Loss of Heterozygosity at 11q22 Correlates with Low Progesterone Receptor Content in Epithelial Ovarian Cancer


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Abstract

Forty-seven epithelial ovarian cancers were analyzed for loss of heterozygosity (LOH) at D11S35 (11q22), close to the progesterone receptor (PR) gene, and for tumoral estrogen receptor (ER) and PR content. Thirty-eight of 47 tumors were informative, and, of these, 14 exhibited LOH. There was a significant association (P = 0.014) between D11S35 LOH and low tumoral PR content. For all informative tumors, there was no correlation between ER and PR; however, exclusion of tumors with LOH from the informative series revealed a linear correlation between tumoral ER and PR (P = 0.013), and established ER (P = 0.025) and PR (P = 0.05) content as significant factors in relation to patient survival. Patients with ER-rich tumors with D11S35 LOH had particularly poor survival compared with ER-rich, D11S35 heterozygous, no loss patients (P = 0.014).

Analysis of the same tumors using two other microsatellites, D11S935 (11p13) and NM23 (17q22), showed no statistically significant relationships, although there were nonsignificant trends for the correlation of ER and PR expression in informative tumors without allele loss at these loci.

We propose that genomic structural alteration at or close to the PR gene locus has biological and clinical sequelae in ovarian cancer.

Introduction

Ovarian cancer is the leading cause of death from gynecological malignancy, and its etiology is poorly understood. Epidemiological and animal studies indicate that ovarian cancer may be an endocrine-related tumor. Chronic estrogen and progesterin administration in animal studies results in ovarian cancer (1, 2). "Incessant ovulation" appears to be a major premenopausal risk factor (3, 4). Nulliparity and a low mean number of pregnancies have been associated with increased risk (5). Increased total pregnancy and lactation time, and the use of oral contraceptives (6, 7) confer a protective effect. The high gonadotrophic milieu of the postmenopausal state may confer additional risk (8). Ovarian cancer has been reported to respond to antiestrogens in about 10–20% of cases and to progestins with an average of 36%, with a range between 0 and 60% (9, 10).

The ovary is the main site of synthesis of estrogen and progesterone, and is also a target organ for these hormones. The actions of these hormones are mediated by specific intracellular receptors that function as hormone-inducible nuclear transcription factors with context-specific, often conflicting effects on proliferation and differentiation of target tissues (11).

PR3 is regulated by estrogen via ER (12); estradiol-induced PR expression has been demonstrated in ovarian cancer cell lines that express ER (13).

That histopathological subtypes of epithelial ovarian cancer differ in their PR expression is suggested by at least six reports demonstrating that endometrioid ovarian tumors contain relatively more PR than other histological types (9), and that PR positivity is associated with well-differentiated ovarian tumors in premenopausal women (14). High PR content in breast (15) and ovarian (16, 17) cancer has been shown to correlate with a favorable prognosis, although the biological relevance of steroid hormones and their receptors to ovarian cancer remains controversial.

The PR gene has been localized to chromosome 11q22 (18, 19), so that it is now possible to determine whether structural or regulatory disturbances of the chromosomal locus affect the PR content of endocrine-regulated epithelial cancers and hence these tumors' relationships with prognosis. In breast cancer, allele losses on chromosome 11p were found to correlate with PR-negative tumors (20). Despite initial studies suggesting that 11q LOH is an infrequent event (21, 22), several recent studies suggest that it is, in fact, a common event in breast cancer (23–25). Furthermore, a recent breast cancer cytogenetic study (26) demonstrated that 11q but not 6q- tumors contained a low PR:ER ratio, whereas 6q- but not 11q- tumors had a low ER:PR ratio, suggesting some role for genomic structural alteration in the regulation of tumoral steroid receptor content.

In ovarian cancer, evidence has accrued to suggest that distal chromosome 11q cytogenetic abnormalities (27) and LOH (28, 29) are frequent events of clinicopathological significance.

In this study, we used a polymorphic microsatellite lying close to the PR gene to determine allelic loss in ovarian cancer. At the time of the study, no intragenic microsatellite with high heterozygosity was available for the PR gene, RFLP analysis for the PR gene being even less informative. D11S35 was selected for loss of heterozygosity (LOH) at 11q22, close to the progesterone receptor (PR) gene, and for tumoral estrogen receptor (ER) and PR content. Thirty-eight of 47 tumors were informative, and, of these, 14 exhibited LOH. There was a significant association (P = 0.014) between D11S35 LOH and low tumoral PR content. For all informative tumors, there was no correlation between ER and PR; however, exclusion of tumors with LOH from the informative series revealed a linear correlation between tumoral ER and PR (P = 0.013), and established ER (P = 0.025) and PR (P = 0.05) content as significant factors in relation to patient survival. Patients with ER-rich tumors with D11S35 LOH had particularly poor survival compared with ER-rich, D11S35 heterozygous, no loss patients (P = 0.014).

Analysis of the same tumors using two other microsatellites, D11S935 (11p13) and NM23 (17q22), showed no statistically significant relationships, although there were nonsignificant trends for the correlation of ER and PR expression in informative tumors without allele loss at these loci.

We propose that genomic structural alteration at or close to the PR gene locus has biological and clinical sequelae in ovarian cancer.
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because it was the closest microsatellite to the PR gene (with the same Kosambi genetic distance of 0.41) and had an acceptably high heterozygosity rate (30, 31). Subsequent to completing the study, a high resolution physical map of chromosome 11 suggested that it lay slightly (3 centirays, approximately 150 kb) telomeric to the PR gene (32). Two other incidental polymorphic microsatellites were randomly selected to demonstrate specificity of the correlation: D11S935 (11p13) (33) and NM23 (17q22) (34). The PR and ER content of these tumors was then measured, and the relationship between allele loss and tumoral steroid receptor content was assessed.

Materials and Methods

Clinical Specimens. Fresh primary ovarian tumor tissue from 47 patients (mean age, 60 years) with epithelial ovarian cancer was transferred directly to dry ice or liquid nitrogen perioperatively, and stored at −70°C until processing. Hepar- inized blood was obtained from 34 patients postoperatively. In the remaining 13 patients, normal tissue was obtained from formalin-fixed blocks. The International Federation for Gynecology and Obstetrics staging, histopathology, and differentiation state were determined and reviewed in a standardised fashion at a multidisciplinary combined Gynecological Oncology Clinic. Treatment was planned and delivered in accordance with standard protocols, which consisted of the best possible surgical debulking followed by adjuvant/palliative chemotherapy where appropriate. Minimum follow-up on living patients is 24 months, maximum follow-up is 60 months, and all deaths that have occurred have been due to ovarian cancer.

Numbers of patients with tumors that were ultimately informative for steroid receptor content and constitutive heterozygosity were reduced to between 31 and 34, depending on the microsatellite used. Patient characteristics are outlined in Table 1.

DNA Extraction. DNA from fresh frozen tissue was extracted using a standard technique as described previously (35). Extraction of DNA from fixed specimens was performed by cutting 3- × 10-μm sections from the block, dewaxing in xylene for 30 min twice, removing the xylene by washing three times in 100% ethanol, and desiccating the specimen under heat. Proteinase K (200 μg/ml) digestion was performed overnight at 37°C, and then the proteinase K was heat inactivated at 95°C for 5 min and debris was removed by centrifugation. The resultant preparation provided adequate DNA template for PCR.

Oligonucleotide Primers. The oligonucleotide primer sequences for D11S35 (30) were S35-S (5'-ACAATTTGGA-TTACTACTAGCACC-3') and S35-AS (5'-AACTATGTATT-TGTATCGATCACC-3'). For D11S935 (33), the primer sequences were amf 254b9-CA (5'-TACTAACCAAAGAGT-TGGGG-3') and amf 254b9-GT (5'-CTATCATTCAAGAAA-TTTGGC-3'). The primer sequences for NM23 (34) were (5'-TTCACCCGGGTAGAAGAACC-3') and (5'-TCTCAGTACTTCCGGTGACC-3').

PCR and Polymorphic Microsatellite Detection. PCR was performed in a reaction volume of 50 μl under the following reaction conditions: 94°C for 3 min/1 cycle, 94°C for 1 min/55°C for 1 min/72°C for 1 min × 35 cycles, and 72°C for 5 min × 1 cycle in a Hybrid thermocycler. Reaction mix contained 50 mM KCl, 10 mM Tris-HCl (pH 9), 1% Triton X-100, 1.5 mM MgCl₂, 200 μM deoxyribonucleotide triphosphate, 1.25 units Taq polymerase, 10 pmol of each primer, and 100 ng DNA.

Ten μl of the PCR reaction product were loaded onto 8% denaturing polyacrylamide gel, separated by electrophoresis, passively transferred to Hybond nylon, and probed with a ³²P end-labeled poly(CA) probe as previously described (36). Two observers visually analyzed the autoradiographs and recorded allele imbalance when there was clear reduction in the intensity of one allele in the tumor DNA.

Determination of ER and PR by EIA. Tumor specimens snap frozen at operation were homogenized in buffer (10 mM Tris, 0.25 mM sucrose, 1 mM EDTA, pH 8.0, plus 1% v/v monothioglycerol and 10% v/v glycerol) as previously described (37). After centrifugation at 105,000 × g, the supernatant cytosol was assayed using EIA kits according to the manufacturer’s method (ER-EIA and PR-EIA kits; Abbott Laboratories, Maidenhead, Berkshire, United Kingdom; Ref. 38). The protein content of the cytosol was determined according to the method of Bradford (39), and receptor concentrations were expressed as fmol/mg protein. The PR antibody used was Kd68, a rat mAb which recognizes both the A and B subunits of PR (and therefore recognizes sequences downstream of codon 165, the start site for subunit A) and binds both free and complexed PR, presumably avoiding the hormone-binding site (40).

Recognizing that cutoff values for steroid receptor levels are controversial, ER poor or PR poor was defined as less than 30 fmol/mg protein and ER rich or PR rich was defined as 30 fmol/mg protein or more. These values derive from reports describing clonogenic assays of human ovarian carcinoma cells from clinical specimens, human ovarian cancer cell lines, and
Table 1. Clinicopathological parameters of the patient series

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<th>Survival (mo)</th>
<th>Alive?</th>
<th>Stage (FIGO)*</th>
<th>Histology</th>
<th>Grade</th>
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*FIGO, International Federation of Gynecology and Obstetrics; A, alive at most recent analysis; U, uninformative/homozygous; L, LOH; H, heterozygous with no loss; NK, not known.

Clinical work showing that these approximate cutoff values for ER and PR represent a reasonable index of hormone sensitivity (13, 41, 42).

Statistical Analysis. The Mann-Whitney U test was used to compare the medians of tumor PR content in populations with and without LOH for the three microsatellite markers. Two-tailed P values were calculated.

Spearman's rank correlation was used to examine correlations between tumor ER and PR content in those with and without LOH. Kaplan-Meier/log rank analysis was performed to determine any relationship between the allele loss and survival in D11S35 informative subgroups.

Results

Allele Loss Analysis. Fig. 1 shows examples of the three microsatellite markers used in this study. Of 47 epithelial ovarian cancer patients, 38 were informative (constitutively heterozygous) at D11S35 (72%). Of these, 14 (37%) exhibited LOH at this locus and 24 retained heterozygosity. There was inadequate tissue for ER and PR quantification in seven of these informative specimens (two with LOH and five heterozygous, no loss), and therefore the final allele loss rate in this group was 12 (39%) of 31. For D11S935, the final allele loss rate was 13 (39%) of 33 and for NM23 (17q22), the final allele loss rate was 19 (61%) of 31 (Table 1). The sample contained nine informative endometrioid ovarian cancers, none of which exhibited LOH at D11S35. Fisher's exact test for endometrioid versus other histologies revealed a significant correlation between D11S35 retention of heterozygosity and endometrioid histology (P = 0.04) in the 38 informative tumors.

PR Distribution and Relationships between Allele Loss and PR. Sufficient material was available for ER and PR measurement in 39 of the 47 tumors (Table 1), and this estimation was performed in a blind fashion at a different institution (by R. A. H.). A summary of PR data distribution by locus and allele loss status is shown in Table 2. The mean tumoral PR concentration for the 12 patients with LOH at D11S35 was 5.2
The mean tumoral PR concentration for the 19 heterozygous patients with no loss at D11S35 was 51.4 fmol/mg, with a median of 21 fmol/mg (range, 1–187 fmol/mg). For D11S935 the median PR of the LOH group was 7.5 fmol/mg (range, 1–1144 fmol/mg). For NM23 the median for the LOH group was 6 fmol/mg (range, 1–187 fmol/mg), and for the no-LOH group 10.5 fmol/mg (3–1144 fmol/mg). Plotting tumoral PR concentration against D11S35 allele loss status as a log scatter plot (Fig. 2) shows the association of low/absent tumoral PR with D11S35 LOH, but this clear difference is not apparent for the other two markers.

The difference between the medians in the D11S35 LOH/no-LOH groups was statistically significant with the Mann-Whitney U test \( P = 0.014 \). These analyses were repeated for a chromosome 11 short arm locus, D11S935 at 11p13; and for NM23, a chromosome 17 long arm locus (17q22). Although large, apparent differences were also noted (see above) for the LOH and heterozygous/no loss groups at these two loci, no
Fig. 3 Scatter plot of natural log-transformed (ln) ER and PR values comparing tumors with allele loss (LOH) and tumors that were heterozygous without LOH (Het). Data presented: a, D11S35 (11q22); b, D11S935 (11p13); and c, NM23.

In PR (fmol/mg protein)

In ER (fmol/mg protein)

In PR (fmol/mg protein)
Relationship between ER and PR. The whole D11S35 informative group was analyzed for linear correlation between tumoral ER and PR content by Spearman's rank correlation. There was no significant correlation for the whole group. However, when patients with D11S35 LOH were removed from the analysis, there was a linear correlation between tumoral PR and ER content (Spearman's rank correlation, \( P = 0.013 \)). Fig. 3a shows a scatter plot of D11S35 heterozygous/no loss group and LOH group in terms of their ER versus matched PR contents. The analysis was repeated for D11S935 (Fig. 3b) and NM23 (Fig. 3c). Although there was no significant correlation between ER and PR content in either the heterozygous/no loss group or the LOH group for either of these latter markers, it is interesting that both had trends to significance in their respective heterozygous/no loss group, but these trends were not different between the D11S935 marker on 11p (\( P = 0.058 \)) and NM23 on 17q (\( P = 0.063 \)).

Survival Analysis. Kaplan-Meier/log rank survival analysis for ER-rich versus ER-poor patients showed no significant difference between the two groups (data not shown). PR-rich versus PR-poor showed a nonsignificant trend in favor of the PR-rich patients (\( P = 0.08 \), data not shown). Comparing patients with tumor D11S35 LOH versus patients with D11S35 heterozygous/no loss tumors also showed no difference (data not shown). However, analysis of patients with D11S35 heterozygous/no loss tumors only showed a survival advantage for patients with PR-rich (\( \geq 30 \) fmol/mg) tumors compared to those with PR-poor (<30 fmol/mg) tumors (\( P = 0.05 \), Fig. 4a) and PR-poor patients did equally as badly whether they had LOH at D11S35 or not (Fig. 4b).

D11S35 heterozygous/no loss patients with ER-rich tumors (\( \geq 30 \) fmol/mg) had a better outcome compared to those with ER-poor (<30 fmol/mg) tumors (\( P = 0.025 \); Fig. 5a).

Patients who had informative ER-rich (\( \geq 30 \) fmol/mg) tumors were then plotted according to whether they had LOH at D11S35 or not (Fig. 5b), and a significant (\( P = 0.014 \)) survival differences of mean and median PR concentration by allele loss status were recorded (Table 2 and Fig. 2).

**Fig. 4** Kaplan-Meier survival curves with log rank analysis for D11S35 heterozygous, no loss (HET/no loss) ovarian cancer patients subdivided by PR content (a) and PR-poor patients subdivided by D11S35 allele loss status (b). Vertical lines, censored patients.
advantage in favor of those with no LOH at D11S35 was observed (although this subgroup comprised only 12 patients).

Discussion

This report demonstrates that allele loss close to the PR gene on chromosome 11q22 is associated with low tumoral PR content, suggesting the possibility that genomic structural disruption including or flanking the PR gene may have a significant role in the dysregulation of the PR content of at least some ovarian cancers.

That allele loss at the PR gene locus may genuinely disrupt PR expression is also suggested by the observation that when tumors with LOH at the PR gene locus are excluded from the informative sample, a significant correlation between tumoral ER and PR content is revealed, indicating that in many of these heterozygous tumors, the regulatory link between estradiol, ER, and PR is intact, and that the converse is true in those with LOH at the PR gene locus. Although trends also are observed for distantly physically linked (D11S935; 11p) and unlinked (NM23; 17q) markers, they do not reach conventional significance levels, nor are the trends substantially different from each other. (One might have expected that substantial deletions involving 11p as well as 11q, resulting in co-loss of the chromosome 11 markers, might be responsible for a higher level of borderline significance with this distant but physically linked marker than for an unlinked marker.)
nonsignificant trends is consistent with the likelihood that PR gene disruption will not be the only mechanism of tumoral PR down-regulation.

Steroid receptor negativity in the literature is variably and arbitrarily defined: in the case of ER from as low as 1–5 fmol/mg protein (indicating the background level of ELA) to between 15 and 20 fmol/mg (indicating the clinical levels below which hormonal therapeutic manipulations are unlikely to yield benefit). Interestingly, all tumors with LOH at D11S35 had PR of 11 fmol/mg or less.

Not all previous literature is supportive of our observations. Fuqua et al. (22) investigated the status of RFLPs within the PR gene in a large series of breast tumors and found no correlation with PR expression. Additionally, none of five informative breast cancer normal/tumor pairs exhibited allele loss using one of these RFLPs, and the authors concluded that PR gene rearrangements were not a significant mechanism for the alteration of tumoral PR content. However, the extent of contaminating normal stroma (containing constitutive DNA) in these tumors was not discussed, and the six human breast cancer cell lines tested in the study were all homozygous at the reported RFLPs.

The observed tight link between D11S35 LOH and loss of PR expression may be due to variations on the classical Knudson two-hit model (43). Loss of imprinting (for which there is some evidence on 11q, although not in ovarian cancer; Ref. 44) of one allele in association with large deletions of the other may occur in concert and may affect regulatory rather than structural regions, perhaps explaining the negative findings of Fuqua et al. (22).

Clearly, analysis of the PR gene locus and RNA species in these tumors is required to demonstrate the nature of these abnormalities, since the Kd68 anti-PR antibody may not detect truncated forms of the PR protein.

Analogous work with the ER in breast cancer has demonstrated an ER variant lacking exon 5 of the hormone-binding domain in ER-negative/PR-positive breast cancers that constitutively activates the ER response (45). A truncated ER species which inhibits the binding of ER to its response element has been identified in ER-positive/PR-negative breast tumors (46), and an ER variant co-species lacking exon 3 of the DNA-binding domain was identified from ER-positive tumors which was unable to function as a transcriptional inducer (47).

Kaplan-Meier survival analysis in the small subgroups of our series must be treated with caution given the sample sizes. Nevertheless, it is interesting that patients with ER-rich tumors and allele loss close to the PR gene had particularly poor survival; all six patients having died within 2½ years, whereas those with ER-rich tumors that retain heterozygosity at the PR gene locus have particularly good survival, with five of the six patients alive beyond 3 years. Clearly, such small numbers inevitably introduce bias, and this observation should perhaps stimulate a prospective analysis in a larger series of patients.

Our finding that none of the endometrioid tumors had LOH close to the PR gene is intriguing, and consistent with the many reports that this histological subtype is associated with higher tumoral PR content (9) and the finding in one study that endometrioid tumors can have a 54% response rate to progesterin therapy (42).

The analysis of PR content in relation to survival may well be complicated by the presence of LOH in association with abnormal PR protein, which is undetected by antibodies. Removing tumors with LOH from our series and examining those that had retained heterozygosity resulted in the survival patterns that would have been expected; patients with ER-rich and PR-rich tumors exhibiting a survival advantage (Figs. 4 and 5).

We suggest that structural alteration at the PR locus on chromosome 11q22 may represent a significant mechanism for the dysregulation of tumoral PR content. Further analysis of the PR gene structure, expression, and associated clinical correlations in these tumors to determine the mechanism(s) involved will be important and is currently under way in our laboratory.

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References

Loss of heterozygosity at 11q22 correlates with low progesterone receptor content in epithelial ovarian cancer.

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