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A Common Polymorphism Renders the Luteinizing Hormone Receptor Protein More Active by Improving Signal Peptide Function and Predicts Adverse Outcome in Breast Cancer Patients

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

Objective: We hypothesized that an LH receptor (LHR) gene variant may affect LHR 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 clinicopathological features was studied in a large cohort of 751 breast cancer patients with complete follow-up. Functional analysis of the insLQ-LHR and non-LQ-LHR (no LQ insertion) was carried out using transfection studies.

Results: We found a significant association between the insLQ-LHR and a shorter disease-free survival (hazard ratio, 1.34; confidence interval, 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 times lower EC_{50} than non-LQ-LHR; $P = 0.02$) and plasma membrane expression (insLQ-LHR has 1.4 times higher B_{max}; $P = 0.0006$) rendering the insLQ-LHR allele more active.

Conclusions: The insLQ polymorphism increases LHR activity, thereby shortening breast cancer disease-free survival, probably by increasing estrogen exposure in female carriers. **(*J Clin Endocrinol Metab* 91: 1470–1476, 2006)**

WORLDWIDE, THE DIAGNOSIS of breast cancer is made one million times per year. Factors contributing to an increased cumulative lifelong 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 is the ovaries, which are under control of the pituitary gonadotropins LH and 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 a 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

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Abbreviations: CI, Confidence interval; DFS, disease-free survival; DTT, dithiothreitol; ECD, ectodomain; EGFP, enhanced green fluorescent protein; EPOS, Eindhoven Perimenopausal Osteoporosis Study; EndoH, endoglycosidase H; HA, hemagglutinin; hCG, human chorionic gonadotropin; HR, hazard ratio; HWE, Hardy-Weinberg equilibrium; LHR, LH receptor; PNGase F, *N*-glycosidase F.

reticulum more efficiently, resulting in a more sensitive mature LHR protein that is expressed at a higher level than its non-LQ counterpart. Moreover, we establish that the insLQ polymorphism is associated with a shorter disease-free survival (DFS).

Subjects and Methods

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 yr (range, 28–94 yr). Analyses of survival and clinicopathological features were performed in 751 cases of which complete follow-ups are currently available. The median follow-up period of patients alive is 130 months from primary surgery (range, 13–255 months). Two population-based cohorts were used to determine the genotype frequency in the general population: the Rotterdam study and the Eindhoven Perimenopausal Osteoporosis Study (EPOS). The rationale and design of these cohorts have been described previously (14, 15).

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 previously described (16), 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 enhanced green fluorescent protein (EGFP) was used to construct the insLQ and non-LQ full-length LHR expression plasmids (17). As described before, neither the HA1 immunotag nor the EGFP extension affect expression and signal transduction of the LHR (17). The LHR cDNA in pSG5 carrying the insLQ insertion (pSG5insLQ-LHR) as originally described in Kraaij *et al.* (18) 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 *Bsu*36II and *Bgl*II, followed by blunting and re-ligation. 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 number 1). This procedure rescues the *Bgl*II 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 (19) 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 *Eco*RI (in the vector at the 5′ side of the insert) and *Eco*47III 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 *Eco*RI/*Eco*47III digested pSG5-insLQ-hLHR-ECD plasmid. The plasmid containing the signal peptide with the deleted hydrophobic region was constructed by digestion with *Pst*I 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 LHR function

Transfection experiments concerning signal transduction and binding in HEK293 cells used a calcium phosphate transfection method as previously described (19). 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

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 (20), 2 μg SV40Renilla (transfection efficiency), and 10 μg of either pSG5-insLQhLHR-GFP or pSG5-non-LQhLHR-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 h 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. B_{max} (single-point determinations) and K_d (Scatchard analysis) were carried out as described previously (19, 21).

LHR 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-ECD constructs for Western blot analysis, because 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 harvested in PBS with 20 mM *N*-ethylmaleimide, snap-frozen in liquid nitrogen, and stored at −80 C. Cell pellets were resuspended in Laemmli sample buffer to a final concentration of 1 μg/μl and denatured at 95 C for 5 min. Some samples were reduced by adding 20 mM dithiothreitol (DTT) before denaturing.

For deglycosylation reactions with endoglycosidase H (EndoH) or *N*-glycosidase F (PNGaseF), the manufacturer's instructions were followed (New England Biolabs, Westburg, Leusden, The Netherlands). After enzyme treatment, the protein was analyzed using gel electrophoresis and Western blotting with a conjugated HA1-horseradish peroxidase rat monoclonal antibody (Roche Diagnostics Nederland BV, Almere, The Netherlands) at 1:5000 dilution.

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 survival (endpoint was death) and DFS (endpoint was recurrence including second primary breast tumor) analyses were carried out using proportional hazards regression analysis. The assumption of proportional hazards was investigated using a test based on the Schoenfeld residuals (22) and was not violated for insLQ genotype. Hazard ratios (HR) for the insLQ allele are presented with their 95% confidence interval (CI). Differences between HR for insLQ carriers *vs.* noncarriers 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. DFS and overall survival probabilities were calculated using the actuarial method of Kaplan-Meier (23). Survival probabilities were compared between

insLQ carriers and noncarriers 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). 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

insLQ-LHR genotype, breast cancer risk, and clinicopathological features

Genotype determination was successful in 1240 breast cancer samples: 631 non-LQ/non-LQ, 516 non-LQ/insLQ; and 93 insLQ/insLQ. Genotypes were found to be in HWE ($P = 0.37$). The genotype distribution in control population cohorts EPOS (n = 1759: 903 non-LQ/non-LQ, 708 non-LQ/insLQ, and 148 insLQ/insLQ; HWE, $P = 0.58$) and ERGO (n = 3640: 1887 non-LQ/non-LQ, 1443 non-LQ/insLQ, and 310 insLQ/insLQ; 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 insLQ-LHR allele. For 751 patients, detailed clinical follow-up is available and patient and tumor characteristics were compared in noncarriers *vs.* 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).

insLQ-LHR and survival analysis

The Kaplan-Meier curves as a function of overall survival and DFS for insLQ carriers *vs.* noncarriers are shown in Fig. 1. The insLQ carriers show shorter overall survival compared with noncarriers (HR = 1.25; 95% CI, 1.03–1.51; $P = 0.03$) (Fig. 1A). Median overall survival is 112 months *vs.* 138 months for insLQ carriers *vs.* the noncarriers (26 months difference). In multivariate analysis, however, the insLQ allele was not an independent factor for overall survival (results not shown). The insLQ carriers show significantly shorter DFS compared with noncarriers by univariate analysis (HR = 1.34; 95% CI, 1.10–1.63; $P = 0.003$) (Fig. 1B). Next, the prognostic value of the insLQ allele was tested in the multivariate model for DFS, including classical prognostic factors age, menopausal sta-

TABLE 1. Characteristics of 751 breast cancer patients stratified by insLQ-LHR genotype

Feature	% of patients	insLQ genotype		*P* value[b]
		Noncarriers (%)	Carriers (%)[a]	
Breast cancer cases	100	379 (50.5)	372 (49.5)	
Age (yr)				
<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
Node 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]				
≤2 cm	35	146 (56.2)	114 (43.9)	
>2 cm	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 because of incomplete information on histological grade and/or receptor status.
[d] Tumors of unknown size (n = 10) are included as tumor size greater than 2 cm.
[e] Cutoff value used was 10 fmol/mg protein.

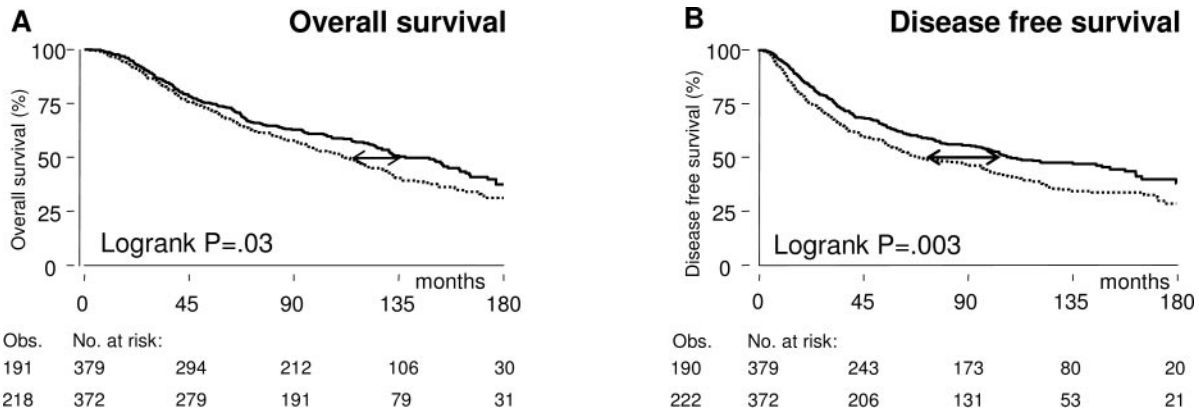

FIG. 1. Kaplan-Meier survival curves for patients homozygous for the non-LQ allele (*solid line*) *vs.* those heterozygous or homozygous for the insLQ allele (insLQ carriers; *dotted line*). Difference in median survival is indicated by a *two-headed arrow*. A and B, DFS and overall survival of noncarriers *vs.* insLQ carriers, respectively.

tus, 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), because 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 non-LQ-LHR transfected HEK293 cells. Cells expressing the insLQ-LHR construct responded to hCG with a slightly higher maximal increase in luciferase activity and a 1.9-fold lower EC_{50} ($P = 0.02$) than non-LQ-LHR-expressing cells (Fig. 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 B_{max} values for insLQ-LHR-expressing HEK293 cells were highly significantly increased ($P = 0.0006$) compared with non-LQ-LHR-expressing cells (Table 2). K_d values for insLQ- and non-LQ-LHRs from Scatchard analyses were not different (results not shown).

The LQ insertion enhances signal peptide efficiency

Signal peptides contain three 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 (24). Because the LQ insertion is located in the hydrophobic region of the signal peptide, the increased receptor sensitivity and cell surface expression may result from altered function of the signal peptide. This was tested using signal peptide-LHR-ECD constructs for Western blot analysis (see also *Subjects and Methods*).

Expression of insLQ-LHR-ECD and non-LQ-LHR-ECD in HEK293 cells yielded two major bands at 51 and 43 kDa (Fig.

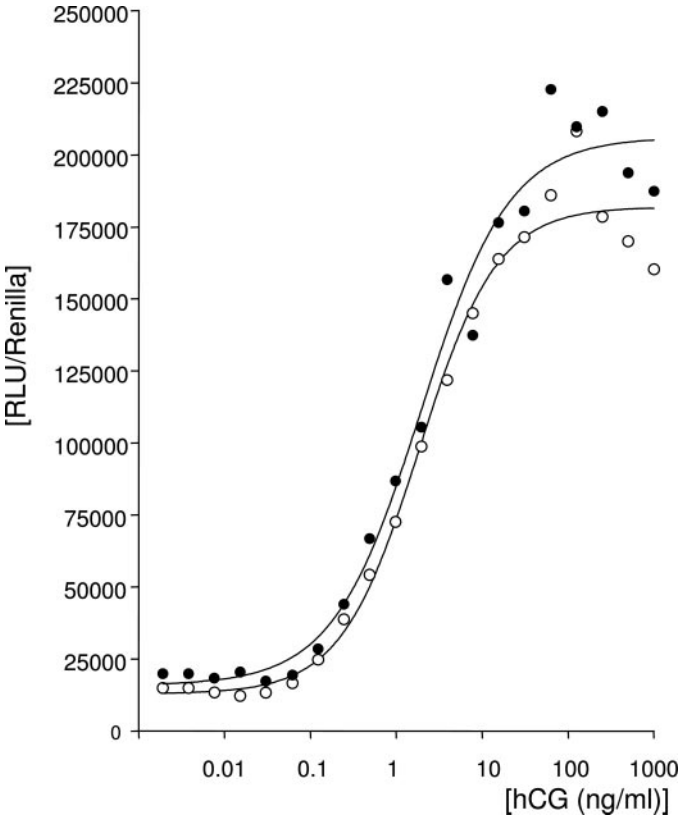

FIG. 2. hCG-induced cAMP response element activation by the insLQ- and non-LQ-LHR variants. HEK293 cells were transfected with pSG5-hLHR-HA1 with the LQ (●) or without the LQ insertion (○) in the presence of a cAMP-responsive luciferase reporter plasmid ($pCRE_6luc$). Subsequently, the cells were incubated 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 EC_{50}) and show a higher maximal response (see also Table 2). This graph is a representative of six independent experiments.

TABLE 2. EC_{50} and B_{max} values for insLQ- and nonLQ-LHR variant

	insLQ-LHR	non-LQ-LHR	n	*P* value[a]
$logEC_{50}$ (95% CI) (ng/ml)	0.15 (0.05–0.26)	0.29 (0.16–0.41)	7	0.004
B_{max} (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.

3). However, the intensity of the 43-kDa band was much lower in the insLQ-LHR-ECD-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 cotranslational 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 PNGaseF, 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 (Fig. 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 (25). 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 fragment 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 more LHR protein to be translocated into the endoplasmic reticulum membrane. To test this hypothesis, we constructed LHR-ECD constructs containing different signal peptides: insLQ, non-LQ; no h-region (delH); the hemagglutinin signal peptide (HA); and finally, no signal peptide (delSP) and subsequently expressed these proteins in HEK293 cells (Fig. 5). A gradual decrease in quantity of the cytosolic fragment from the delSP to delH to non-LQ 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 non-LQ lanes indicates that signal peptide cleavage has not occurred.

Discussion

Here we report our studies on the impact of the insLQ-LHR 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 insLQ-LHR 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 insLQ-LHR is not associated with breast cancer risk and cannot be used to predict the likelihood of female carriers to develop breast cancer, carriership of insLQ-LHR 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 *vs.* noncarriers, the insLQ independently predicts DFS 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 insLQ-LHR, 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 K_d 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

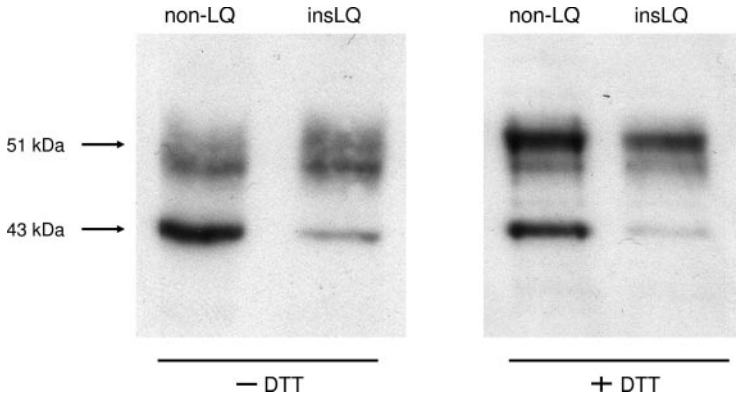

FIG. 3. The non-LQ-LHR-ECD transfected cells express more of a nonfolded 43-kDa LHR-ECD isoform. HEK293 cells were transfected with pSG5-hLHR-ECD-HA1 with (insLQ) or without (non-LQ) insertion. After protein extraction, denatured nonreduced (−DTT) or reduced (+DTT) extracts were run on separate 12% polyacrylamide 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.

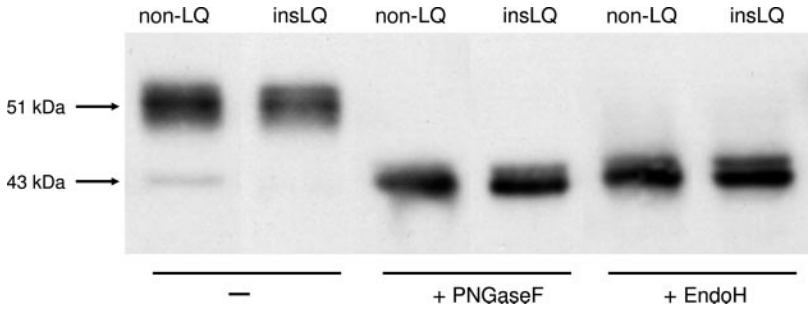

FIG. 4. The 43-kDa LHR-ECD species is not glycosylated, indicating a cytosolic location. HEK293 cells were transfected with pSG5-hLHR-ECD-HA1 with (insLQ) or without (non-LQ) insertion. Protein was extracted, left untreated (–), treated with PNGaseF or with EndoH, and subsequently separated using 8.5% 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.

cytosolic band in the cell lysates from non-LQ 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 (24), 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 insLQ-LHR variant with a decrease in the EC_{50} raises the question of whether increased expression of the LHR 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 non-LQ-LHR were transfected into HEK293 cells, we found that the EC_{50} for both receptor gene variants did not change with receptor density (data not shown). Thus, the elevated insLQ-LHR sensitivity is not caused by the increased expression as such, but the LQ insertion in the signal peptide may affect the quality of the LHR 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 (26, 27). This insertion was found to completely abolish hCG cell surface binding in transfected cells (27). Although no further detailed studies were performed in these reports, the results indicate that absent trafficking of the LHR was caused by a dysfunctional signal peptide.

Notwithstanding the large proportion of postmenopausal women in our breast cancer patient cohort, the effect of the insLQ-LHR polymorphism is strong. This may indicate that the 10–25% of ovarian steroid hormone production that is under LH control has a significant contribution to the recurrence of disease in these patients. 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

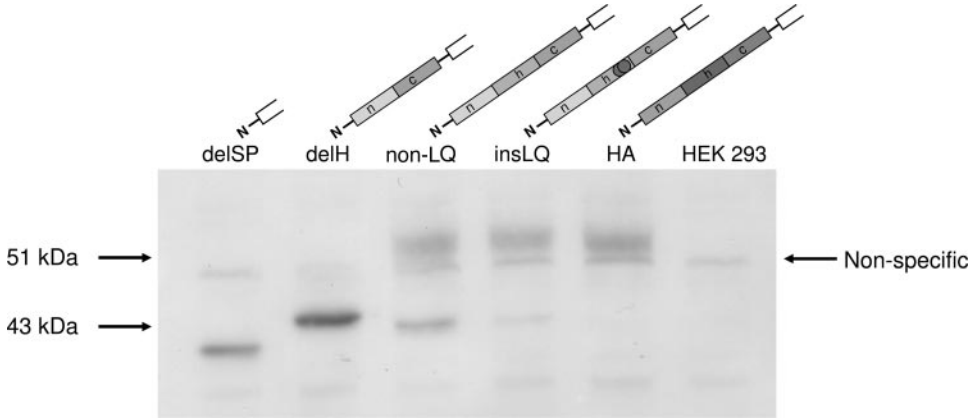

FIG. 5. LHR signal peptide analysis; the increased hydrophobicity of the insLQ signal peptide renders it more efficient. HEK293 cells were transfected using pSG5-hLHR-ECD-HA1 expression plasmids carrying different signal peptides. Protein was extracted, run on 12% polyacrylamide gels, and analyzed using 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; non-LQ, signal peptide without LQ insertion; insLQ, signal peptide with LQ insertion; HA, ECD with hemagglutinin signal peptide; HEK293, nontransfected cells. Nonspecific indicates a band that is present in the nontransfected cells. On the *left side*, the apparent molecular masses of the two LHR protein species are indicated.

transgenic pituitary LH-overexpressing mice (LHβ-CTP mice) develop mammary hyperplasia, but only in the presence of functional ovaries (28). Moreover, LHβ-CTP mice that develop mammary tumors display elevated serum levels of estrogens compared with 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 insLQ-LHR variant is associated with shorter DFS 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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