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Original Article

Biomarkers and endosalpingiosis in the ovarian and tubal microenvironment of women at high-risk for pelvic serous carcinoma

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Abstract: Introduction: BRCA mutations increase the risk for development of high-grade pelvic serous carcinomas. Tissue biomarkers distinguishing women at high-risk (HR) for ovarian cancer from those at low-risk (LR) may provide insights into tumor initiation pathways. Methods: A prospective study of 47 HR women (40% BRCA carriers) undergoing risk-reducing salpingo-oophorectomy and 48 LR controls undergoing salpingo-oophorectomy was performed. Ovarian/tubal tissues were harvested. Immunohistochemical analysis of candidate proteins CSF-1, CSF-1R, ErbB4 is presented, with scores separately analyzed in epithelium and stroma, in ampulla, fimbria, ovary, and ovarian endosalpingiosis (ES). Comparison was performed between HR and LR groups. Results: Elevated levels of CSF-1 ($p=0.005$) or ErbB4 ($p=0.005$) in the ovarian epithelium, or ErbB4 ($p=0.005$) in the ovarian stroma, were significantly associated with both the HR status and carrying a BRCA mutation, as was nuclear ErbB4 staining. Ovarian ES, an entity which likely derives from the tubal mucosal epithelium, was also associated with HR ($p=0.038$) and BRCA mutation status ($p=0.011$). Among the BRCA carriers only, markers also found association when present in the tube as well as in ovarian ES ($p < 0.05$). ROCs were generated including in the regression model both CSF-1 and ErbB4 expression levels. A model including CSF-1 in ovarian epithelium, ErbB4 in ovarian stroma, and younger age achieves AUC=0.87 (73% sensitivity, 93% specificity) of detection of the HR status. In BRCA carriers, CSF-1 in ovarian epithelium alone achieves AUC=0.85. Conclusions: Our data suggest that elevated levels of CSF-1/ErbB4 in the adnexae correlate with HR/BRCA carrier status. CSF-1/CSF-1R signaling is active in ovarian cancer progression; our data suggests a role in its initiation. ErbB4, in particular nuclear ErbB4, may have a role in tumor initiation as well. Ovarian ES, an entity which may represent a latent precursor to low-grade pelvic serous carcinomas, was surprisingly associated with both HR status and the BRCA carrier cohort. In line with these findings, both ErbB4 and CSF-1R expression in ovarian ES correlated with carrying a BRCA mutation. This analysis, which needs to be validated, indirectly suggests a potential link between ovarian ES and the development of pelvic serous carcinoma in women who are BRCA mutation carriers.

Keywords: CSF-1, ErbB4, endosalpingiosis, high-risk

Introduction

Ovarian and fallopian tube carcinogenesis

Epithelial ovarian cancer (in particular the high-grade serous subtype) still presents with widespread disease throughout the peritoneal cavity, accompanied by a poor long-term outcome. Over the last 30 years, there has been no significant improvement in the dismal 15% 10 year survival rate.

It is currently widely accepted that precursors from the fallopian tube epithelium frequently give rise to high-grade pelvic serous carcinomas, in particular in women who are high-risk (HR) for the development of pelvic serous carcinomas [1, 2]. Thus what was previously classified as the serous subtype of epithelial 'ovarian' cancers has as its origins not only the ovary and primary peritoneum, but in particular, the fallopian tube [3, 4]. The most accepted tubal precursor to date remains the serous tubal intraep-

ithelial carcinoma (STIC), characterized by p53 mutations [5]. Unfortunately aberrant p53 staining (p53 signature) in the fallopian tube is not specific to STICs, and can be seen frequently in otherwise normal fallopian tubes from control women who are not HR [6]. Thus, there is a need for specific tissue biomarkers indicative of the HR status. Tubal epithelial proliferation [6] particularly of the tubal secretory cells [7] may also prove to be a harbinger of some forms of pelvic serous carcinoma.

Endosalpingiosis, glands lined by tubal-type epithelium, is believed to result, at least in part, from shedding of tubal epithelial papillae and clusters which implant on the ovary and on peritoneal surfaces. Intraovarian endosalpingiosis, which may represent invagination of those shed tubal structures, has been suggested to be a critical point in the pathway to transition to low-grade ovarian serous carcinoma [8]. Papillary tubal hyperplasia has been suggested as a putative precursor of endosalpingiosis and of low-grade serous ovarian carcinomas [9]. Low-grade serous ovarian carcinomas are molecularly distinct from the more common high-grade pelvic serous carcinomas [10], but the notion that the fallopian tube may serve as a site of origin for both types of serous carcinomas is gaining some favor [11].

High-risk women, in particular those carrying BRCA mutations, are at risk for development of high-grade pelvic (ovarian, fallopian tube, primary peritoneal) serous carcinomas. There is not yet a clear or consistent association of low-grade serous carcinomas with the HR status. Moreover, to date, there are no previous reports examining the association of endosalpingiosis with being HR. Indirectly, there is a report of 7 of 32 HR patients who had cytologic evidence of endosalpingiosis in the peritoneal washings taken at the time of risk-reducing salpingo-oophorectomy. All 7 patients were BRCA mutation carriers [12].

Clinical need for biomarkers

There continues to be a need for relevant biomarkers of ovarian cancer risk [13]. Over 200 serum biomarkers, not all with known biologic functions in ovarian cancer, have been proposed. The 35 most promising comprised a biomarker panel in which blood samples of women up to 2 years prior to development of their ovarian cancer, proved to be no more discriminating in determining risk, than CA125 [13]. To date, screening approaches have not impacted on improved detection or survival [13-15]. In this current study searching for tissue biomarkers of ovarian cancer risk, we chose to initially focus on a select group of biomarkers (CSF-1, CSF-1R, ErbB4) based on their potential mechanistic role in ovarian carcinogenesis. Circulating CSF-1 has previously been studied as part of a small panel including CA125, and there was only a minimal advantage over CA125 in the detection of ovarian cancer [16]. Development of biomarkers based on molecular profiling of ovaries or fallopian tube tissues from women at HR may lead to higher sensitivity or specificity. Expression profiling has been performed of HR fallopian tube epithelium [17], ovarian surface epithelium [18, 19], and ovarian inclusion cysts [19], which has started to give some insights into dysregulated pathways in the HR epithelium. Tissue biomarkers which may result from these efforts may not necessarily be mediators of carcinogenesis: however, CSF-1/CSF-1R and ErbB4 may represent both, i.e., biomarkers based on mechanistic pathways.

CSF-1, the macrophage colony stimulating factor, is a differentiating and survival factor for macrophages. By binding to its receptor (CSF-1R) encoded by the c-*fms* proto-oncogene, the role of CSF-1 has been extensively investigated both in the tumor microenvironment, as well as in cancer cells [20, 21]. The CSF-1/CSF-1R pathway is now established as an important mechanism by which epithelial ovarian cancer cells impart virulent, invasive metastases [20]. Both autocrine and paracrine pathways for activation have been delineated as well as elucidation of post-transcriptional regulatory factors [20, 22, 23]. Their overexpression or activation leads to poor survival of ovarian cancer patients [24, 25]. Interestingly, independent *in vivo* experiments have observed a significant effect of low levels of CSF-1 in ovarian cancer cells on inhibition of tumorigenicity [20, 26]. This provided the first hint that CSF-1 may have a role in ovarian cancer initiation, as well as in its progression.

The tyrosine kinase v-erb-b2 erythroblastic leukemia viral oncogene homolog-4 (ErbB4, or HER4) mutations exist in numerous types of human cancer including ovarian [18, 27, 28] and breast cancer [29]. Although part of the EGF receptor family, ErbB4's role in oncogenesis and in cancer progression remains incompletely understood with conflicting reports;

however, it does appear that ErbB4 may hold a role in both. In mammary tissue, it has a role in maintaining orderly development [30, 31]. The role of ErbB4 in improved survival has been demonstrated in those with breast cancer [32, 33], but at least one study has questioned this association [34].

In ovarian cancer, ErbB4 expression was markedly higher than in benign ovarian tissues [35]. ErbB4 expression in ovarian cancer was also associated with improved long-term survival [27]. However, a recent study found that a higher level of expression of one isoform of ErbB4 to be associated with both poor clinical outcomes and with growth of ovarian cancer cells *in vitro* [28]. There is little known about ErbB4 pertaining to ovarian carcinogenesis. However, recently, expression profiling of ovarian surface kinases comparing normal, HR, and malignant ovaries [18] identified ErbB4 as having a linear trend of expression, increasing from normal, to a higher rate in HR, and the highest in ovarian cancer.

We therefore reasoned that ErbB4 and CSF-1, in addition to CSF-1R as it mediates CSF-1 signaling, may represent candidate biomarkers associated with the HR status. The expression of CSF-1, CSF-1R, and ErbB4 was therefore studied in the fallopian tubes and ovaries of HR women undergoing risk-reducing salpingo-oophorectomy, compared to those of low-risk (LR) women also undergoing salpingo-oophorectomy. Tissues also underwent evaluation of p53 staining. The presence of endosalpingiosis was studied for its association with the HR status.

Methods

Clinical trial eligibility and design

The University of Arizona Cancer Center (UACC) multidisciplinary HR cancer genetics clinic was opened in 2004, first focusing on women at HR for breast and ovarian cancer. Genetic counseling and testing of HR women, if appropriate or feasible, is a key component of this effort. Physicians also provide counseling and offer increased surveillance or preventative recommendations for women at increased risk for breast and ovarian cancer.

This report concerns the comparison of the HR and LR control groups who were part of a study of the anti-androgen flutamide, offered to HR patients who met eligibility criteria. The potential subjects were given the opportunity to participate in the treatment arm of the study, in the study as a control, or to decline to participate. Subjects in both the treatment and control arms of the study completed a reproductive/hormone history questionnaire.

Patients were eligible for the study if they were ≥ 18 years of age. HR patients were eligible who elected to have a prophylactic salpingo-oophorectomy, and agreed to use an acceptable, non-hormonal means of contraception prior to surgery. LR patients were eligible who had a salpingo-oophorectomy as part of their planned gynecologic surgery. The criteria for the HR and LR groupings are defined below. Additional inclusion criteria for HR patients included adequate hepatic function [defined as serum bilirubin ≤ 1.0 x Upper Limit of Normal (ULN), alkaline phosphatase, AST, and ALT ≤ 2.5 x ULN] and adequate renal function [defined as serum creatinine ≤ 1.5 x ULN]. Eligible participants were required to have a granulocyte count ≥ 1500/μL, platelet count ≥ 75,000/μL, and hemoglobin ≥ 9 g/dL.

Exclusion criteria included liver disease (including viral or other hepatitis), current alcohol abuse, or cirrhosis. Additional exclusion criteria included pregnancy or lactation, current use of hormone therapy or active treatment for cancer, or recent, current, or planned participation in another experimental drug study.

The study was approved by the University of Arizona IRB and was conducted in accordance with institutional and federal guidelines. In total, tissues were analyzed from 47 HR control and 48 LR control patients accrued between 8/29/06 and 5/20/11. There were an additional 12 HR patients who received flutamide for 6 weeks; analysis of the effect of flutamide is the subject of a separate paper. Relevant clinical information was abstracted from the medical records such as menopausal status, BMI, personal cancer history, genetic mutation status for BRCA1/2 and Lynch Syndrome mutations, and family history for cancer. Included in this study were 12 control patients who consented to the University of Arizona IRB approved UACC tumor biorepository prior to their salpingo-oophorectomy. Detailed hormonal/reproductive information in the medical records was not available for the majority of those 12 control

patients who consented to the UACC tumor biorepository. The clinical information was consolidated into a database comprising 3 groups: LR control (n=48), HR control (n=47), and HR flutamide treated (N=12). In this report we concentrated primarily on the comparison of the two control groups, with the exception of the analysis of ovarian endosalpingiosis.

Definition of high-risk

Women at HR (n=47) carried a BRCA 1 or 2 mutation (40.4%), a Lynch Syndrome mutation (1 case) and/or were defined by a family history of: ≥ 1 first degree relative with epithelial ovarian cancer (17%), ≥ 1 case of breast cancer age ≤ 40 years old (34%), > 1 case of breast cancer ≤ 50 years old (25.5%), male breast cancer (2 cases), and/or family history of breast/ovarian cancer (89%). The majority (70%) of the HR patients also had a personal history of breast cancer, with median age at diagnosis of 43 years (range 26-60). The BRCA mutation distribution favored BRCA2 over BRCA1, representative of our HR clinic population [36]. Patients who were LR did not fulfill any of the HR criteria. 25% of LR patients had a personal history of breast cancer, with median age at diagnosis of 47.5 years (range 40-70).

Pathology of tubes/ovaries for both HR and LR cases

All patients had removal of at least one ovary and fallopian tube. All fallopian tubes and ovaries chosen for analysis by IHC were morphologically unremarkable. In addition, 83% of the HR, and 85% of the LR patients had concomitant hysterectomy as part of their surgical procedure.

Pathologic examination of adnexae

The University of Arizona procedure for pathologic analysis of fallopian tubes and ovaries from women at HR, is a complete submission of the tissues, with sections of the tubal fimbria taken by optimizing the surface area of the tubal fimbria by the SEE-FIM protocol [37, 38]. The pathology was read by a gynecologic pathologist (WZ), with attention paid to serous intraepithelial carcinoma and dysplasia within the fallopian tube (mainly tubal fimbria), and endosalpingiosis within the ovary. P53 staining to search for p53 signatures was performed in the adnexal tissues of the entire cohort of HR and LR patients, with both ovary and fallopian tube studied for p53 in 84% of cases.

The adnexal tissues from LR patients were processed and analyzed by usual University of Arizona procedures. Two sections of each ovary/tube were taken for standard pathologic analysis, including one additional section of the tubal fimbria.

IHC methods and scoring

5 μm sections of ovary and fallopian tube were mounted on slides and underwent deparaffinization, dehydration, quenching in methanol, rehydration, and antigen retrieval in 10 mM citrate buffer pH 7.0 under high pressure and temperature. Staining for all antibodies was first optimized on control tissues upon review by a gynecologic pathologist (WZ). The antibodies for ErbB4 (ab19391, Abcam), CSF-1R (ab61137, Abcam) and its ligand, CSF-1 (ab9693, Abcam), were utilized. Slides were blocked with serum and stained with primary antibody, incubating overnight. A biotinylated secondary antibody was then added and incubated for an hour the next day. Afterwards, the slides were stained with an avidin-biotin enzyme complex (ABC Kit- Vector Labs). Slides were then stained with a solution of 3,3'-diaminobenzidine (DAB) (Vector Labs), counterstained in hematoxylin, dehydrated, and permanently mounted. Slides were scored by NA and WZ, and based on intensity of stain (0-3) and percentage of area stained (0-100%), with both scores multiplied to yield a product (total score). In addition to scoring cytoplasmic stains, nuclear stains were also scored when present, specifically in reference to the nuclear staining for ErbB4. Within the ovary and fallopian tube (ampulla and fimbria), epithelium and stroma were scored separately. Lastly, attention was paid to the presence of marker staining in ovarian endosalpingiosis.

Statistical methods

Demographic characteristics for high and low risk women were summarized using descriptive statistics depending on the underlying distribution. Correlations between quantitative variables were computed using Kendall's T, for qualitative variables Fisher's exact test was used.

Table 1. Demographic, reproductive, and hormonal characteristics of high-risk and low-risk cohorts

	Low risk	High risk	*p*-value
	Number (%)	Number (%)	
Age (years)	N=48	N=47	P=0.025
Mean ± SD (range)	51.7±11.3 (21-81)	46.9±9.1 (27-66)	
Menopausal	N=44	N=46	P=NS
Yes	25 (57)	20 (43)	
No	19 (23)	26 (57)	
BMI	N=48	N=47	P=NS
Mean ± SD (range)	29.9±7.0 (18.6-45.8)	28.9±7.4 (20.1-56.2)	
Number of Pregnancies	N=46	N=46	P=NS
0	6 (13)	6 (13)	
1-3	23 (50)	27 (59)	
≥ 4	17 (37)	13 (28)	
Hormone Replacement Therapy			
Estrogen only	N=20	N=34	P=NS
Ever	5 (25)	4 (11)	
Never	15 (75)	30 (88)	
Progesterone only	N=17	N=34	P=0.033
Ever	3 (17)	0	
Never	14 (82)	34 (100)	
Combined	N=17	N=32	P=NS
Ever	1 (5)	2 (6)	
Never	16 (94)	30 (93)	
Oral Contraceptive Use	N=34	N=43	P=NS
Ever	24 (71)	33 (76)	
Never	10 (29)	10 (23)	
Endometriosis	N=48	N=47	P=NS
Yes	8 (17)	2 (4)	
No	40 (83)	45 (96)	
Personal History of Breast Cancer	N=48	N=47	P < 0.001
Yes	12 (25)	33 (70)	
No	36 (75)	14 (30)	
BRCA Positive	N=48	N=47	P < 0.001
Yes	0	19 (40)	
No/unknown	48 (100)	28 (60)	
History of Tubal Ligation/Hysterectomy	N=42	N=45	P=NS
Yes	20 (48)	14 (31)	
No	22 (52)	31 (69)	
Endosalpingiosis Present	N=48	N=59	P=0.038
Yes	27 (56)	45 (76)	
No	21 (44)	14 (24)	

Differences between biomarkers for high and low risk women were computed using a Wilcoxon test.

A logistic regression model was used to determine the predictive value of the biomarkers. Univariate and multivariate modeling was used to check the assumptions of the models. The result of the final logistic model was used for the creation of the Receiver Operating Curve (ROC) and also for the computation of sensitivity and specificity. A result with a *p*-value less or

Table 2. Analysis of IHC scores comparing the LR control cohort (N=48), with the HR controls (N=47) or with those with a BRCA mutation (N=19)

Adnexal Site	Biomarker	High-risk (*P* value)	BRCA mutation (*P* value)
Ampulla Epithelium	CSF1	NS	0.043
	ERBB4	NS	NS
	CSF1R	NS	NS
Ampulla Stroma	CSF1	NS	NS
	ERBB4	NS	NS
	CSF1R	NS	NS
Fimbria Epithelium	CSF1	NS	0.023
	ERBB4	NS	NS
	CSF1R	NS	NS
Fimbria Stroma	CSF1	NS	NS
	ERBB4	NS	NS
	CSF1R	NS	NS
Ovary Endosalpingiosis	CSF1	NS	NS
	ERBB4	NS	0.052
	CSF1R	NS	0.013
Ovary Epithelium	CSF1	0.005	0.003
	ERBB4	0.005	0.003
	CSF1R	NS	NS
Ovary Stroma	CSF1	NS	NS
	ERBB4	0.005	0.056
	CSF1R	NS	NS

equal 0.05 was assumed to be significant. All computations were performed using SAS 9.3 (Cary, NC)

Results

Demographic information including hormonal and reproductive factors for the HR and LR patients are shown in **Table 1**. Younger age was associated with HR status (p=0.025). Age was highly correlated with menopausal status ($p < 0.0001$). HR patients were more likely to have a personal history of breast cancer ($p < 0.001$). There are no significant differences between HR and LR patients with respect to menopausal status, prior tubal ligation or hysterectomy, endometriosis, BMI, OCP or other reproductive or hormonal factors, with the exception that LR patients were more likely to have been exposed to progesterone only (p=0.03).

We reasoned that exposure to flutamide for 6 weeks would not alter the presence of endosalpingiosis in the ovary. Therefore, for the analysis of ovarian endosalpingiosis only, we included the entire HR cohort in this study (N=59), whether flutamide treated or not. We found the presence of ovarian endosalpingiosis to correlate significantly with the HR status (p=0.038). Furthermore, carrying a BRCA mutation was significantly associated with the presence of ovarian endosalpingiosis, with 85.7% of BRCA carriers having ovarian endosalpingiosis, compared to 56.2% of LR patients (p=0.011).

The expression of the CSF-1, ErbB4, and CSF-1R biomarkers were analyzed with respect to HR status (**Table 2**). Elevated levels of CSF-1 (p=0.005) or ErbB4 (p=0.005) in the ovarian epithelium or ErbB4 (p=0.005) in the ovarian stroma were significantly associated with the HR status. Their expression in other locations, including the fallopian tube ampulla or fimbria, epithelium or stroma, did not find significance. CSF-1R expression similarly did not find significance in this analysis. There was no significant correlation between overexpression of CSF-1 and that of ErbB4. Correlation of expression of each of these markers in the ovary with their respective expression in the fallopian tube was also studied, and there was no significant correlation.

Several positive findings were noted on correlational analysis of immunohistochemical staining for these markers. For both HR and LR cases, in the ovarian epithelium, CSF-1 expression was significantly correlated with that of CSF-1R ($p < 0.036$). In addition, ErbB4 expression was correlated between epithelium of fimbria and ampulla, or between fimbria epithelium and fimbria stroma, for both HR and LR cases ($p < 0.009$). For HR cases only, there was correlation between CSF-1 in the epithelium of fimbria and of ampulla (p=0.004) as well as in the stroma of fimbria and of ampulla (p=0.009). Similar correlation was noted for CSF-1R, but in the respective epithelial compartments only of

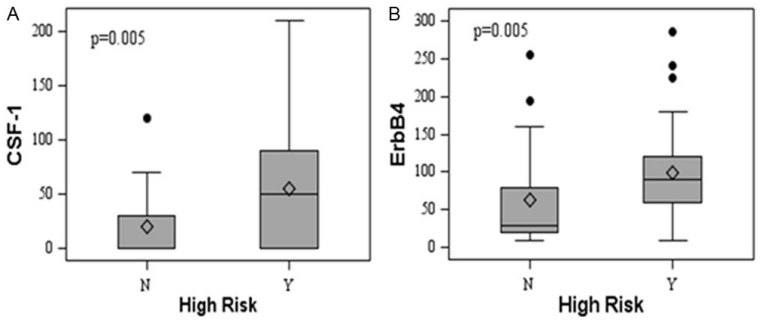

Figure 1. Boxplots of immunohistochemical scores for (A) CSF-1 in ovarian epithelium and (B) ErbB4 in ovarian stroma, by HR status. Median (line) with 25th and 75th percentiles are depicted by the box. ◊indicates the mean.

fimbria and ampulla (p=0.005). ErbB4 expression in ovarian epithelium was correlated with that in ovarian stroma (p=0.002). These correlations were not observed in the LR cases.

We next studied nuclear ErbB4 staining, which we found present only in fimbria epithelium or ovarian endosalpingiosis. In the fimbria epithelium, ErbB4 staining was observed to be in the nucleus in 14.6% of LR cases, compared to 36.2% of the HR cases (p=0.019). Such nuclear staining was also strongly correlated with BRCA mutation status (p=0.023), with 42.1% of BRCA positive cases showing nuclear ErbB4 staining. This finding of significance of nuclear ErbB4 staining in the tubal fimbria epithelium contrasts with the lack of significance of overall ErbB4 staining in the same location (**Table 2**). In ovarian endosalpingiosis, nuclear ErbB4 staining was more than twice as common (14.9%) in HR women, than in the LR cohort (6.2%); however this finding was not significant. P53 signature was observed in 10.5% of the cohort with BRCA mutations, and only in the fallopian tube fimbria. None of the fallopian tubes were found to have STIC. None of the 48 LR cases expressed p53 in either the tube or ovary.

Figure 1 depicts boxplots which highlight the findings for CSF-1 in ovarian epithelium and for ErbB4 in ovarian stroma. Representative examples of immunohistochemical staining of these markers in ovarian tissue for both HR and LR cases are presented in **Figure 2**.

Further analysis of these biomarkers was performed of the HR control cohort found to carry a BRCA mutation (N=19) which represents a subset of the HR patients, compared to the LR cohort (**Table 2**). This analysis confirms the significance of CSF-1 and ErbB4 as described for the analysis of the larger HR cohort (**Table 2**). Of note, among the BRCA carriers only, CSF-1 expression found association with the BRCA carrier status when present in the epithelium of fallopian tube ampulla and fimbria. A representative example of immunohistochemical staining for CSF-1 in the fallopian tube from BRCA carriers is depicted in **Figure 2**. Notably, both ErbB4 and CSF-1R found significance with the BRCA carrier status when expressed in the epithelium of ovarian endosalpingiosis (**Table 2**, with representative ErbB4 staining in **Figure 2**).

Based on these results, ROC were generated including both CSF-1 and ErbB4 variables along with age in the regression model. Ultimate selection defined 3 variables: increased expression of CSF-1 in ovarian epithelium, of ErbB4 in ovarian stroma, and younger age. This model achieves an Area Under the Curve (AUC) of 0.87 with 73% sensitivity and 93% specificity for being HR (**Figure 3**). When the analysis was restricted to the BRCA positive cohort, ROC of CSF-1 in ovarian epithelium alone, achieved an AUC of 0.85 with 84.6% sensitivity and 87.5% specificity for having a BRCA mutation.

Discussion

Endosalpingiosis has been suggested by ourselves and others to be a precursor to the low-grade serous ovarian carcinomas [8, 9]. Our study finds, for the first time, an association between ovarian endosalpingiosis and the HR status. Of note is the finding that ovarian endosalpingiosis is also significantly correlated with having a BRCA mutation. In line with this intriguing finding, is our observation that ErbB4 and CSF-1R expression in ovarian endosalpingiosis correlates with carrying a BRCA mutation. It is well known that HR patients who are BRCA carriers are at significant risk for high-grade pelvic serous carcinomas. However, there has not yet been a clear association of HR patients with low-grade serous carcinomas, nor ovarian endosalpingiosis with high-grade serous carcinomas.

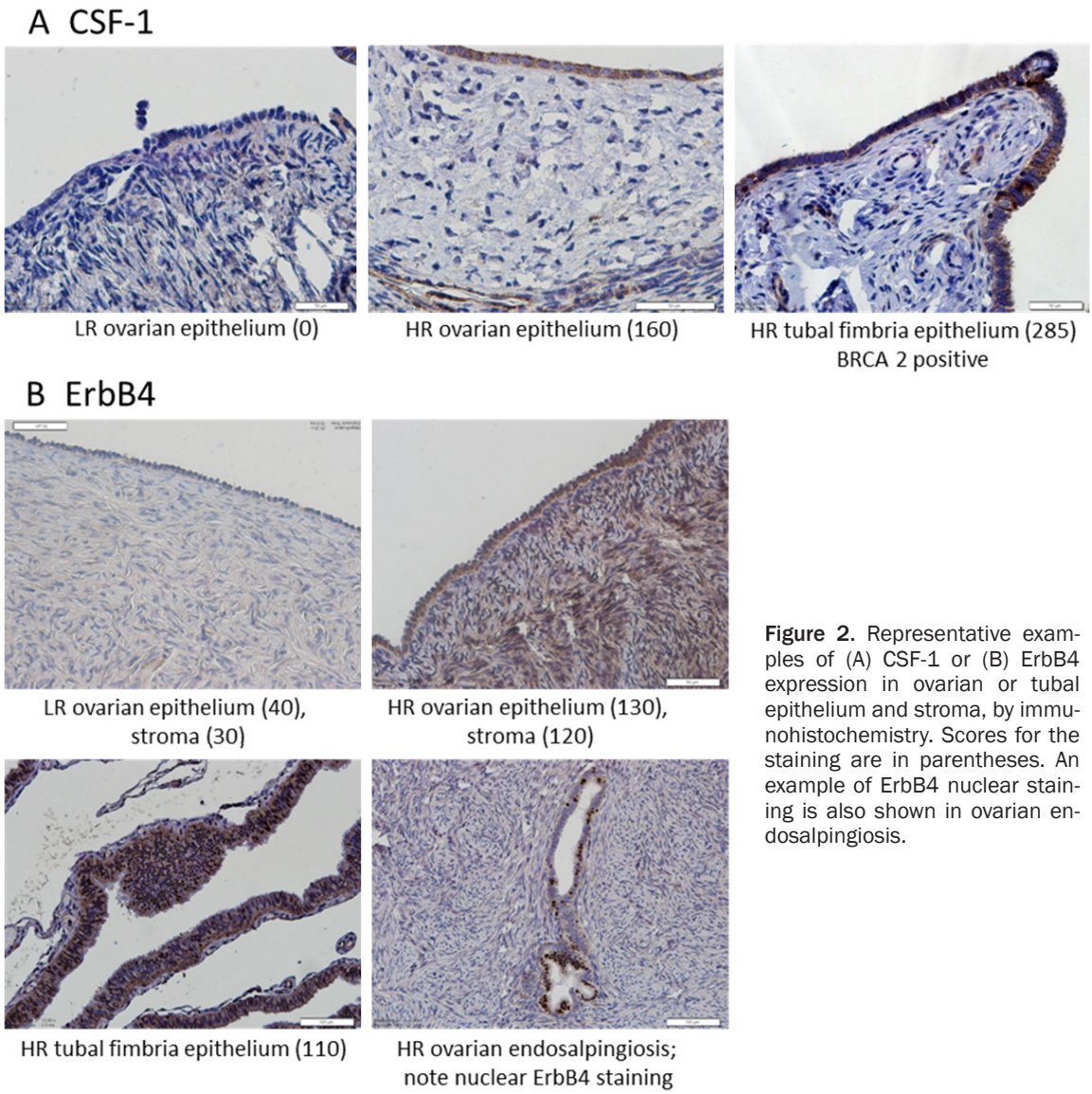

Figure 2. Representative examples of (A) CSF-1 or (B) ErbB4 expression in ovarian or tubal epithelium and stroma, by immunohistochemistry. Scores for the staining are in parentheses. An example of ErbB4 nuclear staining is also shown in ovarian endosalpingiosis.

Our findings indirectly suggest a potential link between ovarian endosalpingiosis and ovarian cancer initiation in women who are BRCA carriers. We can conjecture that the BRCA carrier status may predispose to some low-grade serous carcinomas, via ovarian endosalpingiosis. Alternatively, ovarian endosalpingiosis in the setting of BRCA mutations may lie in the pathway of high-grade as well as low-grade serous carcinomas. Ovarian endosalpingiosis is not a finding which is specific to HR ovaries, and it appears to be relatively common being present in over 50% of LR ovaries; therefore, this entity alone does not appear to be pre-neoplastic. While the clinical significance of this association of ovarian endosalpingiosis and BRCA status is unknown at this time, a mechanism for neoplastic transformation in this setting may involve the two tyrosine kinase receptors, ErbB4 and CSF-1R. CSF-1 found overexpressed in the ovarian and tubal epithelium of BRCA carriers can stimulate CSF-1R overexpression in ovarian endosalpingiosis, as can CSF-1 bearing macrophages infiltrating the ovarian stroma. Prior data suggesting that ErbB4 could have a role in ovarian carcinogenesis was generated by study of this receptor in ovarian surface epithelium of HR patients who did not carry a BRCA mutation [18]. Our findings confirm and expand on the original observation, in that correlation with the HR and BRCA carrier status was observed of ErbB4 expres-

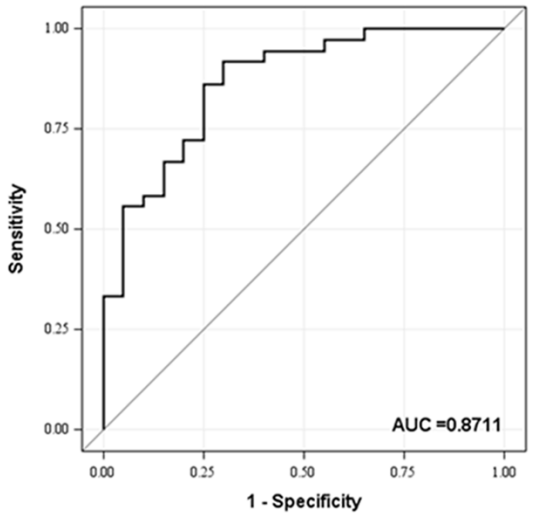

Figure 3. Receiver Operating Curve for the prediction of being HR, based on a model of CSF-1 in ovarian epithelium, ErbB4 in ovarian stroma, and age. This model achieves a C value of 0.87, with 73% sensitivity and 93% specificity.

sion in ovarian stroma as well as ovarian epithelium. Additionally, correlation of ErbB4 in ovarian endosalpingiosis was found with the BRCA positive cohort.

The role of ErbB4 in cancer in the literature has been quite conflicting, especially when it comes to prognosis of cancer cohorts [18, 27, 28, 32-34]. In one report of breast cancer, analysis of nuclear ErbB4 staining was associated with worse prognosis compared to membranous ErbB4 staining [39]. We studied ErbB4 signaling by focusing on nuclear ErbB4 staining. Stimulation of ErbB4 with one of its ligands, neuregulin-1, cleaves ErbB4 releasing the ErbB4 intracellular domain which translocates into the nucleus to control gene expression [40]. Different ErbB4 intracellular domains differentially regulate nuclear translocation and signaling [41]. Expression of ErbB4 is estrogen inducible while ErbB4 binds to and transactivates estrogen receptor in the nucleus [42]. Thus it is likely that the nuclear localization of ErbB4, which we found in this paper to be associated with both HR and BRCA carrier status, may have a specific role in neoplastic transformation.

While there is abundant evidence supporting the fallopian tube epithelium as the initiator of carcinogenesis in a significant subset of ovarian cancers in HR patients [1-4], analysis of our selected markers suggests that the ovarian epithelium and stroma are still important to ovarian carcinogenesis in the HR cohort. Both CSF-1 and ErbB4 expression in ovarian epithelium, and ErbB4 in ovarian stroma, are associated with the HR as well as the BRCA positive cohorts. Analysis of the BRCA carriers however confirms the additional importance of the fallopian tube as a site of initiation of carcinogenesis, as CSF-1 expression in fimbria and ampulla epithelium was associated with BRCA mutation carriers. In HR cases only, (1) levels of CSF-1 became similar throughout the fallopian tube ampulla and fimbria, when comparing within epithelial or within stromal compartments; and (2) levels of ErbB4 became similar in ovarian epithelium and stroma; findings not observed in the LR cases. The tumor microenvironment, in particular the stroma containing inflammatory mediators is an increasingly appreciated key mediator of ovarian/tubal carcinogenesis [43]. An advantage of our work is the focus on staining for tissue biomarkers in both epithelium and stroma.

We present a ROC for prediction of the HR status, with an AUC of 0.87 which combines increased levels of tissue biomarkers CSF-1 and ErbB4 in ovarian epithelium and stroma, with younger age. Expression of CSF-1 and ErbB4 were not correlated with each other. Although younger age was significantly correlated with the pre-menopausal status, we did not find in this cohort association of any of the hormonal/reproductive factors, as we may have expected [44], with HR status. For BRCA mutation carriers, CSF-1 in ovarian epithelium alone was able to achieve similar results with generation of an ROC with an AUC of 0.85.

Our study is limited by small numbers, especially when examining subsets of the cohorts, such as BRCA status, however despite this limitation, several positive findings were observed, which need to be validated. Our data suggest that elevated levels of CSF-1 and ErbB4 in the adnexae correlate with HR/BRCA positive status. Ovarian endosalpingiosis, an entity which may represent a latent precursor to low-grade pelvic serous carcinoma, was also associated with both the HR status and having a BRCA mutation. The current analysis, limited to 3 selected proteins, finds more significance by HR status in marker changes within the ovary (epithelium, stroma, and endosalpingiosis)

than the fallopian tube. When the analysis was restricted to the BRCA carrier cohort, significance was also found for CSF-1 in the tubal fimbria and ampulla, more in line with expectations. Our cohort of fallopian tubes did not appear to contain foci of STIC, despite careful processing of the tubal fimbria. Therefore, we cannot comment on whether these precancerous changes in the fallopian tube overexpressed CSF-1.

Assays of tissue biomarkers of the HR status such as we have observed in ovarian epithelium and stroma cannot be easily translated to clinical application. The significance of these biomarkers lies more in helping to elucidate biological meaningful proteins in ovarian and tubal carcinogenesis. As discussed above, our data expands on the role of ErbB4 in ovarian cancer initiation. CSF-1/CSF-1R signaling is established as having a role in ovarian cancer progression [20, 26]. The evidence presented in this study strengthens the role of such signaling in its initiation, between CSF-1 in the epithelium of both fallopian tube and ovary, and CSF-1R in ovarian endosalpingiosis. A planned global approach will be able to identify a larger array of biomarkers in the ovarian and tubal microenvironment, to build on these findings.

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Disclosure of conflict of interest

None.

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