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Molecular Clustering Based on *ER*α and *EIG121* Predicts Survival in High-Grade Serous Carcinoma of the Ovary/ Peritoneum

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Abstract

Assessment of estrogen receptor expression by immunohistochemistry has yielded inconsistent results as a prognostic indicator in ovarian carcinoma. In breast and endometrial carcinomas, panels of estrogen-induced genes have shown improved prognostic capability over the use of estrogen receptor immunohistochemistry alone. For both breast and endometrial cancer, over-expression of estrogen-induced genes is associated with better prognosis. We hypothesized that analysis of a panel of estrogen-induced genes can predict outcome in ovarian carcinoma and potentially differentiate between tumors of varying hormonal responsiveness. From a cohort of 219 women undergoing ovarian cancer surgery from 2004 –2007, 83 patients were selected for inclusion. All patients had advanced stage ovarian/primary peritoneal high-grade serous carcinoma and underwent primary surgical debulking followed by adjuvant treatment with platinum and taxane agents. The expression of *ER*α and six genes known to be induced by estrogen in the female reproductive tract (*EIG121, sFRP1, sFRP4, RALDH2, PR, IGF-1*) was measured using qRT-PCR. Unsupervised cluster analyses were used to categorize patients as high or low gene expressors. Gene expression results were then compared to those for estrogen receptor immunohistochemistry. Clusters were compared using chi-square analyses, and Cox proportional hazards were used to evaluate survival outcomes. Median follow-up time was 38.7 months (range 1–68). A cluster defined by *EIG121* and *ER*α segregated tumors into distinct groups of high and low gene expressors. Shorter overall survival was associated with high gene expression (HR 2.84

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[1.11–7.30], p=0.03), even after adjustment for other covariates. No difference in estrogen receptor immunohistochemistry expression was noted between gene clusters. In contrast to other hormonally-driven cancers, high expression of *ER*α and the estrogen-induced gene *EIG121* predicts shorter overall survival in patients with high-grade serous ovarian carcinoma. Such a biomarker panel may potentially be used to guide management with estrogen antagonists in this patient population.

Keywords

estrogen-induced genes; ovarian high-grade serous carcinoma; hormone antagonism

Introduction

Ovarian cancer is the fifth most common cause of cancer death in American women (1). Carcinoma of the ovary is typically diagnosed in women older than 50 years of age, and the majority of cancers are advanced at time of diagnosis (1). The majority of ovarian cancer types are epithelial in origin, and of these, high-grade serous carcinoma is the most common. For the majority of patients, the treatment of high-grade serous carcinoma is surgical debulking followed by adjuvant chemotherapy with combination platinum and taxane agents. The five-year overall survival for women with high-grade serous carcinoma is less than 30%, due in part to its asymptomatic progression from localized tumor to metastatic disease, high rate of recurrence, development of chemoresistance, and the paucity of agents effective at treating recurrent disease (1–3).

Because ovarian function is, at least in part, hormonally regulated, it is generally presumed that ovarian carcinoma should be somewhat responsive to hormonal manipulation. Given the success of hormone manipulating agents in the treatment of breast, endometrial, and prostate carcinomas, incorporating agents aimed at antagonizing hormone-dependent proliferative pathways into the ovarian cancer treatment algorithm has been proposed. Agents targeting estrogen pathways are of particular interest, especially since the inhibition of estrogen in estrogen receptor (ER) positive breast cancers translates into reduced risk of recurrence and increased survival (4). Despite the fact that more than 60% of ovarian cancers demonstrate ER positivity (5), inhibition of estrogen-associated pathways has not translated into significant improvements in patient outcomes. In a meta-analysis of 18 ovarian cancer trials which treated patients with tamoxifen, a selective estrogen receptor modulator, the collective response rate was only 13% (6). Pre-treatment determination of tumor ER status has also demonstrated poor predictive ability of response to treatment. For example, Schwartz *et al* (7) performed a prospective trial of chemotherapy with or without tamoxifen in patients with advanced stage ovarian cancer and noted that there was no relation between hormone receptor status and survival outcomes. More recent studies evaluating the activity of aromatase inhibitors in ER-positive patients have reported response rates between 3–17%, with stable disease achieved in up to 26% (8, 9). Taken together, these investigations suggest that there is a subset of women with ovarian cancer who will have some degree of response to hormone antagonism, but ER immunohistochemistry may not be a sufficient means of identifying these patients.

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An alternative to ER immunohistochemical assessment is to evaluate genes known to be induced by estrogen, a strategy which has resulted in improved capability to segregate tumors based on hormone sensitivity in other malignancies. In breast cancer, quantitative examination of estrogen-regulated genes helps to detect subgroups within ER-positive tumors with differing survival parameters, even when accounting for tumor characteristics such as lymph node positivity, tumor size, and the use of chemotherapy (10). Validated using specimens provided by a several different investigators, a gene panel proposed by Oh *et al* (10) accurately predicted patients with invasive breast ductal carcinoma who had markedly different relapse-free survivals. Similar findings have been reported in endometrial cancer. In 2009, Westin *et al* (11) described a panel of estrogen-induced genes in patients with endometrial carcinoma which identified two distinct clusters based on degree of gene expression. Higher estrogen-regulated gene expression was predictive of improved recurrence-free survival and was able to distinguish between high/intermediate- and low-risk tumors with a false negative rate of only 4.8% (11).

Given the findings in breast and endometrial carcinoma that estrogen-regulated genes demonstrate prognostic capability, it is possible that analyzing estrogen-regulated gene expression may have similar utility for ovarian cancer patients. Determining which subset of women with ovarian cancer who may potentially respond to estrogen antagonism would afford the oncologist the ability to initiate such treatment earlier in the disease course, either alone or in combination with other therapies. Our primary aim was to quantify the expression of estrogen-induced genes in a cohort of women with the most common ovarian cancer, high-grade serous carcinoma, and determine if differential expression was predictive of clinical outcomes. Secondarily, we compared gene expression to immunohistochemical assessment of ER, the current standard for judging hormone sensitivity, to determine if immunohistochemistry accurately predicts tumor molecular profiles. We hypothesized that this examination of estrogen-induced genes would identify subsets of patients with different clinical characteristics and distinct survival outcomes. Because higher estrogen-induced gene expression portends improved prognosis in other hormone-sensitive tumors, we expected that a similar relationship would be observed in this cohort of ovarian cancer patients.

Materials and Methods

Patient Selection and Clinical Data Acquisition

After IRB approval, a review of the institutional Tumor Bank identified two-hundred nineteen (219) patients from whom ovarian or primary peritoneal carcinoma specimens were obtained at the time of tumor-reductive surgeries between 2004 and 2007. Pathologic diagnoses were made by gynecologic pathologists after microscopic review of hematoxylin and eosin-stained slides derived from surgical specimens containing ovarian or primary peritoneal carcinomas. Patient clinical characteristics were obtained by a review of electronic medical records and included date of birth, race, anthropometric variables, date of surgical staging, debulking status, primary and secondary chemotherapy regimens, date of recurrence, date of last follow-up, and disease status at last follow-up. Both clinical and pathologic features were utilized to determine inclusion criteria. Patients selected for

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inclusion in the study demonstrated only advanced stage (III or IV), high-grade serous ovarian or primary peritoneal carcinoma. Additionally, all patients received treatment with platinum and taxane agents as first-line adjuvant chemotherapy. Specific exclusion criteria included treatment with neoadjuvant chemotherapy, consolidation/maintenance chemotherapy, and first-line treatment regimens that were experimental protocols or not platinum-based. Body mass indices were categorized by World Health Organization definitions of normal weight, overweight, and obese. Chemotherapy resistance was determined using Gynecologic Oncology Group criteria, which include 1) disease progression while on a first-line platinum-based regimen; 2) tumor progression within six months of completion of platinum-based therapy; and 3) persistent clinically measurable disease with best response as stable disease at the completion of first-line therapy (12).

Gene Selection and RNA Preparation

Seven genes (*ERα, PR, EIG121, IGF-1, RALDH2, sFRP1, sFRP4*) were included for transcript quantification by qRT-PCR. Our research group has previously used a genomics approach to identify that *EIG121, RALDH2, sFRP1*, and *sFRP4* are highly induced by estrogen in the human female reproductive tract (11, 13, 14). *PR* and *IGF-1* are classical estrogen-induced genes (15, 16). Transcript analysis of *ERα* was included for subsequent comparisons to ER immunohistochemistry studies. After microscopic confirmation of tumor histology and presence of greater than 70% viable tumor, RNA was extracted from frozen tissues, prepared using the TriReagent (Molecular Research Center, Cincinnati, OH), and precipitated with isopropyl alcohol. Ultraviolet spectrometry was used to confirm adequate concentrations of RNA in each prepared specimen. RNA was applied to RNeasy spin columns (QIAGEN, Valencia, CA), eluted, and treated with 10% RNAse-free DNAse solution. Specimens were incubated for 30 minutes at 37°C and subsequently treated for 10 minutes at 75°C to inactivate DNAse I. Post-incubation RNA was stored at −80°C until quantitative RT-PCR is performed.

Reverse Transcription and Real-Time Polymerase Chain Reaction

Probes and primers (TaqMan® probe) for the gene panel are shown in Table 1. Aliquots (100 ng) of each RNA were reverse transcribed in quadruplicate (including a no reverse transcriptase control) with 300 nM assay specific reverse primer, 4 mM MgCl2, 500 μM dNTPs and 10 Unit of MMLV Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) at 50°C for 30 min, followed by 72°C for 5 min. Forty μl of PCR mix containing 1X PCR buffer, 300 nM specific forward and reverse primers, 4 mM $MgCl_2$, Taq DNA polymerase and 100 nM fluorogenic probe were then added to each 10-μl RT reaction. Amplification was performed by use of the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) at 95°C for 1min, followed by 40 cycles of a 12-sec step at 95°C and a 1-min step at 60°C. Synthetic RNA or single strand DNA amplicon standards were serially diluted in water containing 100 ng/μl yeast tRNA (Invitrogen, Carlsbad, CA).

Immunohistochemistry

Immunohistochemistry for ERα (clone 6F11, 1:35; Novacastra/Leica Microsystems, Chicago, IL) was performed using standard histological techniques. Antigen retrieval was accomplished with citrate buffer for 30 min at 100 °C. Stained slides were microscopically reviewed in a blinded fashion by the study primary author (MPS) and a gynecologic pathologist (RRB). ERα protein expression was scored semi-quantitatively as per cent tumor with 2+ or 3+ nuclear staining. Tumors were considered positive if 10% of tumor cell nuclei demonstrated strong (2+ or 3+) expression.

Statistical Analyses

Statistical analyses were performed using STATA 10.0 (StataCorp, College Station, TX) and SPSS 17.0 (Chicago, IL). Summary statistics were generated to describe the study population. The Wilcoxon rank-sum test was used to compare mean gene expression in relation to clinical variables, and linear regression was used to compare gene expression with continuous clinical variables (body mass index, age). Chi-square analyses were used to describe the relationship between categorical variables, and logistic regression was used to determine associations between clinical variables and categorical outcomes. Recurrence-free survival (RFS) and overall survival (OS) for each gene was analyzed using Cox proportional hazards models and the Kaplan-Meier method. RFS was defined as the interval between initial surgical treatment and date of recurrence. OS was defined as the interval between initial surgical treatment and date of last follow-up or death. A p-value of <0.05 was considered statistically significant, and all tests were two-sided.

Unsupervised cluster analyses were performed for each gene separately, and in various permutations, to include all six estrogen-induced genes and *ER*α. Specific clusters were selected based on their potential ability to segregate tumors into distinct groups, and analyzed to compare differences in survival, outcome, and demographic characteristics between these groups. Characteristics between cluster groups were compared using chi-square or Fisher's exact analyses, when appropriate. Univariate Cox proportional hazards models were used to analyze gene associations between survival, gene expression clusters, and other patient clinical features. Variables with p-values <0.15 in the univariate analysis were included in the multivariate model. The Kaplan-Meier method and log-rank test were used to compare survival outcomes.

The Wilcoxon rank-sum test was used to compare percentage of ER immunohistochemical expression between tumor samples from the different clusters. Fisher's exact test was used to compare clusters when protein expression was characterized categorically. All tests were two-sided, and a p-value of <0.05 was considered statistically significant.

Results

Two hundred nineteen (219) women underwent surgery for ovarian or primary peritoneal carcinoma during the study interval. Application of the inclusion criteria resulted in a relatively homogenous cohort for study numbering 113 patients of which frozen tissues for 83 were available from the institutional Gynecologic Oncology Tumor Bank. Patients were

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followed for a median of 38.7 months (range 0.5–67.8) from the time of their cancer diagnoses. The demographic features of the cohort of 83 patients are summarized in Table 2. The mean age at diagnosis was 62.6 years (range 34.5–85.9), and the majority of patients were Caucasian. A significant proportion of patients were overweight or obese (42.2%), and more than 18% had a BMI 30 kg/m^2. Fifty-two (62.7%) patients had an optimal surgical debulking, defined as less than 1 cm of residual disease at the conclusion of primary cytoreductive therapy, and more than 90% of the patients recurred during the follow-up interval. No association between either age (p=0.22) or race (p=0.88) and frequency of optimal debulking was observed. However, as BMI increased, the frequency of optimal debulking decreased (p=0.03).

In a univariate analysis, increased expression of *EIG121* demonstrated a statistically significant association with worse OS (HR 1.21 [1.09–1.35], p<0.001) (Table 3). Increased *RALDH2* expression was also associated with reduced OS, though to a lesser degree (HR 1.18 [1.03–1.35], p=0.016). There was a trend towards a negative association between *sFRP1* expression and OS, but the association was not statistically significant. In a multivariate model which included gene expression and patient clinical characteristics, both *sFRP1* (HR 1.04 [1.00–1.07], p=0.028) and *EIG121* (HR 1.20 [CI 1.08–1.35], p=0.001) remained independently prognostic of worse OS. When RFS was considered, greater *EIG121* expression was associated with shorter time to recurrence (HR 1.13 [CI 1.02–1.26], p=0.021). *EIG121* was the only gene in the panel whose expression demonstrated such an effect. When accounting for patient characteristics in a multivariate model, *EIG121* remained predictive of RFS (HR 1.14 [CI 1.02–1.27], p=0.02).

Unsupervised cluster analyses based on gene expression were performed and resulted in a total of 246 unique clusters. All clusters were reviewed, and those which appeared to best segregate patients into two distinct groups were considered for more detailed analysis. Ultimately, the cluster defined by *EIG121* and *ER*α was selected for further examination because that combination appeared to reasonably segregate tumors into distinct groups of high expressors (Cluster 1) and low/intermediate expressors (Cluster 2) (Figure 1). The characteristics of the patients in each cluster are described in Table 4. There was no difference between clusters with regards to BMI, age at diagnosis, frequency of optimal debulking, or recurrence. There was a greater proportion of non-white patients in Cluster 1 than Cluster 2 (41% vs. 20%, p=0.03).

A univariate analysis of patient characteristics and gene expression clusters in relation to OS is shown in Table 5. Age at diagnosis (p=0.56), race (p=0.57), and BMI (p=0.60) were not significantly associated with OS. The presence of residual disease at the time of surgical debulking is a classical indicator of poor prognosis in ovarian carcinoma (17). In our cohort, we similarly found that presence of residual disease following surgical debulking significantly reduced OS (HR 2.65 [1.13–6.18], p=0.02), as did categorization into Cluster 1 (HR 3.02 [1.19–7.65], p=0.02). In a multivariate analysis, both residual disease (HR 2.53 [1.08–5.95], p=0.03) and Cluster 1 gene expression (HR 2.84 [1.11–7.30], p=0.03) remained significantly associated with shorter OS. A post-hoc statistical evaluation determined that the power to detect an association of this magnitude between Cluster 1 gene expression and

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OS was 99%. The Kaplan-Meier curve depicted in Figure 2 demonstrates significantly different OS between patients in Cluster 1 and Cluster 2 (log-rank p=0.014).

Table 5 also shows the results of a univariate analysis evaluating gene expression clusters and patient characteristics in relation to RFS. As was seen in OS, age was not associated with RFS (p=0.87). Interestingly, while gene cluster was not predictive of RFS (p=0.15), nonwhite race (HR 2.01 [1.06–3.84], p=0.03) residual disease (HR 1.85 [1.07–3.21], p=0.03), and BMI (HR 1.05 [1.01–1.09], p=0.02) were all associated with reduced RFS. In a multivariate analysis, however, none of these variables remained statistically significant. As expected, the vast majority (90%) of patients in our cohort had recurrent disease following surgical debulking and first-line chemotherapy. It is possible that the wide variety of treatment approaches employed for recurrent disease is obscuring any effects of gene cluster and other variables on RFS.

ER immunohistochemistry is currently the accepted standard method of determining a tumor's potential sensitivity to ER antagonists or anti-estrogen agents. Eighty-eight percent (88%) of all tumors in this study demonstrated strong (2+ or 3+) positive ER staining in >10% of tumor cell nuclei. The mean percentage of ER positive cells per tumor was not significantly different between Cluster 1 and Cluster 2 (69% vs. 59%, p=0.29). When ER expression was categorized as either negative (10%) or positive (>10%), the proportion of samples staining positive was not significantly different between gene clusters (p=0.14). Finally, ER expression by immunohistochemistry did not correlate with gene cluster assignment (R=0.29, p=0.1).

The results of ER immunohistochemistry were assessed to determine if percent ER-positive tumor cells correlated to survival. When percentage of ER-positive tumor cells is analyzed as a continuous variable to predict outcomes, there is no association between percentage of ER-positive cells and either OS (HR 1.00, p=0.89) or RFS (HR 0.99, p=0.60). The mean percentage of ER-positive tumor cells was quite high overall (62 ± 5.0%; mean ± SE). Using 30% ER-positive tumor cells as a cut-off (because this was the lowest value to generate adequate groups for comparison), univariate analysis demonstrated no significant difference in OS (HR 1.20 [0.33–4.30], p=0.78) or RFS (HR 0.70 [0.27–1.80], p=0.46). Using 10% or 20% ER-positive cells as cut-offs similarly yielded statistically non-significant findings (data not shown); however, the number of subjects in the ER-low group for each of these cut-offs was extremely low. ER immunohistochemical expression was not significantly associated with patient age, BMI, race, surgical debulking status, recurrence, or platinum sensitivity (data not shown).

Discussion

Estrogen is a potent steroid hormone responsible for normal physiologic functions in women, including ovulation and the maintenance of bone mineral density. In the setting of aberrations to normal physiology, most notably in the setting of a cancer diagnosis, estrogen takes on a different role. For hormone-sensitive malignancies, estrogen can stimulate cancer cell proliferation. However, the presence of estrogen sensitivity may also be a positive prognostic marker. In both endometrial and breast cancers, higher expression of estrogen-

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induced genes is associated with tumors that tend to be low-grade and less biologically aggressive (10, 11). Greater estrogen sensitivity and increased expression of genes induced by estrogen are also predictive of improved survival (10, 11, 18). Importantly, the identification of tumor estrogen sensitivity allows physicians to consider using hormone antagonistic treatments which are less toxic than traditional chemotherapy and less significantly impact patient quality of life.

The fact that ER immunohistochemistry did not correlate well with the gene-based clustering approach of high grade serous ovarian carcinoma is important. From previous clinical trials of tamoxifen and aromatase inhibitors in women with ovarian cancer, we can estimate a response rate of 13–26% (6, 8, 9). However, as shown here, 88% of high grade serous ovarian carcinomas have sufficient immunohistochemical expression of ER to be considered "positive" and thus eligible for tamoxifen, letrozole, or other similar inhibitors of estrogen action. Thus, ER immunohistochemistry is significantly over-estimating the subgroup of ovarian cancer patients who may benefit from such hormonal approaches. In contrast, the approach of using transcript quantification of *ER*α and *EIG121* of this same set of tumors demonstrated that only 20% of these patients had higher expression of *ER*α and *EIG121*; this figure more closely approximates the 13–26% response rate of estrogen-directed therapies for ovarian cancer. Therefore, this provides good preliminary evidence that quantification of transcripts from a gene panel may be a more accurate clinical method of identifying women who would benefit most from such estrogen-directed therapeutic approaches. A prospective clinical trial will be necessary to definitively prove this point, however.

Abundant data exist demonstrating the poor prognostic capabilities of hormone receptor immunohistochemistry for ovarian cancer (19–25). For example, Slotman *et al* (21) observed that there was no correlation between ER expression and overall patient survival in a group of women with ovarian carcinoma, whereas other authors have argued that combined ER/PR expression can distinguish between groups of patients with markedly different survivals (20). Even when used to gauge potential response to hormonal antagonistic therapies, receptor positivity correlates poorly with treatment outcomes. Rose *et al* (23) examined 123 ovarian carcinomas for PR and ER expression between 1981 and 1989. In a population of mixed histologies, ER and PR were not significantly associated with survival, chemotherapy response, or second-look findings in a multivariate analysis. An alternative approach, quantification of panels of transcripts, is already widely used in the management of breast cancer. Oncotype DX, a multigene classifier that analyzes RNA expression from paraffin-embedded tumor tissue, is currently recommended by the American Society of Clinical Oncology in early-stage breast cancer as a prognostic tool to both determine risk for disease recurrence and predict response to treatment (26, 27). A second gene classifier for breast cancer, MammaPrint, is currently under investigation, but it has shown similar prognostic capability (27).

In the current study it was initially hypothesized that improved survival would be seen in women whose ovarian tumors expressed high levels of *ER*α and estrogen-induced genes. However, this was not observed. High expression of *ER*α and estrogen-induced genes was not only associated with worse overall survival, but it was a negative prognostic factor

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independent of other patient-dependent covariates such as age, race, and BMI. Such a finding suggests that the molecular mechanisms underlying ovarian tumorigenesis may in fact be quite different in high-grade serous ovarian cancer compared to hormone sensitive tumors at other disease sites, or perhaps even different ovarian histologies. It is known that exposure to unopposed estrogen is associated with an increased risk of developing ovarian carcinoma (28). However, more detailed epidemiological studies have shown that a specific type of ovarian carcinoma, the less common histotype endometrioid adenocarcinoma, is most closely linked to estrogen exposure, while high-grade serous carcinoma is not linked to estrogen exposure (29). Therefore, the findings reported for our current study may be specific for ovarian high-grade serous carcinoma and not applicable to endometrioid-type ovarian tumors.

There is some experimental data that estrogen and/or genes induced by estrogen may actually promote adverse biological properties of ovarian cancer cells. Murdoch and Van Kirk (30) showed that ovarian cancer cells treated with estrogen had a significantly decreased ability to undergo apoptosis. In addition, after causing cellular stress by treatment with cisplatin, ovarian cancer cells exposed to estrogen had significantly increased DNA repair activity (30). The authors suggested that estrogen antagonized the apoptotic pathway triggered by chemotherapy-induced DNA damage. While the pathways by which estrogen achieved these changes were not specifically elucidated, these findings are significant because platinum drugs are one of two primary agents used for the treatment of ovarian carcinoma, including high grade serous carcinoma.

Estrogen’s role in stimulating ovarian cancer progression may also lie in the activation of non-genomic signaling mechanisms. Park *et al* (31) found that when estrogen-sensitive ovarian cancer cell lines are exposed to estrogen, the cells undergo an epithelial-to-mesenchymal transition in which the cells lose expression of E-cadherin, become more spindle-shaped, and develop pseudopodia (31). Estrogen-associated cross-talk with the epidermal growth factor receptor (EGFR) pathway may further promote ovarian tumor progression. In ovarian cancer, EGFR expression is a negative prognostic indicator of survival, and overexpression is associated with poor responses to chemotherapy in both *in vitro* and clinical studies (32, 33). Research on other estrogen-sensitive tissues suggests potential mechanisms to explain this phenomenon. McBean *et al* (34) reported that estrogen treatment increased endometrial tissue expression of *EGFR* mRNA and EGFR protein by IHC. Filardo (35) later described a signaling cascade in breast cancer cells initiated by estrogen’s interaction with a membrane-bound G-protein-coupled receptor that subsequently activated EGFR and downstream Mek (35).

EIG121 was significantly associated with poorer OS in our cohort and has been investigated as a prognostic marker in other hormone-sensitive tumors. Interestingly, in both breast and endometrial cancers, increased expression of *EIG121* predicts improved survival (10, 11). Recent mechanistic studies suggest a function of *EIG121* which may help to support our current findings that its increased expression in ovarian cancer is associated with poor outcome (36). Specifically, EIG121 protein is localized to the late endosome-lysosome compartments and regulates autophagy, a cellular pro-survival mechanism activated when a cell is stressed by lack of nutrients or chemotherapy (36). It was shown that EIG121

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conferred protection against the induction of apoptosis on cells exposed to serum starvation and treatment with taxane chemotherapy (36).

When considering these possible mechanisms to explain our findings of decreased survival associated with increased expression of *ER*α and *EIG121*, it is important to also keep in mind that the first-line therapy of ovarian high grade serous carcinoma is very different from that of ER-positive breast carcinoma or endometrioid-type endometrial carcinoma. Many low grade endometrioid-type endometrial carcinomas are treated with surgery alone; chemotherapy is reserved for recurrences or when there are metastases outside the uterus. Many ER-positive breast carcinomas are well-differentiated and respond well to tamoxifen or other pharmacological methods to antagonize estrogen's action. It is possible that potential hormonal responsiveness in ovarian high grade serous carcinoma is masked by the front-line use of cytotoxic chemotherapy.

Identifying biomarkers is important to adequately counsel individual patients regarding their prognoses, but biomarkers may also provide insight into potential therapeutic interventions. Given the finding that high expression of *ER*α and estrogen-induced genes in high-grade serous ovarian tumors is associated with a poorer prognosis, and that the downstream products of estrogen stimulation may enhance cancer cell survival, it is reasonable to consider the addition of an estrogen antagonist to current treatment regimens. At present, the standard of care for ovarian carcinoma is therapy with platinum and taxane agents, both of which are cytotoxic and function independently of hormone-mediated pathways (37, 38). In selected patients with high *ER*α and *EIG121* expression, supplementing this regimen with an estrogen-antagonizing drug may prevent ovarian cancer cells from activating self-preservation pathways, thus making them more susceptible to the lethal effects of chemotherapeutic agents.

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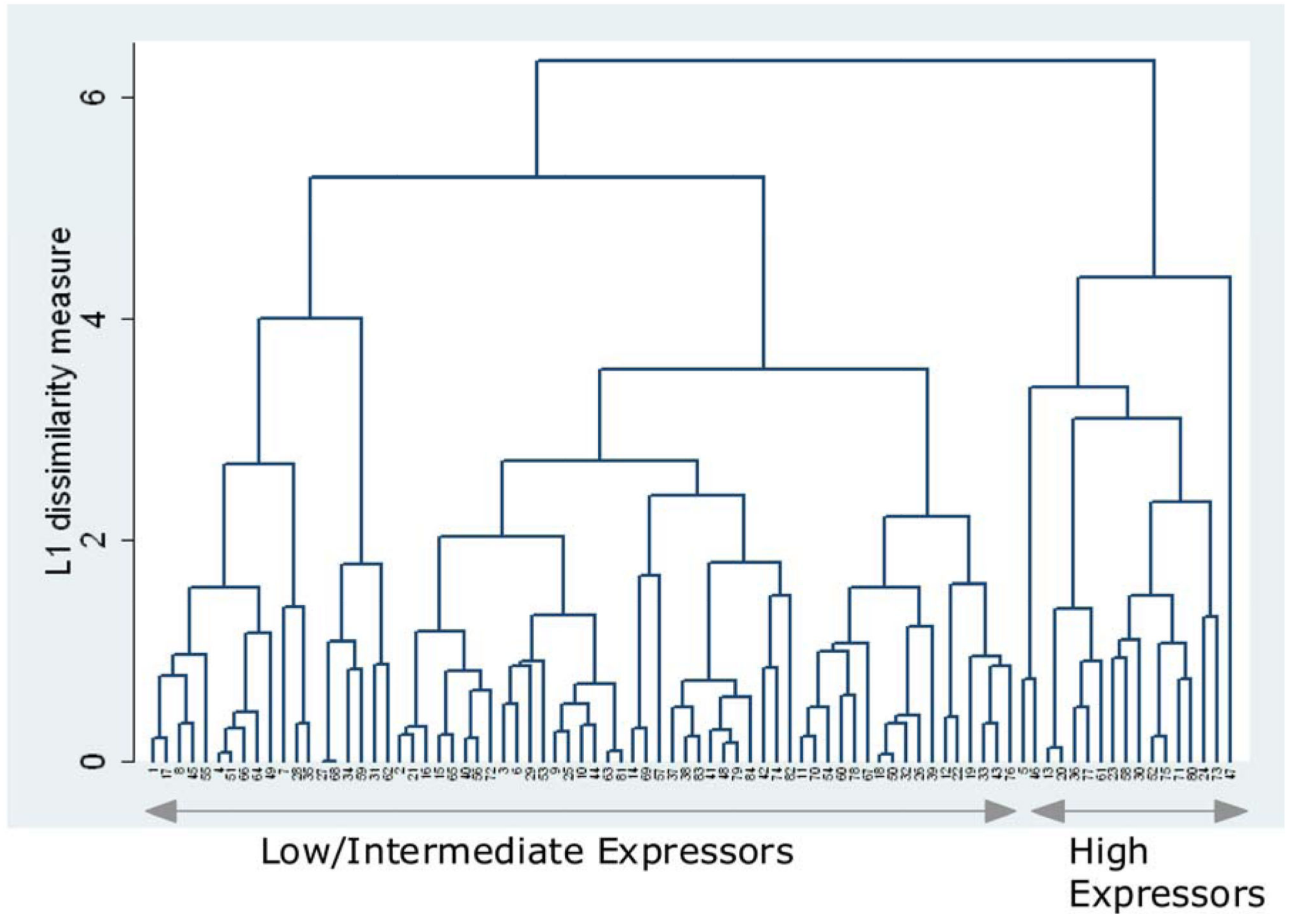

Gene	High Expression Cluster (Cluster 1)	Low/Intermediate Expression Cluster (Cluster 2)
ERα	5.18	4.24
EIG121	3.00	0.15

Figure 1.
Unsupervised cluster analysis using *ER*α and *EIG121*. Cluster 1 has relatively higher expression of these genes, while Cluster 2 has lower expression. Normalized median gene expression (x10^4) is shown in the table below.

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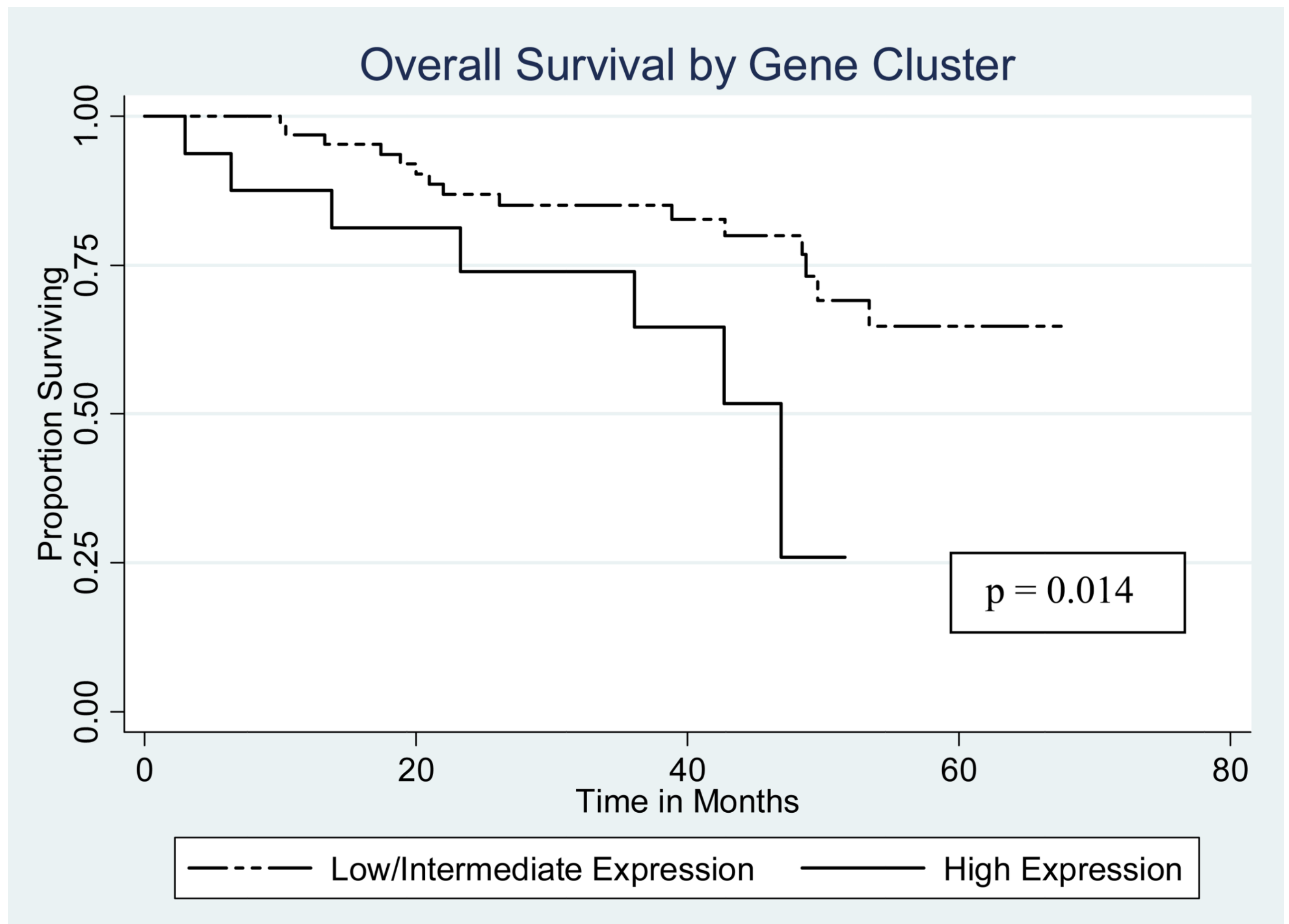

Figure 2.

Kaplan-Meier curve of overall survival by gene cluster. Patients in Cluster 1 (high gene expression) had a significant overall survival disadvantage.

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Table 1

Probes and Primers for Real-Time PCR.

Transcript	Taqman primers and probe	Accession number
hEIG121	3210(+)CTTGCATAGCACCTTTGCAAG	NM_020777
	3135(−)CAGTGGGTGTTGCAGGATG	
	3232(+)FAM-CYGCGGCGATTTGGGTGCC-BHQ1	
	Lowest quantifiable level=240 molecules	
	Average assay efficiency = 90%	
hIGF-1	146(+)GCAATGGGAAAAATCAGCAG	M26544
	237(−)GAGGAGGACATGGTGTGCA	
	217(−)FAM-CTTCACCTTCAAGAAATCACAAAAGCAGCA-BHQ-1	
	Lowest quantifiable level=160 molecules	
	Average assay efficiency = 99%	
hRALDH2	2002(+)AGGCCCTCCTCGCTCAC	NM_003888
	2071(−)TCTGCCCCAGAATGAGCTC	
	2021(+)FAM-ACCCCTCCCTCTCTTCCAAGGAGATC-BHQ1	
	Lowest quantifiable level=210 molecules	
	Average assay efficiency = 96%	
hsFRP1	720(+)GAGCCGGTCATGCAGTTCT	NM_003012
	786(−)CCTCCGGGAACTTGTCACA	
	740(+)FAM-CGGCTTCTACTGGCCCGAGATCG-BHQ1	
	Lowest quantifiable level=210 molecules	
	Average assay efficiency = 95%	
hsFRP4	1175(+)GCGCACCAGTCGTAGTAATCC	AF026692
	1246(−)TTCTTGGGACTGGCTGGTT	
	1202(+)FAM-ACCAAAGGGAAAGCCTCCTGCTCC-BHQ1	
	Lowest quantifiable level=200 molecules	
	Average assay efficiency = 99%	
hERα	1394(+)TACTGACCAACCTGGCAGACAG	NM_000125
	1490(−)TGGACCTGATCATGGAGGGT	
	1466(−)FAM-TCCACAAAGCCTGGCACCCTCTTC-BHQ1	
	Lowest quantifiable level=150 molecules	
	Average assay efficiency = 95%	
hPR	2689(+)GAGCACTGGATGCTGTTGCT	NM_000926
	2754(−)GGCTTAGGGCTTGGCTTTC	
	2710(+)FAM-TCCCACAGCCAGTTGGGCGTTC-BQH1	
	Lowest quantifiable level=220 molecules	

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Transcript	Taqman primers and probe	Accession number
	Average assay efficiency = 93%	
h18s	Primers:	
	(1335+)CGGCTTAATTTGACTCAACAC	M10098
	(1401−)ATCAATCTGTCAATCCTGTCC	
	Probe:	
	(1359+)AAACCTCACCCGGCCCG	
	Amplicon-68 bases in length	

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Table 2

Characteristics of study patients.

Characteristic	n=83
Age at diagnosis (years)	
Mean (range)	62.6 (34.5–85.9)
Median	62.3
Race (%)	
Caucasian	65 (78.3)
African American	7 (8.4)
Hispanic	7 (8.4)
Asian/Other	4 (4.9)
Body Mass Index (kg/m^2)	
Mean (range)	25.9 (15.9–50.8)
Median	24.3
BMI <25 (%)	38 (45.8)
BMI 25-<30 (%)	20 (24.1)
BMI 30 (%)	15 (18.1)
BMI unknown (%)	10 (12.0)
Residual Disease after Debulking (%)	
Yes	31 (37.3)
No	52 (62.7)
Recurrent Disease (%)	
Yes	75 (90.4)
No	8 (9.6)
Platinum Sensitive (n=52)(%)*	
Yes	38 (73.1)
No	14 (26.9)

*Platinum sensitivity not determined for all patients secondary to missing/incomplete date information regarding conclusion of adjuvant platinum/ taxane chemotherapy.

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Table 3

Univariate analysis of overall survival and recurrence free survival by normalized gene expression (x10^4).

	Overall Survival		Recurrence Free Survival	
Gene	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
ER-alpha	0.99 (0.94–1.03)	0.54	1.02 (0.99–1.04)	0.27
EIG121	1.21 (1.09–1.35)	<0.001	1.13 (1.02–1.26)	0.02
sFRP1	1.03 (0.99–1.06)	0.09	1.02 (0.98–1.06)	0.32
sFRP4	1.00 (0.99–1.01)	0.89	1.00 (0.99–1.01)	0.26
RALDH2	1.18 (1.03–1.35)	0.02	1.06 (0.95–1.18)	0.32
PR	0.99 (0.95–1.02)	0.39	0.99 (0.99–1.01)	0.75
IGF-1	1.02 (0.97–1.07)	0.53	1.03 (0.99–1.07)	0.14

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Table 4

Patient characteristics by gene cluster.

Characteristic	Cluster 1 (High-expressors) n=17	Cluster 2 (Low/Intermediate Expressors) n=66	p-value
Age at diagnosis (years)			
Median	60.1	63.3	0.42
Body Mass Index (kg/m^2)			
Mean	25.4	28.3	0.12
Range	15.9–50.8	17.9–47.3	
Race (%)			
White	10 (58.8)	55 (83.3)	0.03
Non-white	7 (41.2)	11 (16.7)	
Optimal Debulking (%)			
Yes	9 (52.9)	44 (67.7)	0.14
No	8 (47.1)	22 (33.3)	
Recurrent Disease (%)			
Yes	14 (82.4)	61 (92.4)	0.75
No	3 (17.6)	5 (4.6)	
Platinum Sensitive (n=52) (%)*			
Yes	7 (18.4)	31 (81.6)	1.00
No	3 (21.4)	11 (78.6)	

*Platinum sensitivity not determined for all patients secondary to missing/incomplete date information regarding conclusion of adjuvant platinum/taxane chemotherapy.

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Table 5

Univariate analysis of overall survival and recurrence free survival by patient characteristics.

	Overall Survival		**Recurrence Free Survival**	
Variable	**HR (with 95% CI)**	**p-value**	**HR (with 95% CI)**	**p-value**
Age at diagnosis (months)	1.00 (0.99–1.01)	0.56	1.00 (0.99–1.00)	0.87
Race (white vs. nonwhite)	1.33 (0.49–3.62)	0.57	2.01 (1.06–3.81)	0.03
Residual disease following debulking surgery	2.65 (1.13–6.18)	0.02	1.85 (1.07–3.21)	0.03
Post-operative BMI	1.02 (0.95–1.09)	0.60	1.05 (1.01–1.09)	0.02
Cluster (1 vs. 2)	3.02 (1.19–7.65)	0.02	1.65 (0.84–3.22)	0.15