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High incidence of ErbB3, ErbB4 and MET expression In ovarian cancer

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Introduction

Ovarian cancer is the second most common gynecologic cancer in the United States after cancers of the uterine corpus. Each year, over 20,000 women are diagnosed with this cancer. It is the most lethal gynecologic cancer and ranks as the fifth leading cause of cancer death among American women. In this year alone, 15,500 women are expected to die of the disease [1]. Early detection is the key to successful treatment by surgery but remains a relatively rare event. The overall prognosis is poor when diagnosed at an advanced stage, after cancer cells have disseminated into the peritoneal cavity or retroperitoneal nodes [2]. Despite initial debulking surgery and aggressive platinum and taxane-based chemotherapy regimens, most patients relapse after achieving a complete clinical response [2].

The high rates of acquired chemoresistance in this disease underscore the need to develop targeted therapies, where patient selection can be based upon well-characterized biomarkers [3]. To date, the most successful approach incorporating biologic therapy for this disease has been through drugs that target the vascular growth factor (VEGF) pathway, although the improvement in progression-free survival (PFS) is underwhelming [4, 5]. For example, bevacizumab is a therapeutic monoclonal antibody that inhibits activation of VEGF receptors through competitive binding to the VEGF ligand. This agent possesses measurable single-agent activity in patients with relapsed epithelial ovarian cancer [6, 7]. When tested in combination with chemotherapy, results show significantly prolonged PFS [8–10]. Other inhibitors targeting the angiogenesis pathway also induce some partial responses or stabilize disease in some patients [11].

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In contrast, trials using targeted therapies against ErbB1 (EGFR) and ErbB2 (Her2) have been disappointing in ovarian cancer [3, 5]. Our goal was to evaluate if this might be attributed to low incidence of expression of ErbB1 and ErbB2 in ovarian tumors and, further, to identify other closely related growth factor receptors that might be more appropriate therapeutic targets. We focused on the closely related family members, ErbB3 (Her3) and ErbB4 (Her4), as well as the receptor for hepatocyte growth factor, MET. Evidence suggests that ErbB3 can mediate resistance to ErbB1 and ErbB2 inhibitors because its phosphorylation is often persistent during treatment, offering tumors the opportunity to escape from current therapies [12–14]. ErbB3-MET crosstalk has been proposed as one mechanism for this resistance [15, 16]. A role for ErbB3 in ovarian cancer was suggested by Tanner, who evaluated ErbB3 expression in 116 patients with primary ovarian cancer and concluded that decreased survival time was associated with the highest levels of ErbB3 [17].

A distinct feature of this report is the evaluation of relative expression for *all four* ErbB family members and MET, using tissue arrays comprising 202 unique tumors from ovarian cancer patients. It is notable that immunohistochemical analysis of ErbB3, ErbB4 and MET is not routinely evaluated in clinical practice and that commercial antibodies to receptors in the ErbB family can be cross-reactive or of poor quality [18, 19]. In our study, antibodies for IHC were carefully validated using well defined positive control tissues. Since global ErbB3 and MET expression was found to be a consistent feature of these samples, phospho-specific antibodies were used to evaluate receptor activation state. Results are discussed in the context of prior studies, that focused on a subset of these receptors within smaller patient sample sizes [17, 20–25] or in cultured ovarian carcinoma cell lines [18, 26]. Based on these studies, we propose the use of these well validated IHC protocols to stratify enrollment of ovarian cancer patients onto trials targeting one or more of these growth factor receptors.

Material and Methods

Reagents and cell culture

ErbB3 antibodies from these commercial sources were tested: MBS301141 (MyBioSource, San Diego, CA), LS-B2126 (LifeSpan BioSciences Inc., Seattle, WA), AP7630a (ABGENT, San Diego, CA), sc-285 (Santa Cruz Biotechnology, Santa Cruz, CA), NBP1-19398 (Novus Biologicals, LLC, Littleton, CO) and BS1654 (Bioworld, St. Louis Park, MN), ErbB4 (sc-283) and MET antibodies (sc-161) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phosphorylated ErbB3 (pTyr1289) and MET (pTyr1349) were from Cell Signaling (Danvers, MA). Antibodies for ErbB1 and ErbB2 were monoclonal 3C6 (source) and rabbit monoclonal 4B5 (source), respectively. SkBr3 breast cancer cells were obtained from ATCC and grown according to their guidelines. Parental SKOV3ip.1 ovarian cancer cells and SKOV3ip.1-GFP cells were gifts of Laurie Hudson and Angela Wandinger-Ness (Univ. of New Mexico). Since SKOV3ip.1 cells express very low endogenous ErbB3, stable transfectants were created that express ErbB3-GFP under the control of a CMV-based expression vector. SKOV3ip.1 cells and their derivatives were maintained in RPMI with 5% heat-inactivated FBS, 1% L-glutamine, 1% sodium pyruvate, 0.5% penicillin/streptomycin (Invitrogen, Grand Island, NY).

Tissue Sources & Processing

Tissue microarrays (TMAs) were prepared from paraffin blocks of 202 human ovarian tumor samples deposited in the UNM Human Tissue Repository in the period from 1997 to 2011. The quality and classification of samples were confirmed by examination of H&E-stained tissue sections. Surgical staging was based on International Federation of Gynecology and Obstetrics (FIGO) criteria. The TMAs included duplicate sections from each tumor block, as well as tissue from the normal ovary, where available. Since many serous, endometrioid, clear cell and mucinous tumors have their origins outside of the ovary, we also included pre- and postmenopausal distal fallopian tube epithelium as a control on the TMAs. The arrays also included positive controls for EGFR and ErbB2 overexpression (breast and lung tumor tissue). Xenograft tumor samples were also excised from mice two weeks after intraperitoneal injection of SKOV3ip.1-ErbB3-GFP cells (5×10^6 cells) for use as controls. The human xenograft tumors were formalin-fixed and paraffin-embedded. Sections were used to optimize conditions for ErbB3 labeling.

Immunohistochemistry

TMAs were stained for ErbB1 (EGFR) and ErbB2 expression using standard clinical IHC protocols at TriCore Clinical Laboratories (Albuquerque, NM). For the other stains, tissue microarray slides were deparaffinized in xylene and hydrated with alcohol before being placed in 3% H₂O₂/PBS blocking solution. Antigen retrieval was achieved with the use of a decloaking chamber in which the slides were heated for 5 min at 120°C (20–25 p.s.i) in 10mM Citrate Solution (pH 6.0). TMA slide sets were incubated overnight at 4°C with primary antibodies using these dilutions: ErbB3, 1:800; ErbB4, 1:400; MET, 1:1000; pErbB3, 1:10; pMET, 1:50. Slides were then washed, incubated with biotinylated secondary antibody and RTU ABC reagent (Vector Laboratories, Inc. Burlingame, CA). Staining was developed using 3, 3-diaminobenzidine (BD Biosciences, San Diego, CA) and sections were counterstained with hematoxylin (Vector Laboratories, Inc.). The majority of the slides were scored independently by 2 investigators, including a board-certified pathologist. The four graded scale was based on these criteria: 0 no or little staining in < 10% of cells (negative), 1+ faint, partial staining in > 10% of cells, staining intensity less than 50% (weak), 2+ weak to moderate, complete staining in > 10% of cells, staining intensity between 50–80% (intermediate), and 3+ strong, complete membrane staining in > 10% of cells, staining intensity between 80–100% (strong). Any staining between 1+ was labeled low and anything between 2+–3+ was labeled as high expression for PFS analysis.

Statistics

PFS was calculated from the date of diagnosis to either the date of first event (recurrence or metastasis) or last follow-up where data was available. The Kaplan-Meier method was used to estimate the PFS probability. Score test (also known as log-rank test) and hazard ratio (HR) based on the univariate Cox regression were used to assess the association of positive staining for each receptor with PFS. Multivariate Cox regression was performed to assess the above association after adjusting for the effect of clinical risk factors (age at diagnosis, stage and debulking status). A regression tree was used to generate a risk classification rule

based on the expressions of antibodies. All statistical analyses were performed with statistical software R.

Results

Since ErbB3 expression is not routinely evaluated by clinical laboratories, our initial goal was to test and validate available commercial antibodies. Of six different sources, MyBioSource's anti-ErbB3 reagents gave the best and most consistent results. As shown in Figure 1A and B, these antibodies were initially validated by immunofluorescence (IF) and immunohistochemistry (IHC) using formalin-fixed, paraffin-embedded cell pellets prepared from SkBr3 breast cancer cells that express >60,000 ErbB3 per cell [19]. Tumors from mice engrafted with +/- SKOV3.ip cells stably transfected with ErbB3 also served as a positive control (Fig. 1C). Finally, prostate tissue provided a third positive control for ErbB3 expression (Fig. 1E). Similar care was taken to validate the specificity of commercial antibodies against ErbB4 and MET, using positive control human tissues and cell lines pretested by western blotting (data not shown).

Human ovarian tumor tissue microarrays were then used to determine ErbB1-4 and MET expression in 202 patient samples from the human tissue repository (HTR) at the University of New Mexico. Clinical characteristics for patients in this retrospective study are reported in Table I. The median age was 61 years - and the majority of patients presented with advanced stage disease [Stage II-IV (74.4%), G3 (66%)].

Images in Figure 2 show results comparing two different ovarian cancers from these arrays along with their assigned score. The figure also shows the staining for each antibody using the appropriate positive control tissue. This figure illustrates both the quality of the samples in the TMAs and shows representative staining patterns for each receptor. All samples in the TMAs were scored based upon membrane staining for ErbB1, ErbB2, ErbB3 and MET. For ErbB4, whose cleavage product is known to translocate from the cytosol to the nucleus, scoring was based upon membrane, cytoplasmic or nuclear staining. The collective results are summarized in Table II, -demonstrating that only 25% of tumors were positive for ErbB1 and 35% were positive for ErbB2 expression. In contrast, ErbB3, ErbB4 and MET expression was marked in 76%, 98% and 96% of cases respectively. Also 79% of the cases have the active form of ErbB3 and 56% have the active form of MET.

Figure 3 shows images of two ovarian cancer cases, after staining for the phosphorylated forms of ErbB3 and MET (phosphotyrosine 1289 ErbB3 and phosphotyrosine 1349 MET) in comparison to their unphosphorylated forms. Where expressed, essentially all of the ErbB3 positive cases also tested positive for ErbB3 phosphorylation. Phosphorylation of MET was more variable, with only 56% staining positive for the active form of MET. It is also notable that phosphorylation patterns were often heterogeneous within the same tumor, as illustrated for patient #076 in Figure 3. This example represents a Stage II clear cell case.

PFS analyses were performed on 126 cases where survival data were available. To evaluate the association of receptor expression with PFS, we examined the difference in PFS between the cases where receptor expression were scored as negative (no expression) and positive

(any expression) (for EGFR, ErbB2) or between the cases where receptor expression were scored as low or high (for ErbB3, ErbB4, MET). We found no sufficient statistical evidence for the association of expression of any of these growth factor receptors with PFS in either univariate analysis (Figure 4) or multivariate analysis adjusting for the effects of age, stage and debulking status.

We also explored the possible role of ErbB3, pErbB3, MET and pMET expression as a predictor of PFS. We found an association of better PFS with high pErbB3 and pMET expression in a univariate analysis groups (log rank test, p-value = 0.017; not shown). However this did not hold as an independent predictor when analyzed against known strong clinical predictors such as age at diagnosis, stage and debulking status using multivariate analysis.

Discussion

Due to the recognized importance of growth factor receptors to cancers of epithelial origin, a number of groups have set out to characterize expression patterns for ErbB and MET/Ron family receptors in ovarian cancer. As pointed out in a 2008 review by Lafky, the wide variation in methods and reagents utilized is a complicating factor in interpreting this literature [18]. We set out to perform comparative analysis of ErbB family and MET expression in a large set of samples encompassing a broad variety of histological types of ovarian cancer, both invasive and borderline cases. For ErbB2, our results are consistent with prior reports that ErbB2 gene amplification and receptor overexpression occurs in 11–30% of cases [23, 24, 27]. Similarly, ErbB1 expression is detectable in only a small subset of patients ([28], Table II, this study) The low frequency of ErbB1 and ErbB2 expression likely explains the poor overall responses of ovarian cancer patients treated with therapeutic antibodies (pertuzumab, cetuximab, matuzumab) or kinase inhibitors (erlotinib, gefitinib, lapatinib or CI-1033) [5, 25, 29–32]. We suggest that prescreening for ErbB1 or ErbB2 expression should be an enrollment criteria for future clinical trials employing these and similar agents in this disease.

Prior studies have reported ErbB3 expression to vary from 3–90% in ovarian cancer [17, 18, 33–37], likely reflecting the inconsistent quality of commercial antibody reagents and standardization of protocols. Amler used ErbB3 mRNA levels as a surrogate for protein expression, implicating high ErbB3 expression in accelerated relapse after treatment of patients with the ErbB2-targeted antibody, pertuzumab [25]. Overexpression of ErbB3 has been suggested to be either a poor prognostic factor in ovarian cancer [17] or to show no significant correlation with grade, age, metastasis or overall survival.

Given this wide variation in prior results and the controversies surrounding ErbB3 expression in ovarian cancer, our initial priority was the validation of ErbB3 reagents for immunohistochemical analysis of the TMAs (Figure 1). Results in Figure 2 and Table II are more in agreement with the conclusion that ErbB3 expression is not directly correlated with PFS, since overall expression was prevalent in over 75% of cases. On the other hand, the high prevalence of ErbB3 cell surface expression in tumor cells indicates that ErbB3-targeted therapies may be broadly applicable in this disease. Consistent with evidence for an

ErbB3-neuregulin autocrine loop in tumor cell proliferation [31], most samples also stain positive for the phosphorylated, active form of the receptor. The low percentage of ErbB2-expressing tumors in this sample set takes on special significance, since ErbB2 is often considered to be ErbB3's preferred heterodimerizing partner [38, 39]. We speculate that another heterodimerizing partner participates in the transactivation of ErbB3. The mostly likely candidate is ErbB4 [40], which we show here is expressed in over 90% of ovarian tumors. Others have also reported high incidence of ErbB4 expression in this disease. [20, 21].

The high frequency of dual expression for MET and ErbB3 in most tumors is also of keen interest, since crosstalk between these two receptors has been reported to drive cancer progression and/or resistance to therapy [16, 41, 42]. Although one co-precipitation study raised the possibility that the two receptors might associate in a complex [16], the extracellular domain of MET is structurally distinct from the ErbB proteins [43] and homology modeling indicates that MET lacks the dimerization arm common to the ErbBs (our unpublished results). Thus, the precise mechanism for Met-ErbB3 crosstalk is presently unknown.

We found MET to be detectable in most of the samples in this study, but these results leave open the possibility that variation in levels may be linked to poor outcome as suggested by others [16, 22]. When activated by its ligand, HGF, MET is mitogenic for cultured ovarian cells [44]. Its inhibition can reduce adhesion, invasion, metastasis and ultimately tumor burden. [22]

Although we failed to link expression of these 5 growth factor receptors with PFS, we acknowledge the limitations of our dataset which include a very heterogeneous mix of ovarian cancer cases (Table I) with only 126 cases with adequate PFS endpoints. Given this mixture, this study set is too small to draw conclusions about PFS. It would have been optimal to have pure grade, either mixed endometrioid and serous or pure serous tumor types included in the array, and the next step would be to consider creating such a TMA for future studies. Although provocative, the possibility that pErbB3 and ErbB3 expression could impact treatment response and PFS will require further investigation.

In summary, ErbB3, ErbB4 and MET are highly expressed in the majority of ovarian carcinomas while ErbB1 and ErbB2 expression is far less common. Small molecule MET kinase inhibitors are currently under evaluation, as are therapeutic antibodies directed at both ErbB3 and MET [31, 44–47]. The availability of standardized IHC protocols for pathologic evaluation, as described here, could improve the design of clinical trial selection criteria for both single agent and combination therapies that target these important growth factor receptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Validation of the ErbB3 antibody from MyBioSource using A and B) SkBr3 breast cancer cells; C and D) xenograft tumors excised from mice engrafted with SKOV3-ip.1 ovarian cancer cells that stably express ErbB3-GFP fusion proteins; and E and F) prostate cancer tissue.

Figure 2. Two representative ovarian cancer samples of different stage and histological types stained for ErbB1, ErbB2, ErbB3, ErbB4 and MET. Magnification is at 40X. Also included are positive control tissues used for each antibody stain.

#014 (Stage IV-B pap serous)

Figure 3.

Two representative ovarian cancer samples of different stage and histological type stained for ErbB3 and MET, with paired images of staining for their phosphorylated forms. The images show the assigned score for each sample. Magnification is at 40X.

Table I

Clinical characteristics of the 202 ovarian cancer patients included in this study Have median age, median age at diagnosis, race, stage, chemotherapy (Yes/No), ascites present (Yes/No), grade and type.

	n		n
<u>Median age (range)</u>	61 (12–94)	<u>Tumor grade</u>	
<u>Median age at diagnosis (range)</u>	55 (4–88)	1	25
		2	22
		3	91
<u>Race</u>		Unknown	64
White/Anglo	89		
Hispanic	59		
American Indian/Alaska native	20	<u>Histology</u>	
Asian	2	EPITHELIAL-Borderline	
Black/African American	1	Serous	17
Other	22	Mucinous	7
Unknown	9	Endometrioid	3
		Mixed	2
<u>FIGO stage</u>			
IA	17	EPITHELIAL-Invasive	
IB	4	Serous	88
IC	23	Mucinous	11
II	2	Endometrioid	22
IIA	2	Transitional/Brenner	4
IIB	4	Mixed	12
IIC	16	NOS	11
IIIA	2	Clear cell	10
IIIB	6		
IIIC	78	STROMAL	
IV	14	Granulosa cell	4
IVA	1	Carcinosarcoma (MMMT)	11
IVB	3		
Unknown	30	<u>Chemotherapy</u>	127
		Ascites	69

Table 2

Summary of ErbB family and MET expression in 202 cases

	EGFR	ErbB2	ErbB3	ErbB4	MET	pErbB3	pMET
Percent of cases positive (IHC)	51/202 (25%)	69/200 (35%)	148/195 (76%)	193/197 (98%)	188/196 (96%)	150/192 (78%)	111/200 (56%)
Low/High cases	43/8	54/15	88/60	52/141	47/141	112/38	99/12