization of the 51-kDa fragment in the endoplasmic reticulum membrane (26). Indeed, the shift of the 51-kDa fragment to a size smaller than the 43-kDa cytosolic band indicates that this protein species has undergone signal peptide cleavage, whereas the 43-kDa has not.

The decreased intensity of the 43-kDa band in the insLQ-LHR-ECD transfected cell lysates indicates that the LQ insertion renders the signal peptide more efficient, causing

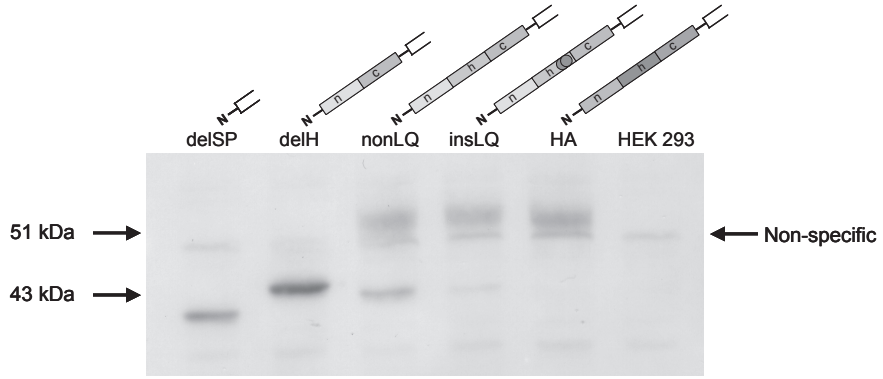

Figure 5. *LH receptor signal peptide analysis: the increased hydrophobicity of the insLQ signal peptide renders it more efficient. HEK293 cells were transfected using pSG5-hLHR-ECD-HA1 expression plasmids carrying different signal peptides. Protein was extracted, run on 12% polyacrylamide gels and analyzed using western blotting with an HA1 antibody. n, h and c, N-terminal, hydrophobic and C-terminal region of the signal peptide, respectively. delSP: no signal peptide; delH: signal peptide without h-region; nonLQ: signal peptide without LQ insertion; insLQ: signal peptide with LQ insertion; HA: ECD with hemagglutinin signal peptide; HEK293: non-transfected cells. Non-specific: indicates a band that is present in the non-transfected cells. On the left hand side the apparent molecular masses of the two LHR protein species are indicated.*

more LHR protein to be translocated into the endoplasmic reticulum membrane. To test this hypothesis we constructed LHR-ectodomain constructs containing different signal peptides: insLQ, nonLQ; no h-region (delH); the hemagglutinin signal peptide (HA) and finally no signal peptide (delSP) and subsequently expressed these proteins in HEK293 cells (Figure 5). A gradual decrease in quantity of the cytosolic fragment from the delSP, to delH, to nonLQ, to insLQ to the HA construct was observed. The smaller size of the cytosolic band in the delSP-lane is in agreement with the expected size of the protein product (40 kDa) whereas the larger size of this band in the delH and nonLQ-lanes indicates that signal peptide cleavage has not occurred.

DISCUSSION

Here we report our studies on the impact of the LHR insLQ polymorphism on receptor activity and on breast cancer outcome in a large series of retrospectively collected breast cancer patients. We validated the previously observed association between the LHR insLQ variant and poor survival (9), in an independent set of breast cancers of Caucasian origin. In addition, we observed a significant association between the insLQ allele and shorter DFS. Whereas LHR insLQ is not associated with breast cancer risk and cannot be used to predict the likelihood of female carriers to develop breast cancer, carriership of LHR insLQ does predict the chance of earlier recurrence of the disease in breast cancer

patients. Although larger tumors were more often observed in the insLQ carriers versus non-carriers, the insLQ independently predicts disease free survival in a multivariate analysis.

Functional studies on the effect of the LQ insertion on LHR protein function *in vitro*, revealed an increase of receptor sensitivity, reflected by a significant decrease of the EC_{50}, and increase in cell surface expression for the LHR insLQ, supporting the hypothesis that the LQ insertion results in increased LHR activity. hCG binding affinity did not appear to be changed, indicating that a subtle change in EC_{50} is not necessarily reflected in a change in Kd as shown previously (10). The changes in LHR function are the result of a more efficient function of the signal peptide carrying the LQ insertion, as indicated by the lower expression of a cytosolic LHR species in cells expressing the insLQ-LHR extracellular domain.

The role of the signal peptide is to promote translocation of the synthesized protein into the lumen of the endoplasmic reticulum. There the protein undergoes signal peptide cleavage, followed by maturation processes such as folding, cystine bond formation and glycosylation. The observed 43-kDa cytosolic band in the cell lysates from nonLQ-and insLQ-LHR transfected cells is consistent with the presence of the intact signal peptide, indicating that it has not been removed. Indeed, deglycosylation of the larger 51-kDa LHR species caused a mobility shift to a smaller apparent molecular mass than the 43-kDa band, indicating that in the 51-kDa protein band the signal peptide had been cleaved. Removal of the h-region from the signal peptide, which is the most essential region for its function (25) had a similar effect as complete absence of the signal peptide, i.e., no translocation into the endoplasmic reticulum at all. Replacing the LHR signal peptide with the very efficient signal peptide of the influenza hemagglutinin protein resulted in translocation of virtually all protein to the endoplasmic reticulum lumen. The slight change in hydrophobicity caused by the LQ insertion in the h-region causes a more efficient function as indicated by our signal transduction and binding experiments. In a previous *in vitro* study on the LQ insertion in the LHR signal peptide, Rodien and co-workers (10) could not identify differences between LHR expression, signal transduction or binding in COS7 cells, although they acknowledged the possibility of a subtle difference in protein maturation due to the location of the insertion in proximity to the signal peptide cleavage site. The association with a higher level of expression of the LHR insLQ variant with a decrease in the EC50, raises the question whether increased expression of the LH receptor is always accompanied by an enhancement of sensitivity of the receptor to ligand. However, in an experiment where increasing amounts of expression plasmid DNA for either inslQ- or nonLQ-LHR were transfected into HEK293 cells, we found that the EC50 for both receptor gene variants did not change with receptor density (data not shown). Thus, the elevated insLQ-LH receptor sensitivity is not caused by the increased expression as such, but the LQ insertion in the signal peptide may affect the quality of

the LH receptor protein, possibly through subtle differences in folding or glycosylation status.Interestingly, an LHR gene variant has been described in patients with complete resistance to LH (Leydig cell hypoplasia), which results in an 11 amino acid insertion in the LHR protein at the same position as insLQ (27, 28). This insertion was found to completely abolish hCG cell surface binding in transfected cells (28). Although no further detailed studies were performed in these reports, the results indicate that absent trafficking of the LH receptor was caused by a dysfunctional signal peptide.

There is still debate on the expression of LHR in normal and tumor breast cells and on the role of local LHR signaling, which may even be different for receptor activation via LH or hCG. Although we cannot exclude direct effects of LH or hCG via locally expressed LHR on breast (tumor) cells, it has been shown that transgenic pituitary LH-overexpressing mice (LHβ-CTP mice) develop mammary hyperplasia, but only in the presence of functional ovaries (29). Moreover, LHβ-CTP mice that develop mammary tumors display elevated serum levels of estrogens as compared to tumor-free transgenic mice. These observations indicate an ovary-dependent role for LHR activity in breast cancer development. Prospective studies including serum estrogen determinations will definitively resolve this issue.

In conclusion, we have shown that the LHR insLQ variant is associated with shorter disease free survival in breast cancer patients. The insLQ effect is most probably mediated by an ovary-dependent increase in estrogen exposure, as a result of the more efficiently acting LHR signal peptide.

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CHAPTER FOUR

GnRH and LHR variants predict adverse outcome in premenopausal breast cancer patients

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ABSTRACT

Breast cancer development and progression are dependent on estrogen activity. In premenopausal women, estrogen production is mainly regulated through the hypothalamic-pituitary-gonadal (HPG) axis.

We have investigated the prognostic significance of two variants of genes involved in the HPG axis, i.e., the Gonadotropin-Releasing Hormone (GnRH) 16Trp/Ser genotype and Luteinizing Hormone receptor (LHR) insLQ variant, in retrospectively collected premenopausal breast cancer patients with a long follow-up (median follow-up of patients alive: 11 years). Carriership was not related with breast cancer risk (the case control study encompassed 278 premenopausal cases and 1758 premenopausal controls). A significant adverse relationship of the LHR insLQ and GnRH 16Ser genotype with DFS was observed in premenopausal hormone receptor positive breast cancer patients. Especially, those patients carrying both the GnRH 16Ser and LHR insLQ allele (approximately 25%) showed a significant increased risk of relapse, which was independent of traditional prognostic factors (HR=2.06; 95% CI 1.21-3.49; P= 0.007).

We conclude that the LHR insLQ and GnRH 16Ser alleles are independently associated with shorter DFS in premenopausal patients. When validated, these findings may provide a lead in the development of tailored treatment of breast cancer patients carrying both polymorphisms.

INTRODUCTION

The diagnosis breast cancer is made one million times each year worldwide. About one quarter of these women are premenopausal at time of diagnosis, which is associated with poor prognosis as compared to postmenopausal women (1, 2). It is anticipated that, as a result of changing demographic and lifestyle factors, more and more women will be diagnosed at a younger age with breast cancer (3). In addition to age and family history, several factors relating to increased or prolonged cumulative estrogen exposure have been identified as important risk factors for breast cancer development and progression (4, 5). Polymorphic variation in genes regulating estrogen production may partly explain differences in susceptibility, clinical presentation and outcome of breast cancer between individuals or populations (5, 6).

In premenopausal women estrogens predominantly arise from the ovaries, where production is regulated by the neuro-endocrine system consisting of hypothalamus, pituitary and gonads: the HPG axis. Internal and external stimuli are integrated in the brain, resulting in the pulsatile secretion of the hypothalamic neuropeptide Gonadotropin-Releasing Hormone (GnRH). GnRH reaches the gonadotroph cells in the anterior pituitary through the hypophysial portal circulation, where it stimulates *de novo* synthesis and secretion of the gonadotropins Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), which reach the ovaries in women through the circulation. LH, acting through the LH receptor (LHR), stimulates production of androgen precursors in theca cells that surround antral follicles. FSH subsequently regulates the granulosa cell enzyme aromatase, which converts these androgens to estrogens. In turn, ovarian sex steroid and peptide hormones (Inhibin A and B) provide negative feedback regulation, either on the pituitary or hypothalamus. The menopausal transition, i.e., the cessation of menstrual cycling, is characterized by disruption of this tightly balanced HPG axis system and is accompanied by continuously increased serum LH and FSH in combination with decreased levels of ovarian sex steroid hormones (7, 8).

We have previously reported, in a training set of 266 Australian breast cancer patients, on association between a common polymorphic CTCCAG insertion (Leu-Gln, LQ) in the signal peptide of the LHR gene and poor overall survival (9). No associations between its ligand, LH, genotype and clinical parameters were observed in this study. In our subsequent validation study, on a large independent breast cancer cohort of 751 retrospectively collected Dutch patients with long detailed follow-up, we have confirmed the association of the LHR insLQ gene variant with poor overall survival, and in addition observed a shorter disease-free survival (10). Furthermore, we have shown the functional importance of the LQ insertion in the signal peptide, i.e., an increased increased activity for the LHR insLQ variant compared with the LHR nonLQ protein. We hypothesized an ovary-dependent increase in cumulative estrogen exposure that may influence breast

cancer outcome in patients with the LHR insLQ genotype (10). Interestingly, the GnRH gene also carries a common signal peptide polymorphism (Trp to Ser at position 16) (11), that has been associated with altered bone mineral density (BMD), which is an indirect marker for estrogen exposure (12).
In line with the hypothesis that possible associations of the above mentioned polymorphisms with outcome would depend on HPG-regulation of ovarian function, we have investigated associations of LHR insLQ and GnRH 16Ser alleles with premenopausal breast cancer outcome in the present study.
We observed that hormone receptor positive premenopausal women carrying either of the variant alleles or the combined variant alleles of both genes had a significant shorter disease free survival; LHR insLQ and the combined alleles were independent of traditional prognostic factors.

MATERIALS AND METHODS

Study populations and subjects

Breast tumor samples

Study design was approved by the Medical Ethics Committee of the Erasmus MC, Rotterdam, The Netherlands. From the DNA samples with complete follow-up described previously (10), we have included 278 premenopausal patients with known estrogen receptor (ER) status and conclusive genotypes. All tumours were invasive (42 had an additional in situ component).
Menopausal status of patients was determined according to the guidelines of the European Organization of Research and Treatment of Cancer (EORTC). The median age of patients at diagnosis is 45 years (range: 22-57 years). The median follow-up period of patients alive is 133 months from primary surgery (range: 13 to 255 months). Other patient characteristics are listed in Table 1.

Control population

As a control cohort we studied pre and perimenopausal women, born between 1941 and 1947 and living in the city of Eindhoven, The Netherlands, from the EPOS study. A prospective population based cohort study collected between 1994 and 1995. Rationale and design have been described previously (13). For the present study we included 1758 (present calls for LHR as well as GnRH genotype) control subjects from this cohort, after excluding women with a history of breast carcinoma. All subjects (median age 49.5 years) are of Caucasian Dutch descent (14). All participants gave their written informed consent, the study was approved by local medical ethical committees.

Genotyping

High molecular weight genomic DNA was used as template for PCR amplification. Exon 1 of the LHR gene was amplified as described by Atger et al, using a 5'-hexachlorofluorescein labeled forward primer (15). Separation and sizing of the PCR fragments and assignment of LHR insLQ genotype was performed using the ABI Prism 3100 automated capillary DNA sequencer and Genescan and Genotyper software packages (Applied Biosystems, Perkin Elmer, Nieuwerkerk aan den IJssel, The Netherlands) as described by us before (10).

The 16Trp/Ser polymorphism in the GnRH gene was determined using the Taqman allelic discrimination assay. Primer sequences used for amplification of the fragment of exon 1 including the SNP were AATTCAAAAACTCCTAGCTGGCCTTA (forward) and CATAGGACCAGTGCTGGCT (reverse). Used probes (with SNP underlined) were 5'-VIC-CACGCACCAAGTCA (anti-sense) and 5'-FAM-AGCCACGAAGTCA (anti-sense). Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems (Perkin Elmer, Nieuwerkerk aan den IJssel, The Netherlands), for details see http://store.appliedbiosystems.com. These reactions were performed on the Taqman Prism 7900HT 384 wells format. Snap frozen primary breast cancer specimens, stored in liquid nitrogen, from which the DNA was obtained for genotyping, contain a relatively high proportion (>40%) of non-tumor tissue. This ensures accurate genotyping, irrespective of the possible loss of heterozygosity (LOH) that may occur in tumor tissue (9). Furthermore, to test for possible LOH we have examined the Hardy-Weinberg equilibrium.

Statistical analysis

Pearson's χ^2 analysis and Fisher's exact test were used to test for independence of the alleles (Hardy-Weinberg equilibrium, HWE) and for association analyses with patient and tumor characteristics, respectively. We allowed for three possible genetic models to explain differences in patient and tumor characteristics between genotype groups, i.e., linear, dominant or recessive effects. A linear effect, assuming a dose-response relationship of the association for the presence of zero, one or two copies of the allele, was tested using a (χ^2) linear trend analysis (16). A dominant effect between hetero- and homozygous combined carriers vs. non-carriers, was tested using χ^2 analysis. Indications for recessive effects were not observed. Univariate and multivariable disease free (endpoint: recurrence including second primary breast tumor) survival analyses were carried out using Cox proportional hazards regression analysis. Hazard ratios (HR) for the LHR insLQ and GnRH 16Ser alleles are presented with their 95% confidence interval (CI). Differences between HRs per LHR insLQ and GnRH 16Ser genotype were tested using the likelihood ratio test associated with the Cox regression analysis. In multivariable analysis, Cox proportional hazard models for DFS were applied to test the genotype variables against traditional factors using a forward stepwise model. The multivariable

model included age, nodal status positive versus negative, differentiation grade, tumor size (larger tumors vs tumors ≤2cm), estrogen receptor status and adjuvant therapy. DFS curves were generated using the actuarial method of Kaplan-Meier (17) and log-rank tests were used to test for equality of survival functions. All computations were carried out using the STATA statistical package, version 9.2 (Stata Corp, College Station, TX, USA). Statistical significance was assumed at $P \leq 0.05$, P-values are two-tailed and relate to data during the total period of follow-up.

RESULTS

LHR insLQ and GnRH 16Trp/Ser genotyping

Genotype analysis for the LHR insLQ polymorphism in the 278 premenopausal patients studied revealed an allele frequency of 0.27. This resulted in 147 (52.9%) nonLQ/nonLQ homozygotes, 113 (40.7%) heterozygotes and 18 (6.5%) insLQ/insLQ homozygotes. The allele frequency for the GnRH 16Ser polymorphism was 0.25. The genotype distribution was 157 (56.5%) 16Trp/16Trp homozygotes, 103 (37.1%) heterozygotes and 18 (6.5%) 16Ser/16Ser homozygotes. Both genotypes were found to be in Hardy Weinberg equilibrium (HWE: P=0.55 and P=0.84 respectively). The genotype distributions and allele frequencies in the control cohort, n=1758, the EPOS study, did not differ significantly from the case distributions. Genotype results for the LHR insLQ polymorphism revealed an allele frequency of 0.29. The genotype distribution was 901 (51.3%) nonLQ/nonLQ homozygotes, 708 (40.3%) heterozygotes and 149 (8.5%) insLQ/insLQ homozygotes. Genotyping for the GnRH 16Ser allele resulted in an allele frequency of 0.25 and revealed 1004 (57.1%) 16Trp/16Trp homozygotes, 627 (35.7%) heterozygotes and 127 (7.2%) 16Ser/16Ser homozygotes. All genotype frequencies and allele frequencies are closely similar in the cases series and in the population controls, which lends support to the genotyping results in tumour material. We conclude that neither LHR insLQ nor GnRH 16Ser genotypes influence the risk of breast cancer development.

Associations with patient and tumor characteristics

The distribution of clinico-pathological characteristics across the GnRH 16Trp/Ser genotype showed a dominant effect of presence of the GnRH 16Ser allele. Carriers of the GnRH 16Ser allele were therefore compared vs. non-carriers. Carriership of the GnRH 16Ser allele was significantly associated with increased lymph node involvement (P=0.001), while LHR insLQ was associated with increasing levels of progesterone receptor (PR) expression (P=0.03). Both polymorphisms were not significantly associated with other clinico-pathological characteristics. No significant interaction between presence of the LHR insLQ and GnRH 16Ser variant was observed in these association analyses.

Table 1. *Cox univariate and multivariable analysis for disease free survival in 278 premenopausal breast cancer patients*

	patients		Univariate analysis			Multivariable analysis		
Factor of base model	No.	%	HR	95% CI	P	HR	95% CI	P
Age (years)								
≤40	67	24	1.00			1.00		
41-50	168	60	0.68	0.47-0.98		0.63	0.43-0.92	
51-60	43	15	0.39	0.22-0.70	0.004	0.33	0.18-0.61	<0.001
Nodal status								
Negative	134	48	1.00			1.00		
Positive	144	52	1.75	1.26-2.44	<0.001	3.18	1.81-5.58	<0.001
Histological Grade								
Poor	153	55	1.00			1.00		
Unknown	59	21	0.56	0.36-0.88		0.60	0.38-0.95	
Well/moderate	66	24	0.61	0.40-0.92	0.006	0.63	0.41-0.96	0.019
Tumor size								
≤ 2cm	113	41	1.00			1.00		
> 2cm	165	59	1.70	1.20-2.39	0.002	1.53	1.06-2.19	0.021
Estrogen receptor status								
Negative	78	28	1.00			1.00		
Positive	200	72	0.89	0.62-1.28	0.54	1.03	0.70-1.50	0.89
Adjuvant therapy								
no	158	57	1.00			1.00		
yes	120[a]	43	1.39	1.01-1.92	0.047	0.45	0.26-0.77	0.004
Factors analyzed								
Carriership								
Non-carriers			1.00			1.00		
GnRH 16Ser	121	44	1.34	0.97-1.85	0.08	1.27	0.91-1.78	0.16
LHR insLQ	131	47	1.59	1.15-2.20	0.005	1.62	1.16- 2.26	0.004
Combined carriership								
Non-carriers	87	31	1.00			1.00		
Only GnRH 16Ser	60	22	1.44	0.89-2.33	0.13	1.33	0.81-2.18	0.26
Only LHR insLQ	70	25	1.71	1.09-2.68	0.02	1.69	1.07-2.66	0.02
LHR insLQ+GnRH16Ser	61	22	2.08	1.31-3.29	0.002	2.02	1.26-3.25	0.004
	278							

[a] Of 120 patients who received adjuvant therapy: 110 chemotherapy, 4 endocrine therapy, 6 both

Associations of LHR insLQ and GnRH 16Ser variants and DFS

We hypothesized hypothalamic-pituitary-gonadal (HPG)-mediated increase in cumulative ovarian estrogen exposure to influence breast cancer outcome. The adverse association of the LHR insLQ allele with DFS was observed only in the premenopausal patients (HR for carriers vs. non-carriers =1.59; 95% CI 1.15-2.20; P=0.005, Table 1), and not in

postmenopausal women (data not shown). In the premenopausal subgroup the LHR insLQ genotype was an independent prognostic factor: addition of LHR insLQ carriership to the multivariable model resulted in an increase of χ^2 from 44.06 to 52.23 ($\Delta\chi^2$=8.17 (df=1); P=0.004) for DFS. The association between presence of the GnRH 16Ser allele and DFS was also tested. An increased HR of 1.34 (95% CI 0.97-1.85; P=0.08; Table 1) for GnRH 16Ser carriers vs. non-carriers was observed.

Interestingly in the biological interestingly hormone receptor (ER and or PgR positive, n=225) subgroup the LHR insLQ genotype retained significance. The adverse association of the LHR insLQ allele with DFS had a HR for carriers vs. non-carriers of 1.59; 95% CI 1.11-2.28; P=0.012. It was also an independent prognostic factor: addition of LHR insLQ carriership to the multivariable model resulted in an increase of χ^2 from 36.28 to 42.70 ($\Delta\chi^2$=6.42 (df=1); P=0.01) for DFS. Moreover, the association between presence of the GnRH 16Ser allele and DFS revealed a significantly increased HR of 1.44 (95% CI 1.01-2.07; P=0.046). In multivariable analysis for GnRH 16Ser carriers vs. non-carriers this was not significant.

Cooperative effect of variants in the HPG axis and DFS

The HPG system in the premenopausal woman involves a cooperative effect of both GnRH and LH action on regulation of ovarian sex steroid production. Therefore, we have examined, in an exploratory study, the combined effect of the GnRH 16Ser and LHR insLQ variants in these premenopausal breast cancer patients. We combined heterozygous and homozygous carriers, providing four groups of similar sizes. Non-carriers, carriers of the GnRH 16Ser allele, carriers of the LHR insLQ allele, and carriers of both alleles, were compared.

The combination of both variants in premenopausal breast cancer patients, present in 22% of this group, resulted in a HR of 2.08 vs. non-carriers of both variants (95% CI 1.31-3.29; P=0.002; log-rank test for trend: P=0.005; Figure 1a and Table 1). In multivariable analysis, including the prognostic factors age, nodal status, differentiation grade, tumor size, estrogen receptor status, and adjuvant therapy, the association was independent: $\Delta\chi^2$=10.0 (df=3); P=0.018; HR=2.02; 95% CI 1.26-3.25; P= 0.004. No significant interaction between presence of the LHR insLQ and GnRH 16Ser variant was observed in these survival analyses.

We next studied the effect in hormone receptor positive patients. The combination of both variants, present in 52 out of 225 patients of this group, resulted in a HR of 2.43 vs. non-carriers of both variants (95% CI 1.42-4.18; P=0.0013; log-rank test for trend: P=0.0055; Figure 1b). In multivariable analysis, including the prognostic factors listed above, the association was independent: $\Delta\chi^2$=8.72 df=3; P=0.03; HR=2.06; 95% CI 1.21-3.49; P=0.007. No significant interaction between presence of the LHR insLQ and GnRH 16Ser variant was observed in these survival analyses.

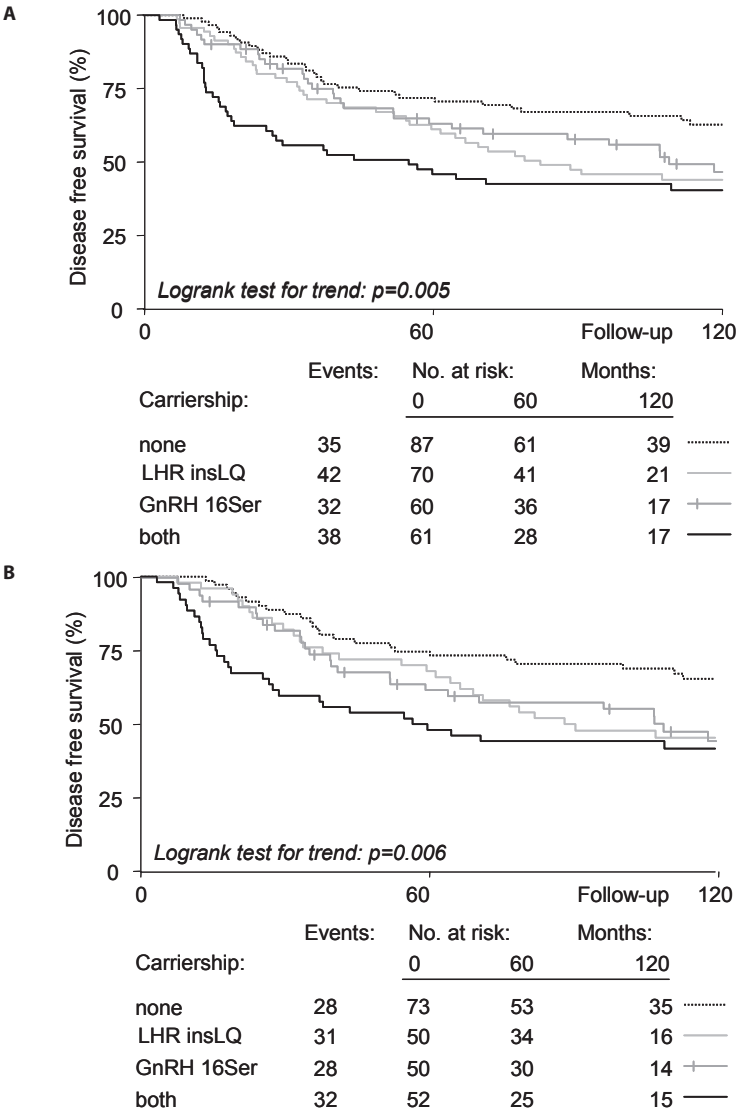

Figure 1 *a and b. Kaplan-Meier curves for disease free survival (DFS) comparing carriers vs. non-carriers in all (Figure a) and in hormone receptor positive (Figure b) premenopausal breast cancer patients. DFS curves are depicted for carriers of both variants, LHR insLQ and GnRH 16Ser vs. non-carriers of both variants.*

DISCUSSION

Breast cancer is a heterogeneous and complex disease. Many gene variants, acting in concert with each other and with environmental factors, may influence its susceptibility, prognosis and response to treatment (18). In light of their possible role in the variability of estrogen exposure, variants of genes involved in the hypothalamic-pituitary-gonadal (HPG) axis

are likely candidates to contribute to differences in clinical phenotype and outcome. In the present study we tested this hypothesis. In a genetic association approach we used stratification for ovarian activity and explored cooperative action of two HPG gene variants. We show that the association between the LHR insLQ allele and a shorter DFS is restricted to premenopausal patients, especially in the hormone receptor positive subset. A common GnRH gene variant, i.e., GnRH 16Ser, showed association with lymph node involvement. In addition, coincident carriership of the GnRH 16Ser variant and LHR insLQ, present in almost one quarter of the patients, resulted in a more than doubled risk of recurrence of disease in (hormone receptor positive) premenopausal patients with a long (>10 years follow-up of patients alive) Multivariable analyses showed that these associations with poor disease-free survival were independent of known prognostic factors.

Whether variants of the GnRH gene differ in function remains to be elucidated. Substitution of Trp by the less hydrophobic Ser amino acid at position 16 may change the efficiency of the GnRH signal peptide. Using the same *in vitro* assay, as described previously by us, for the insLQ signal peptide variant (10), we were unable to detect a difference in signal peptide efficiency between the GnRH 16Ser and GnRH 16Trp signal peptide constructs (data not shown). Furthermore, *in silico* analysis of the variants using on line program SignalP 3.0 did not result in any difference of signal peptide characteristics (http://www.cbs.dtu.dk/services/SignalP/). On the other hand, Iwasaki et al have described an association between the GnRH 16Trp allele and higher bone mineral density (BMD, considered to be an indirect marker for estrogen activity) in 384 Japanese postmenopausal women (12) which suggests higher estrogen activity in women bearing this variant. In contrast, we observed a larger tumor size and a significant increased lymph node involvement and shorter DFS in Caucasian breast cancer patients carrying the other variant, the GnRH 16Ser allele, which we hypothesize to result from a higher level of cumulative estrogen exposure. Possible reasons for the apparent conflicting results are numerous, including differences in sample size, in technical approach, in ethnicity or, so far unknown, differences in the interaction of genetic with environmental factors between Japanese and Caucasian subjects. Consequently, further studies are needed to identify the exact mechanisms of the effect of the GnRH 16Ser polymorphism, including linkage to other polymorphisms in the GnRH gene that may affect regulation of expression.

There are several hypotheses as to how GnRH and LHR gene variants may affect tumor features and clinical outcome in breast cancer as demonstrated in this study. In view of the abundant data on direct effects of GnRH modulation on sex steroid hormone-dependent cancers, a direct effect of locally produced GnRH via GnRH receptors expressed in breast cancer tissue cannot be ruled out (19-22). Local co-expression of mRNAs for GnRH and GnRHR in breast cancer tissue has been shown (23, 24), and direct growth inhibitory of cultured breast cancer cells have been reported as well (25). However, to our knowledge local production of GnRH has not been shown. Hypothalamic GnRH is

unlikely to reach the breast via the peripheral circulation given its low concentration and short half-life (26). Furthermore, the effects of GnRH agonist treatment regimens are most likely explained by down-regulation of pituitary GnRH receptors and subsequent shutdown of the HPG axis (27). A few studies have shown LHR expression in normal and breast tumor cells. However, it is less likely that direct effects of LH explain the adverse association of LHR insLQ with DFS, since this was not seen in the postmenopausal patients in whom circulating LH levels are high.

In premenopausal breast cancer patients with advanced disease, reduction of estrogen levels by a GnRH agonist in combination with the ER antagonist tamoxifen, improves clinical outcome compared with the use of GnRH agonists or tamoxifen alone (27, 28). Randomized trials assessing whether the combination of a GnRH agonist with an aromatase inhibitor improves outcome compared to treatment consisting of a GnRH agonist and tamoxifen are ongoing (29). Regimens for endocrine therapy are still largely empirically based (30). Recently, it has been suggested that, also for adjuvant systemic therapy, the role of genetic factors in breast cancer treatment outcome should be considered (31). Our current study has identified almost 25% of premenopausal patients with a genetic background associated with clearly significant poor outcome and hypothetically associated with altered treatment outcomes. Exploratory studies in our subset of patients that received adjuvant endocrine therapy (n=44) in the current study were underpowered to detect differences in response.

In conclusion, we have shown a strong and independent association with DFS (HR=2.1) in almost one quarter of, hormone receptor positive, premenopausal women carrying LHR insLQ and GnRH 16Ser genotypes. The observations strongly suggest that the adverse outcome in patients with these variants is through enhanced HPG-mediated ovarian estrogen production. Prospective studies, including serum estrogen analyses, are needed. When validated in independent studies, the observed results raise the possibility that LHR insLQ and GnRH 16Ser genotyping may provide additional prognostic information for premenopausal breast cancer patients in clinical practice and may result in tailored endocrine treatment of these patients.

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CHAPTER FIVE

Polymorphic variations in exon 10 of the Luteinizing Hormone Receptor: Functional consequences and associations with breast cancer

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ABSTRACT

Polymorphic variation of the LHR gene may affect receptor function and accordingly may influence ovarian steroid hormone action, including steroid hormone-dependent clinical outcome.

We describe the functional effects of two single nucleotide polymorphisms (SNPs), i.e. LHR 291Asn/Ser (rs12470652) and 312Ser/Asn (rs2293275) in the biologically interesting exon 10 of the LHR gene. Furthermore, ethnic diversity in allele frequencies and genotype distributions of both SNPs was determined. In addition associations with breast cancer were studied in 751 breast cancer patients.

In vitro transfection studies revealed altered glycosylation status and increased receptor sensitivity for the 291Ser LHR variant. No functional consequences were observed for the 312SerAsn LHR SNP. The LHR 312Asn allele was slightly more often present in two independent breast cancer patient cohorts as compared to controls (OR=1.15; P=0.03 and 1.26; P=0.001, respectively). In conclusion, although functional changes of the LHR 291Ser candidate allele were observed, no associations with breast cancer were found, while the LHR 312Asn allele can be regarded as a weak breast cancer risk allele.

INTRODUCTION

The pituitary gonadotropin Luteinizing Hormone (LH) is an essential regulator of ovarian steroid hormone synthesis in premenopausal women. By binding to the LH receptor (LHR), LH stimulates the production of androgens in the theca cells that surround growing follicles. Follicle Stimulating Hormone (FSH) subsequently regulates conversion of androgens to estrogens by the granulosa cell enzyme aromatase. In addition, LHR signaling has an essential role in reproduction through the transduction of the signal of the mid-cycle LH surge leading to ovulation and the subsequent maintenance of progesterone production by the corpus luteum. In case of pregnancy in humans, a second ligand for the LHR, i.e. human chorionic gonadotropin (hCG), takes over the role of sustaining progesterone synthesis by the corpus luteum. In human XY fetuses, hCG-induced LHR activity triggers male sex differentiation (1). Clear evidence for the role of LH and LHR in humans in sex steroid hormone-dependent differentiation and reproduction has been obtained from observations in patients with inactivating mutations in LHβ (chromosome 19q13.33) and LHR (chromosome 2p21) genes. Interestingly, the extent of the functional consequences of various LHR mutations on LHR protein function *in vitro* are reflected in the severity of the phenotypic effects of the mutations *in vivo* (2, 3). More common polymorphic variation in the LHR gene may result in more subtle changes in receptor function, which over a lifetime period may have clinical consequences. Thus, the question arises whether slight changes in LHR activity such as those caused by polymorphisms have subtle effects on disease susceptibility, disease progression or response to treatment. So far, a total of 285 polymorphisms has been identified in the LH receptor gene at an average distance of approximately 300 base pairs (http://SNPper.chip.org; http://gvs.gs.washington.edu/GVS/). The majority of these polymorphisms are located in the large introns. Regarding the potential consequences of LHR gene variants for disease, we have recently shown clear associations between a polymorphic CTCCAG insertion in exon 1 of the LHR gene (rs4539842), which results in the insertion of two amino acids, Leu-Gln (insLQ), with characteristics and progression of breast cancer (4, 5). This polymorphism was clearly shown to improve receptor function (5).

The human LHR gene consists of 11 exons. Exon 1 encodes the signal peptide and the N-terminal cysteine-rich region. Exons 2 to 9 encode leucine-rich repeats involved in hormone binding. Exon 10 constitutes an important part of the so-called hinge region, more specifically, a part of the C-terminal cysteine-rich region which terminates the leucine-rich repast in many protiens. Exon 11 mainly encodes the transmembrane domain and the relatively small C-terminal intracellular domain. Interestingly, a deletion of exon 10 has been described in a patient with normal male sex differentiation, but delayed puberty, apparently because the del(exon10) LHR did not respond to LH, while it was sensitive to hCG (6). Indeed *in vitro* expression of the del(exon10) LHR showed a

slightly decreased response to hCG whereas LH-induced receptor activation was severely impeded (7).

In addition to the LHR insLQ polymorphism, two specific single nucleotide polymorphisms (SNPs), leading to variable amino acids at positions 291 and 312 respectively: 291Asn/Ser (rs12470652) and 312Ser/Asn (rs2293275) are located in exon 10, and may have subtle effects on LHR sensitivity for LH and hCG. The fact that the exon 10 291Asn/Ser and 312Ser/Asn SNPs are located at or near glycosylation sites increases this possibility, since glycosylation plays an important role in the G protein-coupled receptor superfamily for stability, trafficking and cell surface expression (8).

Growth and proliferation of both normal breast tissue and breast tumors is to a large extent dependent on estrogen, as reviewed in (9). Amongst others, variability in estrogen exposure, possibly affected by polymorphic variation in genes involved in regulation of estrogen production and response, may explain differences in breast cancer presentation and outcome between individuals or populations (10-13). Considering its role in the regulation of ovarian estrogen production, the LHR gene is a likely candidate gene contributing to possible inter-individual variability in estrogen exposure. Furthermore, possible direct effects of LHR signaling on breast cancer development and progression cannot be excluded since LHR expression has been shown in breast cancer cell lines as well as in normal and malignant breast tissue (14, 15).

In this report we describe the potential effects of the 291Asn/Ser and 312Ser/Asn SNPs on LH receptor function. We have determined genotype frequencies in a large Caucasian cohort and smaller African-American and Chinese cohorts. In addition, association analyses with both SNPs were performed in 751 breast cancer patients with clinical follow-up. Notwithstanding the functional effects of substitution of 291Asn to Ser, no associations of the LHR 291Ser allele with risk, clinical presentation or outcome of breast cancer were observed, possibly due to the low LHR 291Ser allele frequency. However, the LHR 312Asn allele was identified as a risk allele for breast cancer.

MATERIALS AND METHODS

Transfection studies

Cell culture and transfection

HEK293 cells were cultured in Dulbecco's modified Eagles's medium (DMEM)- Ham's F-12, supplemented with 10% fetal calf serum containing penicillin and streptomycin. Transfection experiments concerning signal transduction and binding in HEK293 cells used a calciumphosphate transfection method as previously described (2). The ectodomain-containing expression plasmids were transfected in HEK293 cells using the Poly-

Fect transfection kit according to the manufacturer's instructions (Qiagen, Westburg, Leusden, The Netherlands).

Construction of hLHR cDNA expression vectors

The pSG5-hLHR-EGFP plasmid expressing the coding region of the hLHR extended with a 3'-hemagglutinin (HA1) immunotag and a 3'-EGFP (enhanced green fluorescent protein), carrying the CTCCAG insertion (insLQ) and Asn amino acids at positions 291 and 312 was used to construct full-length LH receptor expression plasmids (16, 17). Ectodomain expression plasmids (truncated after R365, amino acid numbering by taking the methionine start as number 1 and including the LQ insertion) were constructed from these plasmids by truncation as described previously (5). Full-length and ectodomain pSG5-hLHR-EGFP plasmids were used to construct the 291Ser and 312Ser receptor constructs by site-directed mutagenesis (Quick Change™ site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA), according to manufacturer's protocol. Primer sequence for changing 291Asn to 291Ser were: CCAACAAAAGAACAGAGTTTTTCACATTCC (forward) and GGAATGTGAAAAACTCTGTTCTTTTGTTGG (reverse). Primer sequences for changing 312Asn to312Ser were CAGTAAGGAAAGTGAGTAACAAAA-CAC (forward) and GTGTTTTGTTACTCACTTTCCTTACTG (reverse). Finally, all full-length and ectodomain 291 and 312-LHR variants were subcloned into nonLQ-LHR expression plasmids (i.e. lacking the LQ insertion), as described previously (5).

Analysis of signal transduction and cell surface expression

Recombinant hCG and LH were kindly provided by Organon (Oss, The Netherlands). In order to obtain dose response curves for hCG- and LH-activation of the cAMP-dependent luciferase reporter gene , subconfluent HEK293 cells were transiently transfected with 2 μg of the cAMP-reporter plasmid $pCRE_6$ Lux (18), 2 μg SV40β-galactosidase (transfection efficiency), 10 μg of either pSG5-291Asn-312Asn-hLHR-GFP pSG5-291Ser-312Asn-hLHR-GFP or pSG5-291Asn-312Ser-hLHR-GFP and 6 μg carrier DNA per 75 cm^2. Two days after transfection, the cells were trypsinized and plated in 24-well tissue culture plates (Nunc, Roskilde, Denmark). The following day, cells were incubated for 6 hours in medium containing 0.1% BSA and increasing concentrations of hCG or LH. Cells were lysed and cAMP-dependent luciferase activity was measured as well as β-galactosidase activity as a control for transfection efficiency. Bmax and Kd (Scatchard analysis) were carried out as described previously (2, 19).

LH receptor protein analysis

HEK293 cells were transfected with different LHR-constructs for Western blot analyses of protein expression. In the deglycosylation experiments employing endoglycosidase H and N-glycosidase F (PNGase F) ectodomain LHR expression plasmids were used as de-

scribed in (5). In brief, seventy-two hours after transfection, the cells were washed twice in Phosphate Buffered Saline (PBS) and collected in PBS, 20 mM N-ethylmaleimide, with 1x protease inhibitor cocktail (Sigma-Aldrich, Zwijndrecht, The Netherlands) and centrifuged for 1 min at 2000 rpm. Immediately after pelleting, cells were snap frozen in liquid nitrogen and stored at -80 C before analysis. Cell pellets were resuspended in Laemmli sample buffer to a final concentration of 1 μg/μl and disrupted by 10 passages through a 23-gauge needle. Finally, samples were denatured at 95 C for 5 min and analyzed by gel electrophoresis and Western blotting using a conjugated HA1-horseradish peroxidase rat monoclonal antibody (Roche Diagnostics Nederland BV, Almere, The Netherlands) at 1:5000 dilution.

Study populations and subjects

Breast tumor samples

Genotype analysis and analyses of (disease free) survival and clinico-pathological features were performed in 751 retrospectively collected patients of whom detailed follow-up is currently available, as described previously (5). In brief, 38% of patients was premenopausal, 62% postmenopausal. Data on lymph node status showed that 43% was node negative and 57% node positive. Tumor size distribution was 36% T1, 52% T2 and 12% T3/4. Tumors showed 76% estrogen receptor expression vs. 24% no expression and 67% showed progesterone receptor expression vs. 28% no expression and 5% was unknown (cut-off value: 10 fmol/mg protein). 75% of the patients did not receive any adjuvant therapy, while 6% had received hormonal therapy, 17% chemotherapy and 1% both. Median age in these breast cancer patients is 57 (range: 22-90) year, the median follow-up period from primary surgery is 129 months (range: 13-255 months). For the comparison of LHR 312Asn allele frequencies, a second series 594 patients was selected from the database of the Department of Medical Oncology of the Erasmus MC in Rotterdam, The Netherlands. Of these patients clinical follow-up data were not complete at the time of inclusion in our studies. The median age: 63 years (range: 23-90, n=521). The breast cancer patients included in all studies were mainly form the ethnically homogeneous Caucasian population in the South-Western part of The Netherlands. The study design of the breast cancer cohorts was approved by the Medical Ethics Committee of the Erasmus MC, Rotterdam, The Netherlands.

Eindhoven Perimenopausal Osteoporosis Study (EPOS)

The EPOS study is a population-based cohort study of pre-, peri- and postmenopausal Caucasian women born between 1941 and 1947, living in the city of Eindhoven, The Netherlands (median age 50.0 (range: 46-57) years). Rationale and design have been described previously (20). Study design was approved by the Medical Ethics Committee

of the Erasmus MC. For the present study, 22 women who reported a history of surgery for (suspected) breast carcinoma, were excluded and genotyping was performed on 1806 subjects.

African-American and Chinese subjects

Genotype analyses were conducted in anonymized individuals with self-described ethnic heritage from the Human Variation panel obtained from Coriell Cell Repositories (Coriell Institute, Camden, NJ, USA). Subjects included 110 (Han-)Chinese, (55 male and 55 female) and 60 African-American (14 male and 46 female).

Genotype analyses

High molecular weight genomic DNA was used as template for PCR amplification. The 291Asn/Ser and 312Ser/Asn-LHR polymorphisms were determined using the Taqman allelic discrimination assay. Primer sequences used for amplification of the fragment of exon 10 including the 291Asn/Ser SNP were CTGAAGTCCAAAAGCTCAAATGCT (forward) and TGTGCTTTCACATTGTTTGGAAAAGT (reverse). Used probes (with SNP underlined) were 5'-VIC-CAGACAGAATTTTTC (sense) and 5'-FAM- CAGACAGAGTTTTTC (sense). For detection of the 312Ser/Asn SNP, used primer sequences were TTTTCCAAACAATGTGAAAGCACAGT (forward) and GATACGACTTCTGAGTTTCCTTGCA (reverse) and probes 5'-VIC-TTACAGTGTTTTGTTATTCACTT (anti-sense) and 5'-FAM-CAGTGTTTTGTTACTCACTT (anti-sense). Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems, Perkin Elmer, Nieuwerkerk aan den IJssel, The Netherlands), for details see http://store.appliedbiosystems.com. Reactions were performed on the Taqman Prism 7900HT 384 wells format. Primary breast cancer specimens, flash-frozen and stored in liquid-nitrogen contain a relatively high proportion of non-tumor tissue. This ensures accurate genotyping, irrespective of the possible loss of heterozygosity (LOH) that may occur in tumor tissue, since enough DNA from non-tumor tissue will contribute to the final result (4). Furthermore, we have examined Hardy-Weinberg equilibrium to test for possible LOH.

Statistical analysis

Mean values of *in vitro* transfection studies were compared by one way analysis of variance (ANOVA), EC50 values were log-transformed prior to analysis. Pearson's χ^2 analysis was used to test for independence of the alleles (Hardy-Weinberg equilibrium, HWE) and to compare genotype distributions in breast cancer patients and control populations. Odds ratios were computed to compare allele frequencies between breast cancer patients and EPOS controls. Associations between the various patient and tumor characteristics and the LHR 291Asn/Ser and 312Ser/Asn genotypes were investigated using Pearson's χ^2

and Fisher's exact test where appropriate. Uni- and multivariate overall (endpoint: death) and disease free (endpoint: recurrence including second primary breast tumor) survival analyses were carried out using proportional hazards regression analysis. The assumption of proportional hazards was investigated using a test based on the Schoenfeld residuals (21) and was not violated for LHR 291Asn/Ser and 312Ser/Asn genotypes. Differences between HRs per LHR 291Asn/Ser and 312Ser/Asn genotype were tested using the likelihood ratio test associated with the Cox regression analysis. Disease free and overall survival probabilities were calculated using the actuarial method of Kaplan-Meier (22). Survival probabilities were compared across LHR 291Asn/Ser and 312Ser/Asn genotypes using the log-rank tests for equality of survival functions. All computations were carried out using the STATA statistical package, version 9.2 (Stata Corp., College Station, TX, USA). Statistical significance was assumed at $P \leq 0.05$, P-values are two-tailed and relate to data during the total period of follow-up.

RESULTS

291Ser affects LHR protein function and expression

Possible effects of the LHR polymorphisms on cell surface expression, hCG ligand binding and hCG-dependent LHR signal transduction *in vitro* were investigated using a cAMP-responsive reporter system in transfected HEK293 cells. For the comparison of effects of 291Ser vs. 291Asn and 312Ser vs. 312Asn we used functionally neutral nonLQ constructs (i.e. lacking the LQ insertion in the signal sequence which increases LHR sensitivity and expression (5)). Moreover, the nonLQ-LHR allele is the most frequent variant in the Caucasian population and therefore the most relevant. The insLQ polymorphism is over 30,000 base pairs distant from the exon 10 SNPs. Furthermore, no linkage between the exon 10 SNPs and the insLQ polymorphism is found from analysis of genotype data in Haploview. The maximum D' (a measure of linkage disequilibrium) in breast cancer and EPOS cohorts is 0.25 with a maximum r^2 of 0.03 between the insLQ polymorphism in the signal sequence and the 312Ser/Asn SNP in exon 10.

The effects of the amino acid substitutions at positions 291 and 312 were tested comparing three constructs: 291Asn-312Asn-LHR, 291Ser-312Asn-LHR and 291Asn-312Ser-LHR. The hypothetical 291Ser-312Ser-LHR variant was not considered in the transfection studies since our own data and the data from public SNP databases show that this LHR variant is non-existent in the population. In cells expressing the 291Ser-312Asn-LHR construct, the potency of hCG as represented by the EC50 value, was increased two-fold compared to 291Asn-312Asn-LHR and 291Asn-312Ser-LHR expressing cells (P=0.001 N=3-5; Table 1 and Figure 1). Similar results were obtained with LH (results not shown). Subsequently we determined cell surface expression of cells expressing either one of these

Table 1. *Functional analyses of 291Asn/Ser and 312Ser/Asn LHR variants in HEK293 transfected cells.*

LHR construct	EC50 (mean ±sd) [pM hCG]	n	Bmax (mean ±sd) [fmol/β-gal]	n	Kd ± error [nM]
291Asn-312Asn	61.9 ± 13.6	5	2.13 ± 1.43	10	3.77 ± 0.96
291Asn-312Ser	64.7 ± 9.3	3	2.22 ± 1.89	10	2.16 ± 0.16
291Ser-312Asn	29.3 ± 2.4***	3	2.73 ± 1.58	10	5.67 ± 0.44

EC50 values are determined by CRE receptor assay, Bmax values by radioligand binding assay and Kd value from one Scatchard analysis; *** p = 0.001

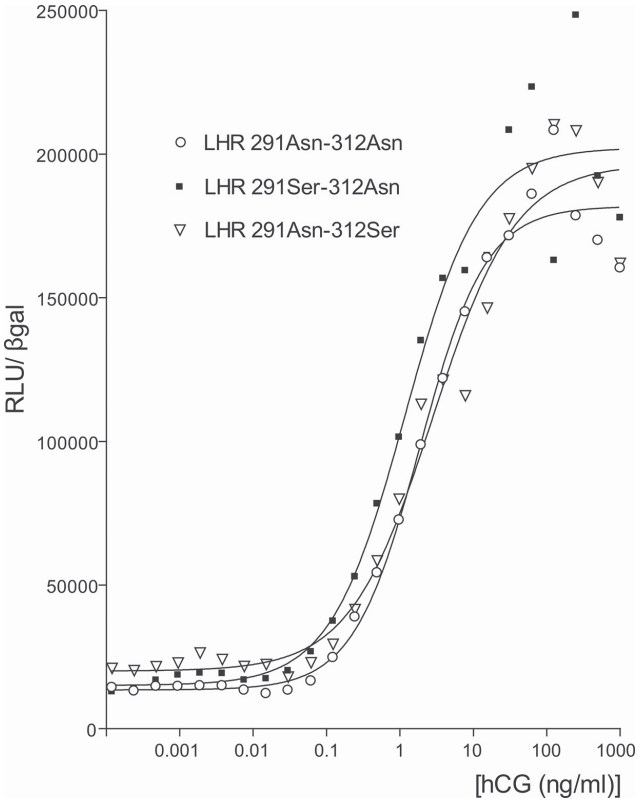

Figure 1. *291Ser-312Asn-LHR is more sensitive to hCG. hCG-induced cAMP response element activation was compared between 291Ser-312Asn (closed squares), 291Asn-312Asn (open circles) and 291Asn-312Ser (open triangles) –LHR variants. HEK293 cells were transfected with the respective expression plasmids in the presence of cAMP-reporter plasmid pCRE$_6$ Lux and SV40β-galactosidase (transfection efficiency). Subsequently, the cells were incubated for 6h with different concentrations of hCG and luciferase activity was measured.*

constructs by [^{125}I]-hCG saturation binding. The Bmax of 291Ser-312Asn-LHR expressing HEK293 cells was not different from 291Asn-312Asn-LHR and 291Asn-312Ser-LHR expressing cells (P=0.69; Table 1). The Kd values for all three LHR constructs from Scatchard analyses were not different (Table 1).

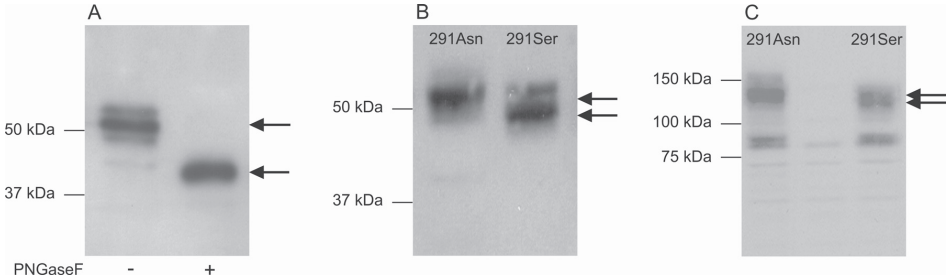

Figure 2. *Changing 291Asn to Ser affects glycosylation of the LHR.*
A. HEK293 cells were transfected with respective ectodomain expression plasmids, whole cell lysates were treated with PNGaseF and subsequently separated using 10% polyacrylamide gels and analyzed by Western blotting with an HA1 antibody. The left and right lanes contain LHR protein before and after PNGaseF treatment, respectively. The numbers on the left indicate apparent molecular mass of protein size markers. After deglycoslyation treatment, the larger band (indicated by upper arrow on right-hand side) of around 51 kDa changes mobility to an apparent molecular mass of around 43 kDa (indicated by lower arrow on right-hand side), indicating that the 51 kDa band is glycosylated. B and C. HEK293 cells transfected with 291Ser LHR constructs (right lane) show increased electrophoretic mobility as compared to 291Asn LHR construct (left lane) by Western blot analysis, both in ectodomain LHR-constructs (B) and in full-length constructs (C; middle lane contains untransfected cells), indicating a decrease of glycan residues.

The Asn (N) at position 291 constitutes a consensus site for N-linked glycosylation: $N^{291}FS$. Presence of carbohydrate chains was shown by deglycosylation with PNGase F (Figure 2A) and endoglycosidase H (not shown) employing truncated LH receptor ectodomain constructs. Substitution of 291Asn by Ser, removing the glycosylation site, results in increased electrophoretic mobility of both truncated (Figure 2B) and full-length (Figure 2C) LH receptor proteins, indicating a change in glycosylation status at 291. The 312Ser/Asn polymorphism is located one amino acid prior to a potential N-linked glycosylation site: $N^{313}KT$. As expected, the amino acid change of Ser to Asn at position 312 did not affect the glycosylation at 313Asn (313N) (results not shown).

Allele frequencies and genotype distributions

The 291Asn/Ser and 312Ser/Asn genotypes were determined in 751 breast cancer patients and in 1806 Caucasian, 110 Chinese and 60 African-American controls. Genotype distributions of both LHR SNPs did not differ from those expected under Hardy Weinberg equilibrium conditions in all cohorts (Table 2).
The minor LHR 291Ser allele has a low frequency ranging from virtually absent in the Chinese and African-American subjects to 6% in Caucasian subjects (Table 2). The minor LHR 312Asn allele showed wide variety in frequency and genotype distribution with an allele frequency ranging from 7% to 72% in the small Chinese and African-American cohorts, respectively (Table 2).

Table 2. *Allele frequencies and genotype distributions for breast cancer patients and non-case cohorts*

	Caucasian		Chinese	African-American
	Breast cancer	Control populations		
		EPOS		
Total number [a]	751	1806	110	60
Median age [b]	60.3	49.5		
Assessment period	1978-1990	1994-1995		
LHR 291Asn/Ser				
Allele frequency [c]	0.05	0.06	0.00	0.01
291Asn/291Asn (%)	673 (89.6)	1580 (89.0)	108 (100)	55 (98.2)
291Asn/291Ser (%)	76 (10.1)	190 (10.7)	0	1 (1.8)
291Ser/291Ser (%)	2 (0.3)	6 (0.3)	0	0
HWE (p) [d]	0.92	0.91	1.0	0.95
Odds ratio LHR 291Ser allele	0.92 (0.71-1.20); p=n.s.			
LHR 312Ser/Asn				
Allele frequency [c]	0.44	0.40	0.07	0.72
312Ser/312Ser (%)	229 (31.8)	628 (35.4)	95 (87.2)	7 (12.3)
312Ser/312Asn (%)	353 (49.0)	862 (48.5)	13 (11.9)	19 (33.3)
312Asn/312Asn (%)	139 (19.3)	286 (16.1)	1 (0.9)	31 (54.4)
HWE (p) [d]	0.89	0.73	0.47	0.15
Odds ratio LHR 312Asn allele	1.15 (1.02-1.30); p=0.03			

[a] Numbers in cells may not add up due to incomplete genotyping

[b] Median age at assessment in years

[c] Frequency of the minor allele is shown

[d] Hardy-Weinberg equilibrium

LHR 291Asn/Ser and 312Ser/Asn genotype and breast cancer risk

Genotype distributions for the 291Asn/Ser SNP and 312Ser/Asn SNPs were compared between breast cancer patients and EPOS controls. The 291Asn/Ser genotype distributions were similar in both Caucasian cohorts (P=0.19; Table 2).

Interestingly, the LHR 312Asn genotype was observed more frequently in breast cancer patients compared to EPOS controls (P=0.03), resulting in an odds ratio (OR) of presence of the LHR 312Asn allele of 1.15 (95% CI 1.02-1.30). To validate this finding, we determined the LHR 312Asn allele frequency in an independent set of 594 breast cancer patients. In this validation set an increased LHR 312Asn allele frequency compared to the EPOS controls was also observed (OR=1.26; 95 % CI 1.10-1.43; P=0.001).

LHR 291Ser and breast cancer clinico-pathological features and survival

Previously we have shown that another LHR variant, LHR insLQ, causes increased LH receptor function and is associated with increased tumor size and poor breast cancer outcome (5). Thus, based on the stimulatory effects of the 291Ser-LHR variant on receptor

sensitivity, this allele was chosen for association analyses in breast cancer patients. Because less than 1% of the patients were homozygous for the LHR 291Ser allele, heterozygous and homozygous carriers of LHR 291Ser allele were combined in the association analyses for sufficient statistical power. Based on the observation that the LHR 312Asn allele was present more frequently in breast cancer patients compared to EPOS controls, differences in patient and tumor characteristics were investigated for this SNP as well.
The LHR 291Ser allele appeared to be differently distributed across the tumor size categories, but the difference was not significant (P=0.06). Furthermore this association was not linear across the successive tumor size categories (P-trend=0.12), suggesting absence of a causal association. Furthermore the LHR 291Ser allele appeared to be associated with progesterone receptor expression (cut-off value >10fmol/mg protein), but this was not significant (P=0.11). The LHR 312Asn allele frequency was slightly higher in the youngest age category (<40 years of age) as compared to patients aged 40 years and older (0.50 vs. 0.44), but this difference was not significant (P=0.17). Both exon 10 SNPs did not show associations with any other patient or tumor characteristics, i.e. menopausal status, nodal involvement, histological grade, estrogen receptor status, history of adjuvant therapy or overall and disease free survival (data not shown).

DISCUSSION

In the present report we present the results of a study of the functional effects, the genotype distribution, and the association with clinico-pathological characteristics and disease outcome of two potentially functional, closely spaced single nucleotide polymorphisms (SNPs) 291Asn/Ser (rs12470652) and 312Ser/Asn (rs2293275) in exon 10 the LHR gene.
Our results indicate that 291Asn in the LH receptor is glycosylated, confirming the data on the pig LH receptor, where it was shown that 36% of all glycan residues in the mature receptor were found at the homologous 291 position (23). The absence of glycan side chains at this position in the 291Ser variant causes a slight decrease in the EC50 of the LH receptor, whereas binding studies, western analysis and signal transduction experiments show that expression levels of both LH receptor variants are comparable. Furthermore, ligand affinity does not seem to be affected as indicated by the absence of a change in the Kd. These results indicate that Asn or Ser at position 291 is involved in the relay of the signal of binding of the hormone to the signal transducing transmembrane domain, rather than binding of the ligand *per se*. Ser at position 277 has been shown to interact with amino acid residues in the second extracellular loop of the LH receptor, showing that this domain of the LH receptor is positioned close enough to allow such interactions (24). Thus, the absence of a glycan side chain at position 291, may facilitate interaction

of the hormone-bound ectodomain with the transmembrane domain of the LH receptor. Changing 291Asn to Ser affects the response of the LH receptor to both ligands. This is not the case when exon 10 is deleted completely, as has been observed in a single patient and in new world monkeys (6, 7).

The presence of an Asn or Ser residue at position 312 did not affect binding or signal transduction *in vitro* at all. It will be interesting to see whether the glycosylation status of the receptor can partially explain the effect of the deletion of exon 10, or that whether other functions of this 27 amino acid stretch are involved.

The LHR 291Asn/Ser and 312Ser/Asn SNPs result from an A/G change at nucleotide 872 and 935, respectively. The observed frequencies for both alleles in this study were in accordance with the reported frequencies for both SNPs (http://SNPper.chip.org; http://gvs.gs.washington.edu/GVS/). The closely spaced SNPs are highly linked, but poorly correlated (r^2=0.05) due to different minor allele frequencies.

Genetic variation in allele frequencies between ethnic groups may widely vary and may affect disease-related outcomes (reviewed in (25 and 26)). In the present study three different ethnic groups, i.e. Caucasians, Chinese and African-American subjects, were included in genotype analyses. Interestingly, the LHR 291Ser allele is virtually absent in the Chinese and African-American subjects, suggesting that the LHR 291Asn/Ser SNP represents a relatively recent Caucasian mutation. The frequency of the LHR 312Asn allele also varied widely, from 72% in African-Americans to 7% in Chinese with an intermediate frequency in the Caucasian women (40-44%). Prevalence, clinical presentation and outcome of breast cancer differ between different ethnic groups (13, 27, 28). Breast cancer incidence is much lower in Chinese women as compared to Caucasian women (29). African-American women show a lower incidence as compared to Caucasian women, but this changes after menopause. Furthermore, they have much higher overall mortality (30). However, conclusions concerning the ethnic diversity in frequency of the LHR variants and the occurrence or clinical phenotype of breast cancer in ethnic groups has to await much more detailed studies using larger ethnically diverse breast cancer cohorts.

Interestingly, the LHR 312Asn allele constitutes a risk allele for breast cancer since it was more often observed in two independent sets of breast cancer patients (i.e. 721 patients v.s. 594 patients mainly from the South Western part of The Netherlands, as compared to 1776 Caucasian EPOS controls). This results in a moderate, but statistically significant, increased risk of breast cancer of 15% tot 26% (OR=1.15 (95% CI 1.02-1.30); P=0.03 and OR=1.26 (95 % CI 1.10-1.43); P=0.001 respectively. Interestingly, the effect size is similar to the increased breast cancer risk following ever use vs. never use of oral contraceptives (31). Furthermore, the trend that was observed of a lower LHR 312Asn allele in the oldest patient categories may support the suggestion that LHR 312Asn is a risk allele. However, these results need to be validated in independent studies. No significant associations of

the 312Ser/Asn SNP with clinico-pathological characteristics or outcome were observed. Since we did not observe functional effects of the 312Asn LHR variant in our transfection studies, linkage with another, perhaps still unknown, functional SNP that drives the association cannot be excluded. Linkage analysis in Haploview, using a minimum r^2 of 0.8, shows linkage of the 312Ser/Asn SNP to rs11125179, a silent SNP (355Asp/Asp) in exon 11 near the intron/exon boundary, as well as linkage to two intron SNPs: rs2349101 and rs17326251 about 1000 bp upstream of exons 10 and 11 respectively. One of these SNPs may affect protein expression or function, possibly through mRNA production or stability (32). The absence of an association with any of the patient and tumor characteristics or with overall or disease free survival was unexpected in view of the increased sensitivity of the 291Ser LHR protein *in vitro*. This is most likely caused by insufficient power to detect an *in vivo* effect in view of the very low LHR 291Ser allele frequency. Compensatory adjustments of LH production in the hypothalamic-pituitary-ovarian axis in premenopausal life masking a subtle functional effect of the polymorphism cannot be excluded either

In conclusion, we have shown that the 291Ser LHR variant results in increased LHR sensitivity, but the presence of a 291Ser LHR allele was not associated with any breast cancer patient characteristic. Interestingly, when comparing breast cancer patients vs. non-case controls, a slightly increased LHR 312Asn allele frequency was observed in breast cancer patients. These results suggest that, either or not via linkage with a functional polymorphism, the LHR 312Asn allele may be a risk allele for breast cancer development. This needs to be validated in a large case control study.

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CHAPTER SIX

Associations of CYP19 and ESR1 SNPs with metastasis-free survival and HER-2/neu amplification in premenopausal breast cancer

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ABSTRACT

Estrogen signaling plays a major role in the development and progression of breast cancer. The purpose of the study was to analyze two candidate SNP, in the CYP19 gene encoding aromatase a key enzyme in estrogen production, and in the ESR1 gene, the final mediator of estrogenic action in the breast.

Genotypes of the 1531T/C SNP in the 3'-UTR of CYP19 and the -397T/C SNP in intron 1 of the ESR1 gene, as well as associations with clinico-pathological characteristics and disease outcome, were studied in 687 breast cancer patients with long follow-up. A case-control study, including 202 prospectively collected cases and 6208 controls, was added to investigate risk.

Neither of the SNPs was associated with breast cancer risk in these predominantly Caucasian populations. Patients with the ESR1 -397CC genotype showed significantly decreased prevalence of HER-2/neu amplification (P=0.02). Premenopausal patients with the combination of both genotypes showed the longest MFS (HR=0.33; 95% CI 0.16-0.65; P=0.001), independent of other prognostic factors and independent of HER-2/neu status (HR=0.36; 95% CI 0.17-0.76; P=0.007).

Our results suggest that the CYP19 1531C allele and ESR1 –397CC genotype are independently associated with longer MFS in premenopausal breast cancer patients. When (prospectively) validated, the CYP19 1531T/C and ERS1 –397T/C genotypes may provide additional prognostic information for premenopausal breast cancer patients. In the future these genotypes may possibly contribute to the development of tailored endocrine breast cancer treatment.

INTRODUCTION

Breast cancer is the most frequent occurring malignancy in females, accounting for almost one third of incident cases of cancer in women in industrialized countries (1). It is a complex disease in which a strong interplay between genetic and environmental factors influences susceptibility, clinical phenotype, prognosis and response to treatment (2). Over a century a growing body of epidemiological and experimental evidence has shown a major contribution of estrogens to the development and progression of breast cancer (as reviewed in (3, 4), both in premenopausal (5) and postmenopausal women (6).
About 25% of patients is diagnosed at premenopausal age, a known poor prognostic factor (7). In premenopausal women one of the rate-limiting steps in ovarian estrogen production is the aromatization of androgen precursors, carried out by the granulosa cell enzyme aromatase. In postmenopausal women adipose and muscle tissue are the main sites for aromatase activity, converting circulating androgen precursors arising predominantly from the adrenals. Polymorphic variation in the gene encoding aromatase, i.e. CYP19 on chromosome 15q21.2, may affect aromatase activity through several mechanisms. Interestingly, several studies have reported associations of a T/C SNP in CYP19 (1531T/C (rs10046), 1531 basepairs downstream from the translation start site in the 3'-UTR) with breast cancer risk (8, 9), as well as with estrogen production in postmenopausal women (10-12). Estrogen action is mediated by two different estrogen receptors, i.e. ESR1 (ER-α) and ESR2 (ER-β). The role of estrogen signaling through the latter in normal and malignant breast tissue has to be solved, whereas, animal studies indicate a clear-cut role for ESR1 in proliferation and differentiation of the mammary gland (13). Moreover, ESR1 expression has clearly been related with prognosis of breast cancer (14) as well as with risk (15). Interestingly, polymorphic variation in the ESR1 gene, located on chromosome 6q25.1, has been associated with steroid hormone-dependent clinical outcome. Several reports have studied an ESR1 SNP in intron 1 (also named intervening sequence 1: IVS1) -397T/C (rs2234693; also referred to as the PvuII restriction site) and the linked -354A/G SNP (rs9340799; also named after the XbaI restriction site). The majority of studies, predominantly performed in Caucasian subjects, indicate a decreased estrogenic action associated with the ESR1 -397T allele, and consequently an increased activity for the -397C allele. This has been concluded by us from associations in The Rotterdam Study (16), between ESR1 -397T and decreased bone mineral density plus increased risk of osteoporotic fracture (17, 18) and increased risk of myocardial infarction and ischemic heart disease in postmenopausal women (19). Moreover, *in vitro* assays indicate decreased transcriptional activity for the ESR1 -397T variant (20). On the other hand, the ESR1 -397T allele has been associated with increased risk of steroid-hormone related cancer such as postmenopausal breast cancer (21, 22), endometrial cancer (23) and prostate cancer (24).

Estrogen receptor signaling has been shown to downregulate HER-2/neu expression at mRNA and protein level *in vitro* (25). In breast cancer an inverse relationship between HER-2/neu amplification and expression of ESR1 has been observed (26). Recently, HER-2/neu was also suggested to be associated with aromatase activity (27).
In the current study we analyzed in a retrospectively collected cohort of 687 Dutch patients the association of two candidate SNPs, CYP19 1531T/C and ESR1 -397T/C with risk and clinical phenotype of breast cancer, including HER-2/neu amplification and clinical outcome.

MATERIALS AND METHODS

Study population

Breast cancer cohort

Complete follow-up is available for 751 of the samples (28), of which 2 have unknown estrogen receptor status. Samples with double primary tumors (n=14) were excluded. Conclusive genotype results for both SNPs were available for 687 tumor samples. Thus, association studies were performed on 687 retrospectively collected tumor samples. Study design was approved by the Medical Ethics Committee of the Erasmus MC, Rotterdam, The Netherlands. Median age in these patients is 57.0 (range: 22-90) years, the median follow-up period from primary surgery of patients alive is 122 months (range: 3-255 months). These patients mainly arise from the South Western part of The Netherlands. HER-2/neu amplification is known for 80% of the samples (29), levels are represented by mean ± SD.

Rotterdam Study

The rationale and design of the Rotterdam Study, a prospective population based cohort study of individuals aged 55 years and over, have been described previously (16). Breast cancer cases were identified as described previously (30). Genotype results were conclusive for 202 of the 258 prospectively collected breast cancer cases, which were used for the nested case-control study. Median age in these cases is 67.4 (range: 55-93) years. A total of 6208 controls with conclusive genotype results were available with median age 68.5 (range: 55-99) years.

Genotype analyses

High molecular weight genomic DNA was used as a template for PCR amplification. DNA was extracted from flash frozen tumor samples, stored in liquid nitrogen as described previously (28). Primary breast cancer specimens, from which the DNA was obtained

for genotyping, contain a relatively high proportion of non-tumor tissue. This ensures accurate genotyping, irrespective of the possible loss of heterozygosity (LOH) that may occur in tumor tissue (31). Furthermore, examining Hardy-Weinberg equilibrium can test for possible LOH. For subjects from the Rotterdam Study genomic DNA was isolated from peripheral leucocytes using standard procedures (19). Primer sequences used for genotyping of the 1531T/C SNP in the CYP19 gene, were AAGGATGGATGATTTG-TATGTGAA (forward) and GACAGGTGTCTGGAACACTAGAGAAG (reverse). Used probes (with SNP underlined) were 5'-VIC- CAGAGTGGGTACTGAC (antisense) and 5'-FAM-CCAGAGTAGGTACTGAC (antisense). Primers used for amplification of the fragment of intron 1 including the –397T/C SNP in the ESR1 gene were CTGTGTTGTC-CATCAGTTCATCTG (forward) and ACTCAGGGTCTCTGGGAAACAG (reverse). Used probes were 5'-VIC-CCAGCTGTTTTATG (sense) and 5'-FAM- CCAGCCGTTT-TAT (sense). Primer and probe sequences were optimized using the SNP assay-by-design service of Applied Biosystems, Perkin Elmer, Nieuwerkerk aan den IJssel, The Netherlands), for details see http://store.appliedbiosystems.com. Reactions were performed on the Taqman Prism 7900HT 384 wells format.

Statistical analysis

Pearson's χ^2 analysis was used to test for independence of the alleles (Hardy-Weinberg equilibrium, HWE), to compare genotype distributions in breast cancer patients and control populations and to investigate associations between the various patient and tumor characteristics and the CYP19 1531T/C genotype and ESR1 -397T/C genotype. Univariate and multivariable overall (endpoint: death) and metastasis-free (endpoint: distant metastasis) survival analyses (OS and MFS) were carried out using Cox proportional hazards regression. The assumption of proportional hazards was investigated using a test based on the Schoenfeld residuals (32). Hazard ratios (HR) are presented with their 95% confidence interval (CI). To test the null hypothesis that HRs were similar across genotypes, z-values were generated. CYP19 1531T/C and ESR1 -397T/C genotypes were tested using the likelihood ratio test associated with the Cox regression analysis. MFS and OS probabilities were calculated using the actuarial method of Kaplan-Meier (33). Survival probabilities across CYP19 1531T/C and ESR1 –397T/C genotypes were compared using the log-rank tests for equality of survival functions, if not stated otherwise, using ESR1 –397TT and CYP19 1531TT genotypes as reference. Interactions between genotypes and prognostic factors were assessed by including interaction terms. Interaction was only observed for both SNPs with menopausal status, therefore we analyzed the association between genotypes and survival in menopausal subgroups. In the premenopausal subgroup associations with the CYP19 1531T/C SNP suggested a dominant effect for presence of the CYP19 1531C allele, which was tested using χ^2 analysis comparing carriers of CYP19 1531C vs. CYP19 1531TT. The analyses across the ESR1 -397T/C

genotype suggested a recessive effect for the ESR1 –397CC genotype, tested by χ^2 analysis comparing the ESR1 –397CC genotype vs. ESR1 -397TC and -397TT. After identification of the main effects of the SNPs, interaction was investigated in the premenopausal patients by including interaction terms of selected CYP19 1531T/C and ESR1 -397T/C genotypes. Non-significant interactions were not included in the model. In multivariable analysis, Cox proportional hazard models for MFS were applied to test the genotype variables added to traditional factors using a forward stepwise model. The multivariable model included age, menopausal status when appropriate, nodal status, differentiation grade, tumor size, estrogen receptor status and adjuvant therapy. HER-2/neu-genotype interactions were also assessed by including interaction terms in the multivariable model. All computations were carried out using the STATA statistical package, version 9.2 (Stata Corp., College Station, TX, USA). Statistical significance was assumed at $P \leq 0.05$, P-values are two-tailed and relate to data during the total period of follow-up.

RESULTS

CYP19 1531T/C and ESR1 -397T/C genotyping and breast cancer risk

The allele frequencies and genotype distributions in breast cancer cases were not significantly different, either in cases from the breast cancer cohort or from the Rotterdam Study, compared to those in controls (Table 1). The genotype distributions in all cohorts were in accordance with those expected under Hardy Weinberg conditions (Table 1). CYP19 1531C and ESR1 -397C were identified as the minor alleles. We conclude from this that the CYP19 1531T/C and ESR1 -397T/C genotypes are not associated with risk of breast cancer development in our study population.

Associations with patient and tumor characteristics

Patient and tumor characteristics for 687 samples with follow-up were related to candidate SNPs in CYP19 and ESR1, involved in estrogen signaling, i.e. the CYP19 1531T/C and ESR1 -397T/C genotypes (see Supplementary table). The distribution of the ESR1 -397T/C genotype across the age decades seemed reversed in the youngest age group (<40 yrs), showing the lowest frequency of the ESR1 -397TT genotype, as compared to the perimenopausal age group (50-60 yrs), which had the lowest frequency of the ESR1 -397CC genotype. Histological grade appeared to be differently distributed across the CYP19 1531T/C genotype, suggesting that the CYP19 1531TT genotype was most frequent in tumors with poor grade, but the overall difference was not significant (P=0.13). Additionally, the candidate SNPs were analyzed for expression of the estrogen receptor (ER) and progesterone receptor (PR) and amplification of HER-2/neu, of which the latter two are downstream effects of ER signaling. Neither CYP19 1531T/C nor ESR1 -397T/C

Table 1. *Comparison of genotype distributions and allele frequencies between case and control population subjects*

	Rotterdam Study			Breast cancer cohort	
	Controls	Cases	P-value[a]		P-value[b]
Total number	6208	202		687	
Median age	68.5	67.4		57.0[c]	
Assessment period	1990-1993	1990-1993		1978-1990	
CYP19 1531T/C					
Allele frequency 1531C (%)[d]	49.5	45.0	0.09	48.5	0.52
Genotypes					
TT, N (%)	1593 (25.7)	57 (28.2)		192 (28.0)	
TC, N (%)	3084 (49.7)	108 (53.5)		323 (47.0)	
CC, N (%)	1531 (24.7)	37 (18.3)	0.12	172 (25.0)	0.34
HWE (P-value)	0.62	0.26		0.12	
ESR1 –397T/C					
Allele frequency -397C (%)[d]	46.4	50.2	0.14	48.2	0.32
Genotypes					
TT, N (%)	1789 (28.8)	50 (24.8)		192 (28.0)	
TC, N (%)	3081 (49.6)	101 (50.0)		333 (48.5)	
CC, N (%)	1338 (21.6)	51 (25.2)	0.31	162 (23.6)	0.47
HWE (P-value)[e]	0.86	0.99		0.45	

[a] Nested case-control study and [b]Breast cancer cohort vs. controls: differences between allele frequencies and genotype distributions tested using χ^2 analysis

[c] Median age at diagnosis in years

[d] Frequency of the minor allele is shown (both C-allele)

[e] Hardy-Weinberg Equilibrium

showed an association with ER expression (P= 0.76 and 0.89, respectively). The CYP19 1531T/C genotype was not associated with PR expression (P=0.56). PR protein expression across the ESR1 -397T/C genotype suggested a linear or additive genetic effect of increased PR expression associated with the –397C allele, but the overall difference was not significant (P=0.16). Interestingly, of patients with HER-2/neu amplification, only 18 (14.5%) had the -397CC genotype, whereas in patients with no detectable HER-2/neu amplification a larger part (n=103, 24.2%) had the -397CC genotype. Indeed, when comparing ESR1 -397CC vs. -397TC and TT, the ESR1 -397CC genotype was associated with a lower prevalence of HER-2/neu amplification (recessive model: P=0.02).

Next, expression levels of ER, PR and HER-2/neu across the genotypes were investigated. Levels of ER and PR expression were not different across the genotypes. Respective ER expression levels (median and range: for CYP19 1531TT are: 61.5 (0-1131), 1531TC: 65.0 (0-1132) and 1531CC: 55.5 (0-1822) fmol/mg protein. Median and range values for the ESR1 –397T/C genotype are respectively -397TT: 61.5 (0-1739), -397TC: 56.0 (0-1225) and -397CC: 68.5 (0-1123) fmol/mg protein. Levels of PR expression across the CYP19

1531T/C genotype are: 48.0 (0-1207), 1531TC: 39.0 (0-1620) and 1531CC: 46.0 (0-1710) fmol/mg protein. Finally median and range values for PR expression across the ESR1 -397T/C genotype are respectively -397TT: 44.5 (0-1710), -397TC: 38.0 (0-1041) and -397CC: 50.0 (0-1757) fmol/mg protein. As expected the level of HER-2/neu amplification was lower in patients with the ESR1 -397CC genotype vs. the -397TC and TT genotypes (1.48 ± 1.62 vs. 1.83 ± 2.30). The difference was borderline significant (P=0.06).

Metastasis-free and overall survival

No significant associations were observed in the association analyses of the CYP19 1531T/C or ESR1 -397T/C genotype with metastasis-free (MFS) or overall survival (OS) (Supplementary Table). HER-2/neu amplification was also not significantly associated with MFS (HR=1.19; 95% CI 0.89-1.59; n.s.) or OS (HR=1.05; 95% CI 0.80-1.38; n.s.).

Stratified analyses in clinically relevant subgroups

In the survival analyses according to CYP19 1531T/C and ESR1 -397T/C genotype, there were no interactions between either SNP and ER, tumor size and nodal status. Interactions were observed for both SNPs with menopausal status.

HER-2/neu amplification has been related to ER expression. As expected HER-2/neu amplification was observed more frequent in tumor samples with ER-negative breast cancer as compared to samples with ER-positive disease (36.4% vs. 17.9%; P<0.001). After stratification for ER-expression, in the ER-positive subgroup a significant association between the ESR1 -397CC genotype and decreased prevalence of HER-2/neu amplification was observed. Of patients with HER-2/neu amplification, only 8 (11.0%) had the -397CC genotype, vs. in patients with no detectable HER-2/neu amplification 82 (103: 24.4%) had the -397CC genotype (recessive model: P=0.01). No difference was observed in the ER-negative subgroup.

HER-2/neu amplification was observed in 25.2% of premenopausal patients vs. 20.9% in postmenopausal patients (n.s.). After stratification for menopausal status, in premenopausal patients a highly significant association between ESR1 -397CC genotype and decreased prevalence of HER-2/neu amplification was observed (recessive model: P= 0.001; Table 2). No difference was observed in postmenopausal patients.

Considering the biological differences in etiology and phenotype between pre- and postmenopausal breast cancer and the observed interactions of the SNPs and menopausal

Table 2. *Distribution of HER-2/neu amplification in 214 premenopausal patients.*

	ESR1 –397T/C genotype		
HER-2/neu amplification, n (%)	ESR1 –397TT or –397TC	ESR1 –397CC	P-value[a]
Negative	120 (75.0)	40 (25.0)	
Positive	51 (87.9)	3 (12.1)	0.001

[a] Fisher's exact test

status, we next explored associations of the genotypes with metastasis-free survival after stratification for menopausal status. In the premenopausal patients both CYP19 1531TC and 1531CC genotypes showed longer MFS as compared to the 1531TT genotype (CYP19 1531TC: HR=0.59; 95% CI 0.38-0.91; P=0.02 and 1531CC: HR=0.66; 95% CI 0.40-1.08; P=0.10 vs. reference CYP19 1531TT: HR=1.00). Carriers of the CYP19 1531C allele (i.e. 1531CC and 1531CT genotype) were combined according to the dominant model, showing a significant improved MFS as compared to the CYP19 1531TT genotype, as shown in Table 3. Kaplan-Meier curves for MFS are shown in Figures 1A and 1B. In multivariable analyses, including adjustment for age, nodal status, differentiation grade, tumor size, estrogen receptor status and adjuvant therapy, the association of carriership of the CYP19 1531C allele and improved MFS was significant (HR=0.56; 95% CI 0.37-0.83; P=0.005; Table 3). When the continuously timevarying effect for ER was included in the model the proportional hazards assumptions were not violated, this did not influence the estimates for the SNPs and HER-2/neu amplification. In multivariable analysis, carriership of the CYP19 1531C allele was significantly associated with longer OS in premenopausal patients in the (HR=0.64; 95% CI 0.42-0.98; P=0.04). In the postmenopausal patients no associations between the CYP19 1531C allele and MFS or OS were observed (data not shown).

When we stratified for menopausal status in survival analyses with the ESR1 -397T/C genotype, the results indicated a recessive effect for the ESR1 -397CC genotype (-397TT: HR= 1.00; -397TC: HR= 1.08; 95% CI 0.69-1.67; P=0.74; -397CC: HR=0.56; 95% CI 0.31-1.04; P=0.065). Indeed, analyzing the -397CC genotype vs. -397TC and -397TT (i.e. carriers of the -397T allele), according to the recessive model, showed resulted in a significant association of the -397CC genotype with longer MFS (see Table 3). Kaplan-Meier survival curves for MFS in premenopausal patients according to ESR1 -397T/C genotype are shown in Figure 1C and for -397CC vs. -397TC and -397TT in Figure 1D.

In the multivariable model, adjusting for traditional prognostic factors age, nodal status, differentiation grade, tumor size and estrogen receptor status and adjuvant therapy, the -397CC genotype proved to be an independent prognostic factor (HR=0.55; 95% CI 0.32-0.95; P=0.03; Table 3). The association with OS was not significant (HR=0.71; 95% CI 0.42-1.20; P=0.20). Again in the postmenopausal subgroup with the -397CC genotype no associations with MFS nor OS were observed (data not shown).

Combined effect of ESR1 and CYP19 genotypes in premenopausal subgroup

Thus, significant associations with improved MFS were observed in premenopausal patients with either the ESR1 -397CC genotype or carrying the CYP19 1531C allele (i.e.1531TC and 1531CC). Since the actions of aromatase and ESR1 cooperatively result in estrogen signaling, the combined effect of both genotypes was further explored. The presence of either the CYP19 1531C allele or ESR1 -397CC genotype was associated with

Table 3. *Cox univariate and multivariable analysis for metastasis-free survival in 268 premenopausal breast cancer patients*

	patients		Univariate analysis			Multivariable analysis		
Factor of base model:	No.	%	HR	95% CI	P	HR	95% CI	P
Age (years)								
≤40	67	25.0	1.00		0.05	1.00		0.02
40-50	160	59.7	0.75	0.49-1.14		0.71	0.46-1.10	
50-60	41	15.3	0.45	0.22-0.88		0.39	0.19-0.80	
Nodal status								
Negative	126	47.0	1.00			1.00		
Positive	142	53.0	2.36	1.57-3.56	<.001	3.60	1.87-6.93	<.001
Histological grade								
Poor	147	54.9	1.00		0.01	1.00		0.03
Unknown	59	22.0	0.59	0.41-1.15		0.68	0.41-1.15	
Well/moderate	62	23.1	0.51	0.30-0.86		0.52	0.30-0.89	
Tumor size								
≤ 2cm	113	42.2	1.00			1.00		
> 2cm	155	57.8	2.50	1.62-3.85	<.001	1.96	1.24-3.10	0.004
Estrogen receptor status								
Negative	80	29.9	1.00			1.00		
Positive	188	70.2	0.83	0.55-1.24	0.36	0.90	0.58-1.38	0.62
Adjuvant therapy								
No	151	56.3	1.00			1.00		
Yes[a]	117	43.7	1.70	1.16-2.48	0.33	0.53	0.29-0.96	0.04
Factors added to the model:								
Genotype								
CYP19 1531T/C								
1531TT	76	28.4	1.00					
1531TC/ CC	192	71.6	0.61	0.41-0.91	0.01	0.56	0.37-0.83	0.005
ESR1 -397T/C								
-397TT/ TC	206	76.9	1.00					
-397CC	62	23.1	0.54	0.31-0.91	0.02	0.55	0.32-0.95	0.03
CYP19 1531T/C and ESR1 -397T/C								
1531TT and -397TT/ TC	63	23.5	1.00		0.004			0.002
1531TC/ CC and -397TT/ TC or								
1531TT and -397CC	156	58.2	0.63	0.42-0.96		0.59	0.39-0.91	
1531TC/ CC and -397CC	49	18.3	0.35	0.18-0.68		0.33	0.16-0.65	
	268							
HER-2/neu amplification								
Negative	160	74.8	1.00			1.00		
Positive	54	25.2	1.72	1.11-2.66	0.02	1.51	0.96-2.36	0.08
	214							

[a] of whom 108 (40%) patients received adjuvant chemotherapy, 3 (1%) endocrine therapy and 6 (2%) both.

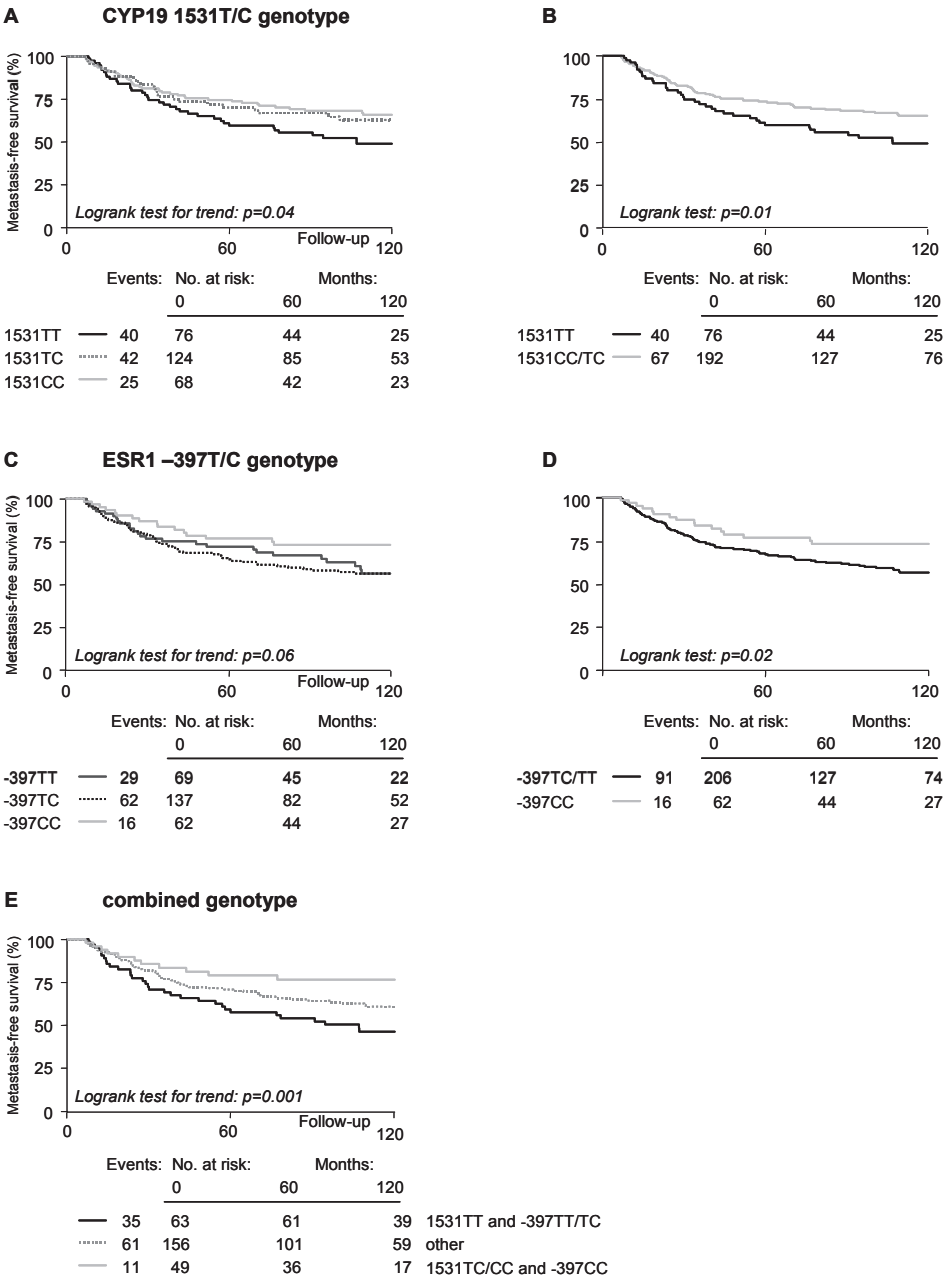

Figure 1. *Kaplan-Meier curves for metastasis-free survival in premenopausal patients. A. MFS according to CYP19 1531T/C genotype (CYP19 1531TT, 1531TC, 1531CC). B. MFS for carriers of CYP19 1531C allele, i.e. CYP19 1531TC/CC vs. non-carriers (1531TT). C. MFS according to ESR1 –397T/C genotype (ESR1 -397TT, -397TC, -397CC). D. MFS for patients with ESR1 –397CC vs. –397TC/TT. E. MFS for carriers of both positive genotypes, i.e. CYP19 1531TC/CC and ESR1 –397CC; either, CYP19 1531CC and ESR1 –397CC or CYP19 1531TC/CC and ESR1 –397TC/TT vs. CYP19 1531TT and ESR1–397TC/TT.*

longer MFS, showing a HR of 0.63 (95% CI 0.42-0.96; P=0.03). A HR of 0.35 (95% CI 0.18-0.68; P=0.002) was observed for these premenopausal patients with both carriership of the CYP19 1531C allele and the ESR1 -397CC genotype vs. patients with neither the CYP19 1531C allele nor ESR1 -397CC genotype (Figure 1E and Table 3). No significant interaction between the two genotypes was observed. A similar association was seen for OS with a HR of 0.48 in carriers of both genotypes (95% CI 0.24-0.94; P=0.03).
In multivariable analysis including the traditional prognostic factors and adjuvant therapy, this combined genotype showed an independent association with MFS (Table 3). Finally HER-2/neu amplification was added to the multivariable model, the estimates for HRs of all prognostic factors in the model were similar. In the multivariable model, including HER-2/neu amplification, the associations between MFS and the combined genotype remained. Presence of either the CYP19 1531C allele or ESR1 -397CC genotype showed a HR of 0.54 (95% CI 0.34-0.85; P=0.008). Having both the CYP19 1531C allele and ESR1 –397CC genotype revealed a HR of 0.36 (95% CI (0.17-0.76; P=0.007) for MFS and a borderline significant association with OS (HR=0.52; 95% CI 0.25-1.07; P=0.07).

DISCUSSION

Polymorphic variation in genes involved in estrogen signaling may partly explain differences in susceptibility, clinical presentation and outcome of breast cancer between individuals or populations (34). Aromatase and ESR1 are involved in the estrogen signaling pathway and are clear targets of breast cancer therapy. In the current study we have evaluated associations between candidate SNPs in the 3'-UTR of CYP19 and intron 1 of the ESR1 gene and breast cancer in a retrospectively collected cohort with long follow-up. Previous reports on these SNPs have primarily focused on risk of breast cancer development, most often in postmenopausal patients. New findings in our study are significant associations of the ESR1 -397CC genotype and carriership of the CYP19 1531C allele with longer metastasis-free survival (MFS) in premenopausal breast cancer patients. In addition, we are the first to show that the ESR1 -397CC genotype is significantly associated with lower prevalence of HER-2/neu amplification.
Similar to other studies (10, 11) we did not observe an association between the CYP19 1531T/C SNP and breast cancer risk, neither in the retrospective cohort, nor in the prospectively collected nested case-control study. A positive association of the CYP19 1531T allele with breast cancer risk was previously reported in two Caucasian cohorts including predominantly postmenopausal patients (8, 9). The ESR1 -397T/C SNP was not identified as a possible risk genotype for breast cancer either. Previous reports on the ESR1 -397 T/C SNP and breast cancer showed inconclusive results when studying risk in all age groups (22) or in postmenopausal patients only (21, 35). Explanations for inconclusive

results from different studies may be due to differences in technical approach of genotyping, in ethnicity of study populations, cohort size and so far unknown, differences in the interaction of genetic with environmental factors. A possible limitation to our study may be that we did not apply haplotype analyses. However, previously published studies suggest that the CYP19 1531T/C SNP is functional (8) and tags the most common haplotype in the Caucasian population, which was associated with increased estradiol levels (11). In addition, a polymorphic variant comprised of a $(TTTA)_n$ repeat, that has been extensively studied in relation to breast cancer risk, has been shown to be strongly linked to the CYP19 1531T/C SNP (8, 12, 36, 37). Although it cannot be excluded that the ESR1 -397T/C SNP is merely linked to another polymorphism responsible for the functional effect, this is unlikely since extensive association studies have not shown stronger associations with other polymorphisms and it does not appear that there are other so far unrecognized polymorphic sequence variations in the vicinity (38).

In the current analyses neither of both SNPs showed a significant association with ER or PR expression. Previous studies relating the ESR1 –397 T/C SNP to receptor expression have shown inconsistent results (39-41). The ESR1 -397CC genotype showed a clear association with decreased prevalence of HER-2/neu amplification. An inverse relationship between ER action and HER-2/neu expression has been shown both *in vitro* and *in vivo* (25, 26). After stratification for ER expression, the association between -397T/C genotype and HER-2/neu amplification only remained in ER-positive tumors. This may suggest a causal relationship between the ESR1 -397C variant and HER-2/neu amplification, although further investigation is needed.

In premenopausal patients we observed a modest, but significant association between improved breast cancer MFS and carriership of the CYP19 1531C allele, independent of traditional prognostic factors (HR=0.61; 95% CI 0.41-0.91; P=0.01). This finding appears to agree with previous reports indicating that the counterpart allele, CYP19 1531T, is a risk allele for large, late stage breast tumors (8). So far, one report has investigated breast cancer overall survival (OS) associated with CYP19 polymorphisms, the authors did not find an association with for the 3'-UTR 1531T/C SNP (42). A significant association with improved MFS was also observed in the premenopausal subgroup for the ESR1 -397CC genotype, showing an almost 50% reduced risk of metastasis (HR=0.54; 95% CI 0.31-0.91; P=0.02). This association was independent of traditional prognostic factors age, nodal status, differentiation grade, tumor size and estrogen receptor status, and adjuvant therapy. The majority of studies performed in a predominantly postmenopausal population, comparable to the current study cohort regarding ethnic background, indicate increased estrogenic activity associated with the ESRC –397C allele (17-19). This appears to contradict our results, since increased ESR1 signaling is hypothesized to increase proliferation of breast tumors (43). On the other hand, previous association studies have indicated that the ESR1 -397C allele may be protective for development of

steroid-hormone related cancer (22-24). The exact effects of the ESR1 -397T/C genotype in premenopausal breast cancer remain to be elucidated.

The actions of aromatase and ESR1 cooperatively result in estrogen signaling. Polymorphisms in ESR1 and CYP19 have been shown to interactively influence bone mineral density, an indirect parameter of estrogen exposure (44). The greatest reduction of risk, i.e. 40% vs. 80% metastasis-free after ten years of follow-up, was observed in premenopausal patients with the ERS1 -397CC genotype and carrying the CYP19 1531C allele, showing a HR of 0.35 (95% CI 0.18-0.68); P=0.002). No evidence for interaction between the genotypes was observed, however it is possible that the power of our study was too low given the small number of premenopausal patients with the specific genotypes.

The question remains how the CYP19 1531T/C SNP can possibly influence premenopausal breast cancer outcome? The CYP19 gene consists of at least 10 different first exons (45), each associated with specific 5' promoter regions that are tissue-specifically regulated by distinct endocrine and paracrine signals. A positive correlation between local aromatase expression and malignant phenotype of breast carcinoma has previously been described, irrespective of menopausal status (46). An association has been shown between the CYP19 1531T allele and higher aromatase mRNA levels and increased use of a breast cancer specific promoter, suggesting that polymorphic variation in the 3'-UTR may influence promoter regulation (8). In addition 3'-UTR gene variants have been shown to influence mRNA stability as reviewed in (47). It can be hypothesized that polymorphic variation in the CYP19 gene, for instance the CYP19 1531T/C SNP, may tissue-specifically alter aromatase expression, either in the ovaries, in normal breast and/or in malignant breast tissue. If androgen precursors are not limiting, then increased aromatase expression will result in increased estrogen production. Recently an association between CYP19 genetic variation and age at onset of menarche, a reproductive feature positively correlated with estrogen exposure during puberty (48), was described (49). This observation indicates that polymorphic variation in the CYP19 gene may upregulate ovarian aromatase activity resulting in increased estrogen production.

In conclusion our results suggest that the ERS1 -397CC genotype is associated with decreased prevalence of HER-2/neu amplification, most obvious in the premenopausal subgroup. More important, the ERS1 -397CC genotype and carriership of the CYP19 1531C allele are associated with longer MFS in premenopausal breast cancer patients, independent of classical prognostic factors. After adjustment for classical risk factors and HER-2/neu amplification in the multivariable model, the combined genotype, i.e. carriership of the CYP19 1531C allele and ESR1 -397CC genotype, showed an independent association with longer MFS. Moreover, the effect on MFS, as reflected by the hazard ratio, was superior to what could be predicted based on absence of HER-2/neu amplification. When validated by independent studies, the observed results raise the possibility that analysis of the ERS1 -397T/C and CYP19 1531T/C genotypes may provide additional

prognostic information for premenopausal breast cancer patients. In the future, these candidate genes may provide predictive value for tailored treatment of premenopausal patients.

Supplementary Table. *Baseline characteristics in 687 patients with per ESR1 –397T/C and CYP19 1531T/C genotypes*

		CYP19 1531T/C				ESR1 –397T/C			
Feature	n (%)	TT	TC	CC	P[a]	TT	TC	CC	P[a]
Breast cancer cases	687 (100)	192 (27.9)	323 (47.0)	172 (25.0)		192 (27.9)	333 (48.5)	162 (23.6)	
Age in years									
<40	68 (9.9)	15 (22.1)	34 (50.0)	19 (27.9)		12 (17.7)	39 (57.4)	17 (25.0)	
40-50	170 (24.8)	50 (29.4)	72 (42.4)	48 (28.2)		48 (28.2)	82 (48.2)	40 (23.5)	
50-60	149 (21.7)	36 (24.2)	83 (55.7)	30 (20.1)		51 (34.2)	72 (48.3)	26 (17.5)	
60-70	166 (24.2)	48 (28.9)	77 (46.4)	41 (24.7)		50 (30.1)	71 (42.8)	45 (27.1)	
> 70	134 (19.5)	43 (32.1)	57 (42.5)	34 (25.4)	0.35	31 (23.1)	69 (51.5)	34 (25.4)	0.15
Menopausal status									
Premenopausal	268 (39.0)	76 (28.4)	124 (46.3)	68 (25.4)		69 (25.8)	137 (51.1)	62 (23.1)	
Postmenopausal	419 (61.0)	116 (27.7)	199 (47.5)	104 (24.8)	0.95	123 (29.4)	196 (46.8)	100 (23.9)	0.49
Nodal status									
Negative	296 (43.1)	82 (27.7)	143 (48.3)	71 (24.0)		84 (28.4)	137 (46.3)	75 (25.3)	
Positive	391 (56.9)	110 (28.1)	180 (46.0)	101 (25.8)	0.81	108 (27.6)	196 (50.1)	87 (22.3)	0.54
Histological grade[b]									
Well/moderate	144 (21.0)	30 (20.8)	71 (49.3)	43 (29.9)		44 (30.6)	66 (45.8)	34 (23.6)	
Poor	386 (56.2)	114 (29.5)	172 (44.6)	100 (25.9)	0.13	105 (27.2)	195 (50.5)	86 (22.3)	0.62
Tumor size[c]									
≤ 2cm	244 (35.5)	70 (28.7)	113 (46.3)	61 (25.0)		69 (28.3)	111 (45.5)	64 (26.2)	
> 2cm	443 (64.5)	122 (27.5)	210 (47.4)	111 (25.1)	0.94	123 (27.8)	222 (50.1)	98 (22.1)	0.40
Estrogen receptor status[d]									
Negative	168 (24.5)	50 (29.8)	75 (44.6)	43 (25.6)		45 (26.8)	84 (50.0)	39 (23.2)	
Positive	519 (75.6)	142 (27.4)	248 (47.8)	129 (24.9)	0.76	147 (28.3)	249 (48.0)	123 (23.7)	0.89
Progesterone receptor status[b,d]									
Negative	200 (29.1)	61 (30.5)	91 (45.5)	48 (24.0)		66 (33.0)	94 (47.0)	40 (20.0)	
Positive	461 (67.1)	122 (26.5)	224 (48.6)	115 (25.0)	0.56	122 (26.5)	224 (48.6)	115 (25.0)	0.16
HER-2/neu amplification[b]									
Negative	425 (61.9)	113 (26.6)	206 (48.5)	106 (24.9)		115 (27.1)	207 (48.7)	103 (24.2)	
Positive	124 (18.0)	36 (29.0)	61 (49.2)	27 (21.8)	0.73	38 (30.7)	68 (54.8)	18 (14.5)	0.07
Adjuvant therapy									
No	516 (75.1)	140 (27.1)	244 (47.3)	132 (25.6)		143 (27.7)	244 (47.3)	129 (25.0)	
Yes	171 (24.9)	52 (30.4)	79 (46.2)	40 (23.4)	0.68	49 (28.7)	89 (52.1)	33 (19.3)	0.30
Survival									
metastasis-free HR (95% CI)		1.00	0.92 (0.71-1.20)	0.82 (0.60-1.12)		1.00	1.09 (0.84-1.43)	0.88 (0.63-1.23)	
overall HR (95% CI)		1.00	0.88 (0.69-1.13)	0.89 (0.67-1.18)		1.00	1.09 (0.85-1.39)	0.91 (0.67-1.24)	

[a] P-value from χ^2 test

[b] Numbers in cells may not add up due to incomplete information on histological grade, progesterone receptor status or HER-2/neu amplification or rounding

[c] Tumors of unknown size (n=11) are included as tumor size > 2cm

[d] Cutoff value used: >=10 fmol/mg protein

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CHAPTER SEVEN

General Discussion

7.1 POLYMORPHIC VARIATION IN THE HPG AXIS IS ASSOCIATED WITH BREAST CANCER

The objective of the studies described in this thesis was to elucidate the role of polymorphic variation in candidate genes within the HPG axis, regulating estrogen exposure and action, on susceptibility, clinical phenotype and outcome of breast cancer. A candidate gene approach was undertaken and variants of the GnRH, LH, LHR, CYP19 and ESR1 genes were selected for the analyses. The LHR 312Asn allele was significantly more often observed in 751 breast cancer patients compared to 1806 controls. In an independent set of 594 breast cancer patients, the finding of an increased odds ratio (OR) was repeated, with an observed P-value of 0.0008. Lohmueller et al (1) have calculated, after a meta-analysis of 301 studies covering 25 polymorphism associations (i.e., an average of 12 replication studies per association), that an initial finding from an association study followed by a study with $P<0.001$ is strongly predictive of future replication. According to this, our finding of the LHR 312Asn allele as a breast cancer risk allele is likely to be repeated in the future. Since the LHR 312Ser/Asn single nucleotide polymorphism (SNP) was not shown to result in functional alterations regarding binding and signal transduction *in vitro*, the association of the LHR 312Asn allele with increased breast cancer risk may be due to linkage with a, so far undetected, functional polymorphisms. Data from the HapMap database shows that the 312Ser/Asn SNP appears in one haplotype block with LHR 291Asn/Ser. The SNPs are highly linked with each other, but poorly correlated and have different minor allele frequencies. In addition to the 291Asn/Ser and 312Ser/Asn SNPs the total haplotype block contains a few intron SNPs and one other coding, but silent, SNP (D355D; rs11125179). An alternative option is that the LHR 312Asn variant holds functional alterations that were not tested. It can be hypothesized that the variant receptor couples to non-cAMP induced cellular effects, including the proliferation-associated MAPK pathways, which have a major role in intracellular signaling in breast cancer (2).

The LHR insLQ allele, although not associated with breast cancer risk, was associated with younger age at diagnosis, larger tumor size and poor overall survival (OS) in our pilot study. Subsequently potential effects of the insertion LQ polymorphism, were investigated *in vitro*. Transfection studies using HEK293 cells, followed by dose response experiments using both hCG and LH, revealed increased receptor sensitivity, as reflected by decreased EC50 values. In addition, binding studies revealed that LHR insLQ receptor variants were expressed at a higher level at the cell surface. Using transfection analyses in HEK293 cells with specific signal peptides targeting the LHR ectodomain to the endoplasmic reticulum, the LQ insertion was shown to improve signal peptide function as compared to its nonLQ-counterpart. The HEK293 cell system is suitable to determine receptor sensitivity for the different LH receptor variants. Hirakawa et al (3) have shown

that the EC50 response to hCG of HEK293 cells transfected with the hLHR, is similar to the response in MA-10 cells. Furthermore, we have repeated the transient transfections in a more physiologically relevant ovarian granulosa tumor cell line, COV434, and obtained similar results. Despite the obvious hypothesis of the LQ insertion affecting signal peptide function it cannot be excluded that possible linkage with nearby functional polymorphisms, for instance in the promoter region, explains the associations observed with breast cancer characteristics. The associations with larger tumor size and OS were validated in a second study in an independent cohort of Caucasian breast cancer patients. More importantly, when focusing on distant and loco-regional recurrence of disease, the LHR insLQ allele was associated with shorter disease free survival (DFS). The association with DFS revealed an allele dose-response relationship, which can be considered to improve evidence for causality of the observed association, especially if the allele is functional, as is the case for LHR insLQ (4). In this second study the association between the LHR insLQ allele and younger age of onset was not replicated. It cannot be excluded that in the initial study the finding of an association was due to chance. Secondly, it is possible that the effect was exaggerated by the relatively small sample size and therefore not picked up in the larger validation study. A third possible explanation, inherent to breast cancer being a complex disease, arises from potential interactions with other, as yet to be defined, genes or exogenous factors that may differ between the two study cohorts.

Notwithstanding the increased *in vitro* sensitivity of the LHR 291Ser variant, no associations with risk, clinical presentation or outcome of breast cancer were observed. Considering the very low LHR 291Ser allele frequency, the sample size of the cohort probably did not provide sufficient power to observe associations *in vivo*. Using the PS: Power and Sample Size Calculation software it was estimated that the study had <20% power to detect an association between LHR 291Ser and breast cancer, assuming a similar effect size as described for the other HPG-variants in this thesis (http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize/). Alternatively, it can be hypothesized that in patients carrying the LHR 291Ser allele, the difference in ligand-responsiveness is overcome by compensatory adjustment of LH production in the hypothalamic-pituitary-ovarian axis in premenopausal life, while in postmenopausal women, circulating levels of LH may exceed EC50 values and overcome any differences in receptor sensitivity for LH. If this would be the case, it is likely that the effect of the insLQ polymorphism is predominantly through its effect on LH receptor cell surface expression.

The GnRH 16Ser allele showed an association with positive lymph node status, and when simultaneously present with the LHR insLQ allele showed an important decrease of DFS in premenopausal patients. Although selected as a potentially functional SNP, using the same *in vitro* assay as applied to test the LQ insertion in the LHR signal peptide, no functional effects were observed for the substitution of 16Trp to the less hydrophic 16Ser. In fact, *in silico* analysis of the variants using on line program SignalP 3.0 did not result

in any difference of signal peptide characteristics either (http://www.cbs.dtu.dk/services/SignalP/). The functional significance of the 16Trp/Ser SNP therefore remains to be elucidated, or it may reside in linkage with other possible functional genetic alterations in or near the GnRH gene.

An aromatase variant resulting from a T to C at position 1531 in the 3'-UTR of the CYP19 gene, CYP19 1531C, showed an association with improved metastasis-free survival (MFS). This SNP has previously been suggested to result in increased estrogen production (5-7). Estrogens act in the breast via ER-α, encoded by ESR1. An additive effect on MFS was observed when a particular genotype of the ESR1 gene is also present. This T/C SNP, at position -397 in intron 1 of the ESR1 gene, showed an association with less HER-2/neu amplification. Presence of both the CYP19 1531C allele and the ESR1 -397CC genotype was independently associated with longer MFS. The effect, as reflected by the hazard ratio, was larger than what could be predicted based on absence of HER-2/neu amplification. Analysis of HapMap data in Haploview and data from the genome variation server (http://gvs.gs.washington.edu/GVS/) shows strong linkage disequilibrium (LD) between several SNPs throughout the CYP19 gene with the 1531T/C SNP in the 3'-UTR. Although it cannot be excluded that the1531T/C SNP is linked to functional SNPs that may drive the associations, the choice of this single locus approach focusing on CYP19 1531T/C, instead of a haplotype approach, is not likely to cause loss of information. The 1531T allele was previously shown to tag the most common haplotype in the Caucasian population, which was associated with increased estradiol levels (6). In addition, a polymorphic variant comprised of a $(TTTA)_n$ repeat, that has been extensively studied in relation to breast cancer risk as well, has been shown to be strongly linked to the CYP19 1531T/C SNP (7-10). Similarly, the ESR1 gene is known with a variety of polymorphisms. The first identified and most studied polymorphism in the ESR1 gene is the -397T/C SNP (also named PvuII). Several studies have indicated associations with estrogen-dependent disease (11-17). Moreover, *in vitro* assays indicate decreased transcriptional activity for the ESR1 -397T variant (18). It cannot be excluded that the ESR1 –397T/C SNP is merely linked to another polymorphism responsible for the functional effect. However, this possibility seems less likely since extensive association studies have not picked up stronger associations (19) and it does not appear that there are other, so far unrecognized, polymorphic sequence variations in the vicinity (20).

Although genetic association studies are very effective to study the genetic influences on complex disease, they are often subject to the criticism that significant findings are often not replicated. The main fear is that multiple testing, either using multiple polymorphisms in the association analyses or investigating multiple outcome variable measurements, may result in false positive results, i.e., type-I-error, under nominal significance thresholds (21, 22). Most statistical methods dealing with multiple testing, result in loss of information when applied to genetic association studies due to overcorrection. However,

the chance of obtaining false positive results can be decreased by increasing the prior probability that a candidate polymorphism is functional (23, 24). In addition, a solid biological rationale relating the gene to the phenotype, can reduce the number of tests on forehand. Since our association analyses were based on strong reasoning that the genes are involved in breast cancer biology and the polymorphisms are likely to be functional and/or well-described in literature, no corrections for multiple testing were performed. Nevertheless, replication remains the golden standard to be established.

7.2 HOW DO THE POLYMORPHISMS INFLUENCE BREAST CANCER CHARACTERISTICS AND OUTCOME?

7.2.1 Ovarian estrogen production

The choice of the candidate gene polymorphisms in the GnRH, LH, LHR, CYP19 and ESR1 genes was mainly based on their possible involvement in increased estrogen exposure or response. The feedback system of the HPG axis, however, is expected to result in inhibition of GnRH and gonadotropin production as a result of sensing of increased estrogen levels. This negative feedback has been demonstrated in several cell (25) and animal models as reviewed in (26), including female rhesus monkeys (27) as well as in women (28) and men (29). However, as observed by de Ronde et al (30) in men, it is possible that small alterations in estrogen circulating levels, such as mediated by polymorphic variation in HPG genes, do not affect pituitary function, as reflected by LH levels. The CYP19 1531T allele studied by de Ronde et al was associated with 9% higher levels of total and bioavailable estradiol in elderly men, whereas their LH levels were not significantly different across the genotypes, in fact, LH appears to be higher in the CYP19 1531TT genotype (30). A similar observation was reported by Genarri et al (31), who studied another CYP19 polymorphism associated with circulating estradiol levels in men. Moreover, Greb et al (32) have observed increased follicular phase estradiol levels associated with a specific FSHR genotype, but similar LH levels in a group of young women. The authors have concluded from this that different levels of ovarian estradiol production do not have to affect LH levels (32). Furthermore, the dynamics of the feedback system as reflected by LH secretion in response to testosterone infusion and depletion differ between ethnic groups (33, 34). From this it can be hypothesized that polymorphic variation in genes involved in the HPG axis may influence the setpoint and dynamics of the feedback system. In addition, several changes in external factors can be expected to alternately influence the set point and equilibrium-state within the HPG axis. Examples of such external factors are those influencing diurnal rhythm (e.g., working night-shifts), reproduction (e.g., methods of hormonal contraceptive) and steroid hormone synthesis (e.g., use of alcohol and drugs). Interactions between these external

factors and gene variants are likely to introduce another level of complexity in HPG axis dynamics.

Since serum estrogen levels in the breast cancer cohort are not known, it is not possible to test the hypothesis of increased ovarian estrogen production directly. However, an exploration of the possible effect of increased LHR activity on ovarian estrogen production was carried out in a group of 21 healthy young women, not using oral contraceptives or other steroid hormones. This group was previously selected to study the influence of an FSHR SNP on levels and dynamics of ovarian hormone production (32). One woman was homozygous (insLQ/insLQ), eight women in this cohort were heterozygous for the LHR insLQ allele (nonLQ/insLQ), and twelve homozygous for the LHR nonLQ allele. No differences in the distribution of anthropometric and reproductive characteristics, nor of FSHR genotype distribution were observed across the LHR insLQ genotypes. The results did not show differences in serum levels of LH or estradiol between LHR nonLQ/nonLQ and nonLQ/insLQ subjects. However, the one homozygous LHR insLQ/insLQ subject showed highly increased estradiol levels in the midluteal phase (Figure 1). In agreement with this, the thickness of the endometrium, which is dependent on estrogenic activity was maximal in the LHR insLQ/insLQ subject. In addition, the endometrium thickness

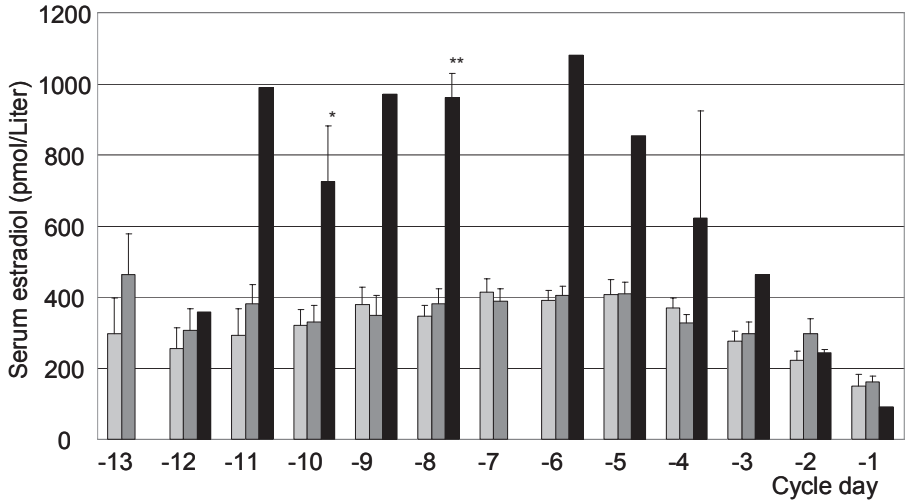

Figure 1. *Serum estradiol levels in pmol/L according to LHR insLQ genotype. The measurements of two consecutive luteal phases (with average duration of 13 days) are averaged and mean levels are shown for subjects with the nonLQ/nonLQ (n=12; light gray), nonLQ/insLQ (n=8; dark gray) and insLQ/insLQ genotype (n=1; black bars). Daily hormone measurements started at variable time points of the luteal phase and continued on average until cycle day 8, after which point hormones were measured every other day. Data were aligned based on the day of onset of menses, counting back from day zero (cycle day 0, onset of menses), i.e. showing the luteal phase. The average luteal phase length, calculated from the duration between LH surge until onset of the next menstruation, was 13 days, therefore a luteal phase period of 13 days is depicted. Hormone measurements from corresponding days of both luteal phases were averaged. *p=0.01; **p=0.004*

showed a clear, but borderline significant linear trend with LHR insLQ genotype (P-trend =0.05). Serum LH levels were not different across the LHR insLQ genotype groups. Apparently, different levels of ovarian estradiol production do not have to affect LH levels (32). These results may suggest that granulosa cells of the corpus luteum expressing only the LHR insLQ variant produce more estradiol during the luteal phase. Interestingly, the luteal phase, during which the corpus luteum is the main site of LHR-dependent estrogen and progesterone hormone production (35), can be considered as a period of risk for carcinogenesis in the breast (36). Despite these exciting preliminary results, it must be stressed that further investigation is warranted before any conclusions can be drawn.

Obesity has been related to poor DFS and mortality in breast cancer patients (37, 38), including recurrence and contralateral relapse (39), regardless of menopausal status. Adipose tissue shows high expression of aromatase, responsible for conversion of androgen precursors to estrogens. Despite the more frequent diagnosis of larger and lymph node positive tumors in obese patients, as compared to lean breast cancer patients, it is hypothesized that the influence of adipose tissue on hormone exposure is an independent factor (37, 39). Especially in postmenopausal obese women increased levels of estrogens and decreased levels of sex hormone-binding globulin (SHBG) are a likely causative mechanism (37, 39). Indeed, in postmenopausal women it has been shown that the peripheral conversion of androgens to estrogens increases as a function of body weight and BMI (40). In premenopausal women an independent role of obesity has been suggested as well. In these women lower levels of SHBG and higher androgen levels were observed, although estrogens were relatively unchanged or decreased (37). It is noteworthy that in premenopausal women the association between obesity and breast cancer risk shows a different pattern than outcome, since in general obesity is protective for breast cancer development in premenopausal women (41).

It can be hypothesized that an additional factor influencing androgen production, such as genetic variation in breast cancer candidate genes, may interact with the effect of increased aromatase activity resulting from an increased mass of adipose tissue (42). A likely candidate is polymorphic variation of the LHR gene. Therefore the breast cancer patients studied in Chapter 3 were stratified for body mass index (BMI), defined as weight in kilograms divided by height in meters squared, as a surrogate measure for adipose tissue mass. BMI ranged from 16.7-42.2 with median 25.3. Patients in the upper BMI quartile, with BMI >27.5, were compared vs. the rest of the patients (BMI ≤27.5). The association of the LHR insLQ allele with adverse DFS, showed a more pronounced effect in the upper BMI quartile (HR=1.72; 95% CI 1.10-2.68; P=0.02) as compared to the lower BMI patients (HR=1.29; 95% CI 1.02-1.64; P=0.03; see Figures 2A and B). In addition, the difference in (median) DFS between LHR insLQ-carriers and non-carriers was larger in high BMI patients (102 months) than in patients with a low BMI (33 months). Although estrogen levels are not known, the effect of stratification for BMI

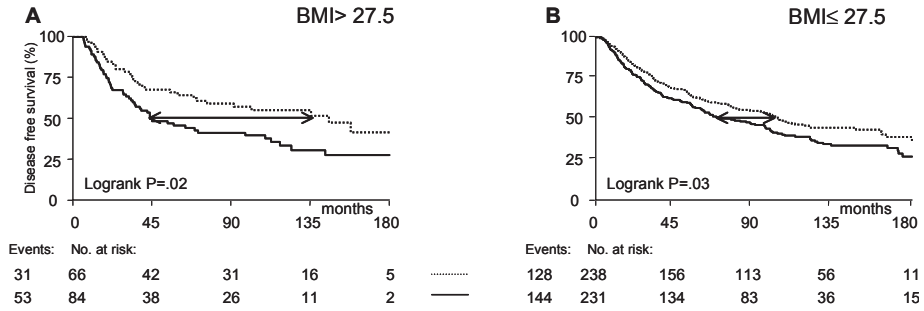

Figure 2A-B. *Kaplan-Meier DFS curves for non-carriers (dotted black line) vs. carriers of LHR insLQ carriers (solid black line) for patient subsets with BMI > 27.5 (A) and BMI ≤ 27.5 (B). The difference in median survival is indicated by a two-headed arrow.*

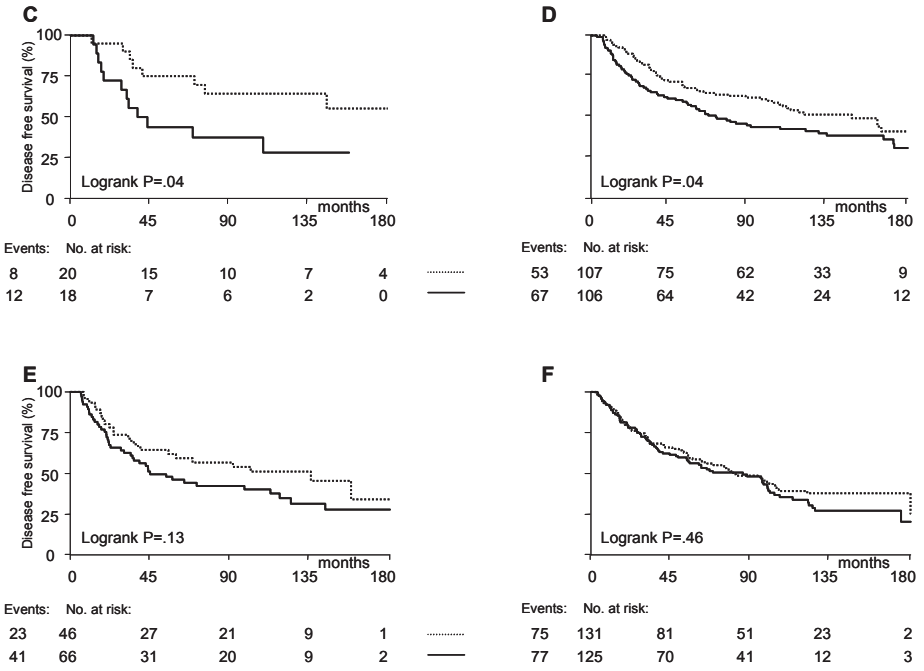

Figure 2C-F. *Kaplan-Meier DFS curves for non-carriers (dotted black line) vs. carriers of LHR insLQ carriers (solid black line), respectively for premenopausal (C) and (D) and for postmenopausal patient subsets patient subsets with BMI > 27.5 (E) and BMI ≤ 27.5 (F).*

on the associations between LHR insLQ and DFS may suggest an interaction between the effect of LHR insLQ, i.e. on production of androgen precursors, and the extent of aromatase enzyme in adipose tissue.

Since the association of LHR insLQ with poor DFS was dependent on premenopausal status (Chapter 4) an exploratory study was undertaken following the hypothesis that,

in addition to adrenal androgens, ovarian androgens may escape the ovary and be peripherally converted. The combined premenopausal-obese subset showed the highest HR (HR=2.56; 95% CI 1.03-6.37; P=0.04) (see Figures 2C vs. 2D). This may support the hypothesis that the LHR insLQ variant causes increased levels of ovary-derived androgens, which in obese premenopausal women may be converted to estrogens at a higher level than in non-obese women. Although no significant association between LHR insLQ and poor DFS was observed in postmenopausal patients, stratification for BMI had a similar effect on the association in this subset (see Figures 2E and F).

7.2.2 Direct effects on breast tissue

Although HPG effects on estrogen exposure were hypothesized to explain the observed associations with breast cancer, a possible role of direct effects on breast tissue cannot be excluded. Estrogen-induced development and chemically induced tumorigenesis of the mammary gland is definitely dependent on expression and function of ESR1, as demonstrated in the αERKO mice (43). Direct involvement of local aromatase activity in the breast tumor development is also clear, as supported by the fact that increased aromatase activity and estradiol levels have been shown in breast tumors as compared to normal tissues in postmenopausal women (44, 45).

Epidemiological data show protective effects of pregnancy on development of breast cancer, which may be mediated by LHR signaling induced by the pregnancy hormone hCG. This observation has led researchers to study LHR expression and action in mammary cancer cell lines and breast tumors. Presence of the LHR has been detected in different breast cancer cell lines (46) and *in vitro* treatment with hCG (LH was not tested) has been shown to inhibit cell proliferation (47, 48). Furthermore, LHR mRNA and protein expression has been shown in normal and malignant breast tissue (46). In addition, Meduri et al (49) have observed a correlation of LHR expression in breast cancer tissue with premenopausal status, higher degree of differentiation, estrogen receptor expression and a tendency towards longer metastasis-free survival. The authors suggested that presence of LHR is associated with lower grade, more differentiated tumors, but this was not further investigated. Possibly LHR expression is an unrelated physiological feature of the human breast, which is downregulated at some point during development or progression of mammary carcinoma. On the other hand the observations may suggest that LHR signaling in the breast is associated with less aggressive breast tumor behavior. In line with this, Russo et al (50) were able to show a protective effect of hCG against development and progression of mammary carcinoma induced by treatment with the carcinogen 7,12-dimethylbenz(*a*)anthracene (DMBA) in a rat model. According to these observations, increased LHR signaling in breast cancer would have positive effects on breast cancer outcome. This is in apparent contrast to the negative associations we observed between the more active LHR insLQ and larger tumor size and shorter disease free survival. However,

the results from *in vitro* studies and models described above may not be appropriate for the situation in human breast cancer patients. Furthermore, the underlying hypotheses are built on possible effects of hCG, whereas LH-induced signaling was not investigated. Prospective studies involving determination of LHR expression in the tumors of the patients in the current association analyses will clarify this issue (see future plans).
Similarly, expression of GnRH receptors in breast cancer tissue and direct effects of GnRH on sex steroid hormone-dependent cancers have been shown (51-56). However, to our knowledge expression of GnRH protein in breast cancer tissue has not been shown, and hypothalamic GnRH is unlikely to reach the breast via the peripheral circulation given its low concentration and short half-life (57). In addition, the effects of GnRH agonist treatment regimens are most likely explained by down-regulation of pituitary GnRH receptors and subsequent shutdown of the HPG axis (58). Therefore, although direct effects cannot be excluded, the observed associations between the GnRH 16Ser allele and breast cancer are most likely to result from involvement of the HPG axis because of the observation of an additive effect with the LHR insLQ variant, in premenopausal patients only.

7.3 FUTURE RESEARCH

The results that have been obtained in the course of this thesis naturally evoke further questions that can only be answered by new research. Such studies could be directed to obtain a more comprehensive understanding of the contributions of the candidate genes to the hormonal carcinogenesis of female breast cancer. In addition, future research may focus on the possible clinical applicability of the polymorphisms in therapeutic settings. Finally, some of the candidate genes may be involved in other steroid hormone-related complex traits.

7.3.1 Expression profiles associated with HPG polymorphisms

To better understand the etiology of the observed associations, a study of the relation between comprehensive gene expression data using RNA expression methodology of breast tumors according to certain HPG genotypes is proposed. It has been observed that expression profiles of metastases closely resemble those of primary tumors (59). Following this observation it has been hypothesized that baseline genetic properties of the breast tumor determine the ability to metastasize, rather than late multistep events (60). This hypothesis also puts forward the possibility that both expression profiles and prognosis may be determined by germline genetic variation (61). In previous studies expression profiles have been shown to be prognostic for metastasis (62-64) and to predict the response to adjuvant systemic therapy (65, 66). A specific expression profile,

consisting of proliferation-associated genes, was shown to be informative especially for tumors with relatively high ER expression (67). It can be hypothesized that polymorphic gene variants, such as those involved in the HPG axis and described in this thesis, correlate with distinctive expression profiles. Information derived from genotyping is then expected to have prognostic and predictive value as well (68). In addition, RNA expression information in relation to the genotypes may yield important insight in the biological effects of the genotypes on breast cancer biology.

7.3.2 Associations with response to treatment

The studies described in this thesis work have resulted in the identification of a possible diagnostic genotype, in addition to several prognostic genotypes. In this section suggestions are made for prospective research studying the diagnostic and prognostic value of the specific genotypes in breast cancer patients. Translational research, e.g. combining results from RNA expression studies and including measurement of serum estrogen levels, is expected to contribute significantly to the outcome of such clinical studies.
The association studies were performed in a retrospective patient cohort, collected partly at a time when it was common practice in The Netherlands that node-negative pre- and postmenopausal patients did not receive systemic adjuvant therapy. Therefore, studying outcome in this cohort offers a way to provide insight in prognostic mechanisms, without confounding effects of treatment (69). When validated prospectively, the findings described in this thesis may contribute to diagnostic and prognostic evaluation of patients and possibly to further optimization of tailored endocrine treatment for breast cancer. Prognostic factors may select patients that are most likely to experience recurrence of disease without adjuvant therapy and therefore potentially benefit most from therapy (70). Systemic chemotherapy as well as hormonal therapy for breast cancer shows several side-effects. This highlights the need for careful selection of high-risk women for breast cancer (71), for classification of patient groups based on prognostic factors (69) and for fine-tuning of therapy within these specific patient groups (72). Amongst others, translational research and improved understanding of (molecular) biological mechanisms, including polymorphic genes and gene-environment interactions, are expected to contribute to optimal treatment (73, 74). The studies described in this thesis have identified gene variants associated with progression of breast cancer in premenopausal patients. It can be expected that the outcome and efficacy of hormonal therapy that interferes with the HPG axis, for premenopausal breast cancer, may be influenced by genetic variation in genes involved in the HPG axis. For example, as described in chapter 4, almost 25% of these patients could be identified, carrying the LHR insLQ and GnRH 16Ser variants, with a more than doubled risk for recurrence of disease. Patients with this combined genotype hypothetically experience an upregulation of ovarian estrogen production. Thus, these patients may require an adaptation of the current hormonal therapy regimens.

To clarify potential predictive value of the LHR insLQ and GnRH 16Ser genotypes, genotyping of retrospectively collected samples is proposed for premenopausal patients that have received adjuvant therapy, preferably using GnRH agonists, either or not in combination with tamoxifen, and to assess whether or not outcome is associated with these genotypic variants. Next, I propose measurement of estrogen levels in women receiving GnRH agonists, as well as genotyping of the LHR insLQ and GnRH 16Ser variants, to study associations between degrees of ovarian suppression across genotypes. If it is proven that ovarian estrogen production is variably suppressed between genotypes, it should be tested whether an increase or alteration of the dosage schedule can further suppress estrogen levels. If so, patients should be randomized to prospectively study the effect of increased administration of GnRH agonists, depending on the genotype of these HPG variants. Alternatively, the LHR may be a drugable receptor and it can be hypothesized that LHR antagonists can be added to treatment interfering in the HPG axis. The same concept can be employed for the CYP19 1531T/C and ESR1 –397T/C variants in trials studying the addition of an aromatase inhibitor, compared to addition of tamoxifen, to a GnRH agonist. These ongoing studies should include genotyping of the CYP19 1531T/C and ESR1 –397T/C SNPs to study whether schedules of aromatase inhibitor and tamoxifen treatment can be further individualized.

In premenopausal patients chemotherapy is often preferred over hormonal therapy based on its rapid effect, although side effects in general are worse than those of hormonal therapy. In addition, it can be asked whether and to what extent the effect of chemotherapy is mediated by ovarian suppression. Several indirect and direct comparisons of ovarian suppression (either or not combined with tamoxifen) indicate that endocrine treatment may equal or even excel polychemotherapy (75-79). This concept has led to the establishment of the PROMISE study, an open label randomized trial in premenopausal patients comparing combined hormonal therapy (ovarian suppression plus tamoxifen) vs. polychemotherapy followed by the same combined hormonal therapy as adjuvant therapy. Interestingly, genetic factors, such as the LHR insLQ, have been suggested to play a role in chemotherapy-induced ovarian suppression (72). Genotyping and comparison of the degree of ovarian suppression (based on estrogen levels and menstrual cycle pattern) and treatment outcome across genotypes would therefore be interesting to add to the protocol. In view of the importance of suppression of ovarian function for the success of adjuvant breast cancer treatment, an interesting additional point of study is the role of serum anti-Mullerian hormone (AMH) as a response marker for ovarian function (80).

7.3.3 Other associations

Several complex traits can be thought of that are likely to show variety in healthy populations as a consequence of polymorphic variants of genes involved in the HPG axis.

Some examples that are listed here are polycystic ovary syndrome, prostate carcinoma, endometrial cancer and effects on bone.

Polycystic ovary syndrome (PCOS) is a heterogeneous syndrome, defined by two out of three of the following criteria (defined by the ESHRE/ASRM): oligo- and/or anovulation; clinical and/or biochemical hyperandrogenism and polycystic ovaries, with the exclusion of other aetiologies. In addition, PCOS is characterized by significantly elevated levels of LH and an increased LH/FSH ratio. Biochemical hyperandrogenism is reflected by higher basal testosterone and androstenedione levels in women with PCOS, as compared to healthy control women. Hyperandrogenism appears to be the strongest genetically inherited characteristic in familial cases (81). Furthermore, it has been shown that after stimulation with hCG, testosterone and androstenedione levels rise earlier in women with PCOS, which may result from a more active LHR (82). Since LHR activation stimulates ovarian androgen production, genetic variation in the LH receptor is a possible mediator in the pathophysiological pathway of PCOS. Therefore, the contribution of polymorphisms in the LHR, such as the LHR insLQ polymorphism, to PCOS should be assessed.

Another interesting question is whether other sex steroid hormone-dependent cancers, such as prostate cancer would show similar associations with tumor size and DFS. Preliminary results from the Rotterdam study indicate an association of the LHR insLQ allele with occurrence of prostate cancer. Incorporation of the LHR insLQ genotype as a continuous term in a binairy logistic regression model, including adjustment for age, resulted in a borderline significant association (P=0.07), showing an OR of 1.2 (95% CI 1.0-1.5). The genotype distribution is shown in Table 1. Considering these preliminary results and the clear relation between testosterone exposure and development and progression of prostate cancer, it will be highly interesting to study associations between HPG variants and prostate cancer in a larger cohort.

The gonadotropin hypothesis of ovarian cancer pathogenesis was first postulated by Cramer et al (83). According to the hypothesis, entrapment of surface epithelium within the ovarian stroma is proposed as the initial event, followed by differentiation, prolif-

Table 1. *LHR insLQ genotype distribution in men of the Rotterdam Study, classified by diagnosis of prostate cancer*

	Mean age	LHR insLQ genotype, n (%)			P-value
Rotterdam Study		non/nonLQ	non/insLQ	ins/insLQ	
All men[a]	68.1 ± 8.2	1308 (51.8)	1102 (39.7)	215 (8.5)	
Prostate cancer	68.7 ± 7.9[b]	111 (46.6)	102 (42.9)	25 (10.5)	
No prostate cancer	68.0 ± 8.2[b]	1197 (52.3)	900 (39.4)	190 (8.3)	0.05[c]

[a] HWE: p=0.24

[b] Age is not different between subjects with and without prostate cancer (p=0.26)

[c] χ^2 test for trend

eration, and eventual malignant transformation of the entrapped epithelium, stimulated by a cooperative effect of excess estrogens and gonadotropins. Epidemiological data to support this hypothesis is found in the increased development of ovarian carcinoma after ovulation induction, which is accompanied by an increase in gonadotropin levels (84, 85). *In vitro* data shows that FSH and LH are able to enhance cell growth in normal and malignant human ovarian surface epithelial cells, the progenitor cells for ovarian carcinoma (86). In addition, transgenic mice with chronically elevated LH develop granulosa cell tumors (87). Interestingly, the ability of LH to induce ovarian tumors was later shown to be highly dependent on the genetic background of mice (88). Therefore HPG gene polymorphisms are interesting candidates to study with respect to ovarian cancer.

Estrogen-induced proliferation of the endometrium plays a role in the development of endometrial cancer, especially when unopposed by differentiation induced by progestins (89, 90). Variants of genes involved in sex steroid biosynthesis are therefore likely to play a role in the etiology of endometrial cancer. Interestingly, a recent large genetic association study has shown that interactions between estrogen replacement therapy and functionally relevant gene variants involved in sex steroid hormone metabolism are likely to influence risk of endometrial cancer (91).

Developmental changes in HPG axis function, such as during puberty and menopause, are accompanied by clear alterations in skeletal metabolism. It is generally assumed that sex steroid levels play a major role in bone metabolism, as suggested by the clear loss of bone mass following estrogen depletion in postmenopausal life. Considering the high levels of gonadotropins accompanied with menopause and the expression of LHR in extragonadal tissues, including primary human osteoblasts (92), a direct role of LHR signaling in bone turnover has been suggested. hCG-overexpressing mice showed an increase of bone mineral density (BMD) and histomorphometric parameters of bone tissue. However, hCG was unable to induce LHR signaling in osteoblasts *in vitro,* as measured by alterations in second messenger levels. In addition, ovariectomy (OVX) in the hCG-overexpressing mice resulted in BMD and histomorphometric parameters similar to wild-type OVX-mice (92). This suggests that the effect of overexpression of hCG is not on bone metabolism directly, but rather through effects on ovarian hormone production. It can by hypothesized that possible increased signaling by the LHR insLQ variant upregulating ovarian estrogen production results in increased BMD and/or affects bone morphometric parameters. However, any differences in BMD and/or morphometry are expected to be found only in premenopausal life, when (lack of) estrogenic effects on bone does not play a role in clinical practice. Therefore, although it would be interesting to study whether women with the LHR insLQ genotype enter menopause with a more favorable bone phenotype protective of osteoporosis, the study did not have major priority so far.

7.4 CONCLUDING REMARKS

The results presented in this thesis may have contributed to the identification of HPG gene variants as prognostic factors in premenopausal breast cancer patients. Questions have been raised about the causality and etiological aspects of the HPG gene variants regarding ovarian estrogen production and effects on carcinogenesis of the breast. In addition, possible predictive value of the HPG gene variants has been suggested for the response to hormonal treatment, interfering in this HPG system, in premenopausal patients. Finally, suggestions are made to study these aspects in subsequent research.

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CHAPTER EIGHT

Summary/ Samenvatting

8.1 SUMMARY

Breast cancer is a frequently occurring disease in women, with high mortality rates. The probability and timing of development, the tumor characteristics upon presentation and the outcome of breast cancer, show wide variability between and within populations. It is expected that common low-penetrance genes, involved in several carcinogenic pathways, play a major role in this common variability. A well-known risk factor in development and progression of breast cancer, as indicated by epidemiological and animal studies, is increased or prolonged cumulative estrogen exposure. Therefore, interference in the production or action of estrogens takes up a central role in breast cancer treatment. Accordingly, common sequence variations (polymorphisms) in genes regulating estrogen exposure and response may influence breast cancer susceptibility, patient and tumor characteristics, prognosis and treatment outcome. Estrogen production and action is regulated within the hypothalamic-pituitary-gonadal (HPG)-axis. Genes involved in the HPG-axis are therefore likely candidates. Several key genes in the HPG-axis were identified and potential candidate polymorphisms were selected and associations with breast cancer risk, clinico-pathological characteristics and survival were studied. In addition, functional consequences of some polymorphisms were investigated.

Common variants of Luteinizing Hormone (LH) and its receptor, the LHR were studied in a cohort of 266 Australian Caucasian breast cancer patients in **chapter 2**. A polymorphism in LH, known with higher *in vitro* activity and associations with several aspects of HPG-function, was selected. In the LHR gene a potentially biologically interesting insertion polymorphism in the signal peptide was chosen. Although the polymorphic variants in LH and its receptor did not appear to be risk alleles for breast cancer, the LQ insertion polymorphism (LHR insLQ) in the signal peptide of the LHR gene was shown to be linearly associated with younger age at diagnosis of breast cancer. Furthermore, the LHR insLQ allele was associated with increased tumor size and a shorter overall survival was observed in carriers of the LHR insLQ allele. The association with younger age at diagnosis was not validated in a larger independent breast cancer cohort, consisting of 751 Dutch Caucasian patients, in **chapter 3**. However, the association with increased tumor size was indeed validated, as was the association with shorter overall survival, independent of other prognostic factors. Moreover, we established an independent association of the LHR insLQ allele with shorter disease free survival (DFS), an important prognostic feature of the disease. In addition, when the LHR insLQ variant was investigated *in vitro* in transfection studies, increased receptor sensitivity and plasma membrane expression was shown as compared to the nonLQ counterpart.

The breast cancer cohort studied in **chapter 3** consisted of pre- and postmenopausal patients. We hypothesized that the more active LHR insLQ variant will result in an increased cumulative ovarian estrogen exposure, which influences breast cancer outcome.

Although it has been calculated that 10-25% of postmenopausal estrogens are produced in the ovary, partly under the influence of LH, the contribution of LHR-mediated ovarian estrogen production is expected to predominate in premenopausal life. Therefore the association studies were extended in **chapter 4**, including stratification for premenopausal status, i.e. the presence of an active ovary and HPG axis. Indeed, the association of the LHR insLQ allele with shorter DFS was shown to be dependent on ovarian function, as reflected by premenopausal status, and was found to be augmented by the simultaneous presence of a single nucleotide polymorphism in the signal peptide of the gonadotropin-releasing hormone gene (GnRH 16Trp/Ser). The GnRH 16Ser allele revealed an association with increased lymph node involvement. However, the association of presence of both the LHR insLQ and GnRH 16Ser alleles with DFS in premenopausal patients was independent of this and other known prognostic factors, suggesting an upregulating effect on HPG-regulated ovarian estrogen production.

In **chapter 5** another functional LHR variant was identified, a single nucleotide polymorphism (SNP), resulting in an Asn to Ser substitution at position 291, abolishing a glycosylation site in the hinge region of the ectodomain. *In vitro* transfection studies revealed altered glycosylation status and increased receptor sensitivity for the 291Ser LHR variant. Despite the clear functional consequences of the 291Asn/Ser SNP, no associations with breast cancer risk or clinical phenotype was observed, illustrating the problem of association analyses with infrequent SNPs. In contrast, using a population-based study, the nearby 312Ser/Asn LHR SNP was identified as a possible minor susceptibility breast cancer gene. This association was validated in an independent set of breast cancer samples.

Chapter 6 describes two candidate genes in estrogen signaling pathway endpoints. Two SNPs in the genes encoding aromatase and estrogen receptor (ER)-α, respectively CYP19 and ESR1, were chosen based on solid literature reports on associations with estrogen-related complex traits. The combined presence of specific genotypes of the CYP19 and ESR1 genes was associated with a reduced risk of metastasis in premenopausal women, independent of other prognostic factors. In addition, the CYP19 SNP showed a modest association with histological grade. The ESR1 genotype was weakly associated with dichotomized progesterone receptor status and strongly associated with gene amplification levels of HER-2/neu, an important predictive factor.

The general discussion in **chapter 7** addresses to some principles of genetic association studies in respect to the major findings described in this thesis. The functional *in vitro* results are discussed in a broader perspective and related to the *in vivo* associations. An etiological pathway via increased ovarian estrogen production is suggested as evidenced by some preliminary results. Finally, suggestions for future research, which may place the current findings in a wider applicable clinical perspective, are presented.

8.2 SAMENVATTING

Borstkanker is een frequent optredende ziekte onder vrouwen met een hoog sterftecijfer. De kans op ontwikkeling, het moment van optreden, de tumorkarakteristieken bij presentatie en het beloop van de ziekte, verschillen tussen en binnen bevolkingsgroepen. Naar verwachting spelen algemene, zogenaamde "laag-penetrante" genen, betrokken bij de ontwikkeling van kanker, een belangrijke rol in deze algemene ziekte-variatie onder mensen. Een bekende risicofactor uit epidemiologisch en dierexperimenteel onderzoek, in het ontstaan en de ontwikkeling van borstkanker, is toegenomen blootstelling aan oestrogenen. Het ingrijpen in de productie en werking van oestrogenen heeft daarom een centrale plaats in de behandeling van borstkanker. Algemene variaties in de DNA-sequentie (polymorfismen) van genen die de productie van en blootstelling aan oestrogeen reguleren, zouden de kans op ontwikkeling, de tumorkarakteristieken, prognose en uitkomst van de ziekte kunnen beïnvloeden. De productie en werking van oestrogenen wordt gereguleerd in de Hypothalamus-Hypofyse-Gonade (HPG)-as. Genen die betrokken zijn bij de HPG-as zijn daarom belangrijke kandidaat-genen. Verschillende belangrijke genen van de HPG-as werden uitgezocht en mogelijke kandidaat-polymorfismen in de genen werden geselecteerd. Vervolgens werden associaties met borstkankerrisico, patiënt- en tumorkarakteristieken en overleving bestudeerd. Daarnaast onderzochten we de functionele gevolgen van een aantal van deze polymorfismen.

Polymorfismen van het hormoon Luteïniserend Hormoon (LH) en diens receptor, de LH receptor (LHR), werden bestudeerd in een cohort van 266 Australische Kaukasische patiënten in **hoofdstuk 2**. Een polymorfisme in LH, waarvan een hogere *in vitro* activiteit en verschillende associaties met HPG-functie bekend zijn, werd hiervoor geselecteerd. In het LHR gen werd een potentieel biologisch interessant LQ insertie-polymorfisme in het signaal peptide gekozen. Hoewel geen van deze polymorfe allelen risico-allelen voor borstkanker bleken te zijn, was het LQ insertie-polymorfisme in het signaal peptide van de LHR (LHR insLQ) lineair geassocieerd met een jongere leeftijd bij het optreden van borstkanker. Daarnaast bleek dit LHR insLQ allel geassocieerd met grotere tumoren en er werd een kortere overleving gezien bij patiënten die draagster waren van dit LHR insLQ allel. De associatie met jongere leeftijd van diagnose werd niet gevalideerd in een grotere onafhankelijke borstkanker-studie, bestaande uit 751 Nederlandse Kaukasische patiënten, zoals beschreven in **hoofdstuk 3**. Echter, de associatie met grotere tumoren werd wel gevalideerd, evenals de associatie met kortere overleving, onafhankelijk van andere prognostische factoren. Bovendien stelden we een onafhankelijke associatie tussen het LHR insLQ allel en kortere ziektevrije overleving vast, een belangrijk prognostisch gegeven van de ziekte. Daarnaast werd, bij bestudering van de *in vitro* gevolgen van de LHR insLQ variant in transfectie studies, een toename van de receptor gevoeligheid en

expressie op de plasma membraan gevonden. Dit in vergelijking tot de zogenaamde LHR nonLQ variant.
Het borstkanker-cohort dat bestudeerd werd in **hoofdstuk 3** bestond uit pre- en postmenopauzale patiënten. De hypothese was, dat de actievere LHR insLQ variant zou leiden tot een toegenomen cumulatieve oestrogeen-blootstelling vanuit het ovarium, met bijkomstige gevolgen voor het beloop van borstkanker. Hoewel naar schatting zo'n 10-25% van de postmenopauzale oestrogenen geproduceerd wordt in het ovarium, gedeeltelijk onder invloed van LH, speelt de LHR-gemedieerde ovariële oestrogeen-productie voornamelijk een rol in de premenopauzale levensfase. Daarom werden de associatiestudies beschreven in **hoofdstuk 4** uitgebreid met stratificatie voor premenopauzale status, oftewel de aanwezigheid van actieve ovaria en HPG-as. Zoals verwacht bleek de associatie van het LHR insLQ allel alleen aanwezig in de premenopauzale situatie en de associatie werd beïnvloedt door de gelijktijdige aanwezigheid van een SNP polymorfisme in het signaal peptide van het Gonadotropine-releasing Hormoon gen (GnRH 16Trp/Ser). Het GnRH 16Ser allel toonde een associatie met positieve lymfeklier status. Echter, de associatie tussen gelijktijdige aanwezigheid van de LHR insLQ en GnRH 16Ser allelen met ziektevrije overleving in premenopauzale patiënten was onafhankelijk van lymkfeklier status en andere prognostische factoren. Dit suggereert een effect via HPG-gestimuleerde ovariële oestogeen-productie.
In **hoofdstuk 5** werd een andere functionele LHR variant beschreven, een één-nucleotide verandering (SNP), resulterend in de verandering van Asn naar Ser op positie 291, en het verdwijnen van een glycosylatie-site in de linkerregio. *In vitro* transfectie studies toonden een verandering van glycosylatie status en toegenomen receptor gevoeligheid aan voor de 291Ser LHR variant. Ondanks deze functionele consequenties van dit polymorfisme werden geen associaties met borstkanker-risico of –presentatie gevonden, wat illustratief lijkt voor associatie-studies met weinig frequente polymorfismen. Hier tegenover stond dat de populatie-studie aantoonde dat een nabij gelegen polymorfisme op het LHR gen, de 312Ser/Asn SNP, een mogelijk borstkanker risico-allel bleek te zijn. Deze associatie werd gevalideerd in een onafhankelijke groep borsttumor-monsters.
Hoofdstuk 6 beschrijft twee kandidaat-genen in de laatste fase van de oestrogeen productie en signaal transductie. Twee SNP's in de genen die coderen voor aromatase en oestrogeen receptor α, respectievelijk CYP19 en ESR1, werden geselecteerd naar aanleiding van fundamentele publicaties over oestrogeen-afhankelijke aandoeningen. De gelijktijdige aanwezigheid van bepaalde genotypes van deze CYP19 en ESR1-genen bleek geassocieerd met een lager risico op uitzaaiïng in premenopauzale patiënten, onafhankelijk van andere prognostische factoren. Daarnaast toonde de CYP19 SNP enige associatie met histologische gradering. Het ESR1 genotype was zwak geassocieerd met de aanwezigheid van progesteron receptor expressie en sterk geassocieerd met een hoger

niveau van HER-2/neu gen amplificatie, een belangrijke voorspellende factor voor het beloop van borstkanker.
De algemene discussie in **hoofdstuk 7** is gewijd aan enkele principes van genetische associatiestudies met betrekking tot de belangrijkste bevindingen beschreven in dit proefschrift. De functionele *in vitro* resultaten worden in een breder perspectief besproken en gerelateerd aan de *in vivo* associaties. Een etiologische route via toegenomen ovariële oestrogeen-productie wordt gesuggereerd aan de hand van enkele voorlopige resultaten. Ten slotte worden aanbevelingen gedaan voor toekomstig onderzoek, dat de huidige bevindingen mogelijk een algemene, klinisch toepasbare, plaats zal kunnen geven.

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CURRICULUM VITAE

The author of this thesis was born on November 1st, 1976 in Sneek, The Netherlands. In 1995 she graduated from secondary school at the Magister Alvinus College in Sneek, after which she moved to Antwerp, Belgium, to study medicine. In 1996 she was able to start the medical study in Rotterdam, The Netherlands. Her graduation research in 2000 was performed at the laboratory of dr. E.M.J.J. Berns, studying the role of genetic variants in breast cancer. This was concluded by an essay entitled "Early onset breast cancer: LH variants, BRCA1 and TP53 mutations". In 2002 she obtained her medical degree cum laude. End 2002 she started to work as an MD-PhD student at the laboratory of dr. A.P.N. Themmen. The research described in this thesis was also supervised by Prof.dr. H.A.P. Pols and dr. E.M.J.J. Berns. As part of the MD-PhD track, the research work was interrupted for one year of the residency internal medicine in 2005. This took place at the Erasmus MC, supervised by dr. J.L.C.M van Saase. In 2005 she received a Travel Grant Award from "The Endocrine Society" and in 2006 from "Women in Endocrinology". In January 2007 she resumed her residency in internal medicine at the Sint Franciscus Gasthuis Rotterdam, under supervision of drs. A.P. Rietveld and H.C.T. van Zaanen.

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