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BRCA1/BRCA2 polymorphisms and prognosis in women with optimally resected stage III epithelial ovarian cancer treated on GOG protocol 172: A Gynecologic Oncology Group study

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Objectives: Mutations in BRCA1 or BRCA2 appear to be associated with sensitivity to platinum agents and improved prognosis, but little is known about the clinical relevance of polymorphisms in BRCA1/BRCA2 in epithelial ovarian cancer (EOC). The Gynecologic Oncology Group (GOG) examined whether common variants of BRCA1/BRCA2 are associated with prognosis in sporadic EOC.

Methods: Genotypes for BRCA1 (Ex12+1485C>T, P871L-RS799917) and BRCA2 (Ex10+321A>C, N372H-RS144848) were determined using Sequenom's MALDI-TOF platform (iPLEXTMGOLD Assay; San Diego, CA, USA) in DNA extracted from blood from women who participated in GOG Protocol 172, a phase III trial of women with optimally resected stage III EOC treated with either intraperitoneal or intravenous cisplatin and paclitaxel. Adjusted Cox regression modeling was performed to assess the associations between polymorphisms and progression-free survival (PFS) and overall survival (OS).

Results: Among the 232 women in this study, the genotype distribution for BRCA1 P871L was 37, 48 and 16% for CC, CT, and TT, respectively. After adjustment for cell type, residual disease and chemotherapy regimen, each C→T transition was associated with a 1.3-fold increased risk of disease progression (95% CI=1.06-1.65, $P=0.013$) or death (95% CI=1.02-1.64, $P=0.031$). The genotype distribution for BRCA2 N372H was 51, 43 and 6% for AA, AC and CC, respectively. BRCA2 N372H genotype was not associated with PFS or OS.

Conclusions: The BRCA1 P871L, but not the BRCA2 N372H, polymorphism, appears to be associated with worse prognosis in women with optimally resected stage III EOC treated with cisplatin and paclitaxel and requires validation.

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Dual targeting of mammalian target of rapamycin and phosphoinositide 3-kinase using NVP-BEZ235 as a novel therapeutic approach in human ovarian carcinoma

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Objectives: The oncogenic phosphoinositide 3-kinase (PI3-kinase/Akt/mammalian target of rapamycin (mTOR) pathway is upregulated in 50-70% of ovarian cancers. Inhibition of either mTOR or PI3-kinase is therefore currently being exploited as a therapeutic strategy. This study investigates the effect of NVP-BEZ235, a novel small molecule inhibitor of both PI3-kinase and mTOR, on the growth of human ovarian cancer cells in culture and in an immunocompetent animal model.

Methods: Experiments were performed in 15 human ovarian cancer cell lines, including SKOV3, IGROV1, and OVCAR5. Cells were treated in culture with increasing concentrations of either RAD001 (mTOR inhibition alone) or NVP-BEZ235 (dual mTOR and PI3-kinase inhibition). Immunoblotting was used for target validation. Cell viability and cell proliferation were determined after three days in culture. Fluorescence-activated cell sorting (FACS) was performed for cell cycle analysis. The effect of NVP-BEZ235 in vivo was assessed using the immunocompetent LSL-K-Ras G12D+/PTEN loxP/loxP murine ovarian cancer model, which is based on Cre-mediated conditional deletion of the tumor suppressor gene PTEN and concomitant expression of constitutively active K-Ras. Mice with established tumor disease were treated with NVP-BEZ235 (40 mg/kg) daily and followed for survival. Tumor tissue was analyzed by immunohistochemistry for protein expression.

Results: In culture, NVP-BEZ235 caused a significant reduction in proliferation in all 15 ovarian cancer cell lines. Interestingly, this effect was independent of the cellular, basal level of PI3-kinase/Akt/mTOR pathway activation, and greater than the effect of treatment with RAD001 alone. NVP-BEZ235 treatment inhibited phosphorylation of Akt, 4-EPB1, and S6. In contrast, RAD001 induced pAkt expression in some cell lines. FACS demonstrated NVP-BEZ235-mediated cell cycle arrest in G1/S phase. In vivo, treatment of mice with established ovarian tumors using NVP-BEZ235 caused downregulation of pAkt and conferred a significantly longer survival compared with that of control animals (118 days vs 80 days, $P<0.05$).

Conclusions: Our results indicate that targeting mTOR and PI3-kinase simultaneously has a significantly greater effect on inhibiting growth of ovarian cancer cells in culture and in vivo compared with mTOR inhibition alone. We propose that dual inhibition of mTOR and PI3-kinase is a promising new therapeutic strategy in ovarian cancer.

Hedgehog pathway inhibitor cyclopamine suppresses GLI1 expression and inhibits serous ovarian cancer xenograft growth

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Objectives: The goal of this study was to determine whether inhibition of the hedgehog (Hh) pathway has an effect on the growth of human serous ovarian cancer xenografts.

Methods: With approval from both the human and animal IRB committees, serous ovarian tumor tissue was harvested at the time of primary surgery and cells were isolated and injected into six-week-old female NOD/SCID mice. Following serial transplantation, tumor-bearing mice (300-600 mm³) were treated with vehicle or with the Hh inhibitor cyclopamine (CYC, 25 mg/kg). Tumors were harvested after 0, 6, 12 and 24 hours, and the RNA was subjected to reverse transcription polymerase chain reaction to determine levels of Hh pathway mediators including PTCH, SMO, and Gli1. The second experiment involved two cohorts of six mice randomized by tumor size. They received either CYC 25 mg/kg or vehicle everyday for 15 days, with tumors measured with calipers every three days. The third experiment employed four cohorts of five mice each matched by tumor size that received (1) single-agent CYC 25 mg/kg administered by gavage every 24 hours along with intraperitoneal vehicle; (2) paclitaxel 15 mg/kg and carboplatinum 50 mg/kg (T/C) intraperitoneally every five days with oral vehicle by gavage every 24 hours; (3) CYC 25 mg/kg by oral gavage every 24 hours + intraperitoneal T/C; (4) oral vehicle by gavage every 24 hours + intraperitoneal vehicle every five days. This four-arm trial lasted 15 days, and tumor measurements and mouse weights were assessed every three days.

Results: Inhibition of the Hh pathway resulted in a decrease in Gli1 mRNA levels after 12 hours. Compared with vehicle, daily CYC treatments precluded tumor growth after 10 days ($P < 0.005$) without significant changes in weight between cohorts ($P = 0.53$) or within cohorts ($P = 0.30$). The four-arm experiment confirmed that single-agent CYC impeded growth ($P = 0.05$), though T/C had improved ability to preclude growth compared with CYC alone ($P < 0.02$). The synergy of CYC and T/C combined produced the most robust inhibitory effect compared with T/C alone ($P < 0.01$), shrinking the tumors a median of 60%. Postharvest tumor weights revealed that tumors treated with CYC and T/C decreased markedly in mass compared with tumors in all other arms of the trial ($P < 0.005$). All arms in which T/C was administered exhibited decreased mouse weight compared with the other arms, though this effect did not reach statistical significance ($P = 0.10$).

Conclusions: CYC inhibits Gli1 expression levels and negatively affects serous ovarian xenograft tumor growth with a significant synergy with conventional intraperitoneal chemotherapy, suggesting that the Hh signaling pathway is a candidate for targeted therapy in ovarian cancer.

Development of a mouse ovarian cancer model with human tumor vessels to test novel antivascular therapeutics

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Objectives: The expression profile of tumor vasculature is unique from that of normal vasculature, and recent studies suggest that different tumor types express distinct tumor vascular markers (TVMs). TVMs represent a novel target for anticancer therapy; however, development of these therapies has been limited by the lack of an animal model system expressing human tumor vessels and TVMs. We therefore sought to establish a mouse tumor model expressing human ovarian TVMs, to test the safety and efficacy of anti-TVM immunotherapeutics.

Methods: H9 human embryonic stem cells (ESCs) were injected subcutaneously into mice and allowed to grow into a teratoma. HEY1 ovarian tumor cells were then grown within ESC-derived teratomas. Resultant vascular cells are derived from the ESCs and are human vessels, as previously described. Tumors were collected and analyzed for human ovarian TVM mRNA and protein expression using reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry, respectively. To investigate the impact of the tumor microenvironment on TVM expression, we cultured human dermal microvascular endothelial cells (HDMECs) together with either ovarian or breast tumor cell lines in mice. Tumors were harvested and specimens collected for RT-PCR and immunohistochemistry.

Results: RT-PCR confirmed the expression of dozens of human ovarian TVMs in ESC-ovarian tumors. In contrast, TVMs were not expressed by ESC-derived teratomas or HEY1 flank tumors grown separately. Immunohistochemistry confirmed the expression of tumor endothelial-specific proteins Tem7 and Thy-1 in the tumor neovasculature. This demonstrates that vessels in this model express human TVMs and suggests that TVMs may be induced in the presence of tumor cells. Interestingly, studies with HDMECs grown with either ovarian or breast tumor cells revealed differential expression of some but not all ovarian versus breast TVMs.

Conclusions: We have developed a mouse tumor model expressing human ovarian TVMs. This represents a critical tool for the development of novel anti-human vascular immunotherapeutics. Furthermore, our data suggest that at least some TVMs are induced by specific tumor types; thus, the distinct expression profile of vascular cells from different tumors may impact the development anti-TVM therapies.

Ovarian cancer tumor-infiltrating regulatory T cells are associated with a metastatic phenotype

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Objectives: Regulatory T cells (T-regs) are a distinct sub-population of T cells with an immunosuppressive role. An attenuated immune response related to T-reg tumor infiltration may be a mechanism that facilitates metastasis in patients with epithelial ovarian cancer. The objective of this study was to determine the relationship between the number of tumor-infiltrating T-regs in ovarian cancers and clinical features and outcome. We also used microarrays to characterize the genomic signature of cancers with high T-reg infiltration.

Methods: Tumor-infiltrating T-regs and cytotoxic T cells (CTLs) were quantitated in 232 primary epithelial ovarian cancers by immunostaining paraffin sections for FOXP3 and CD8, respectively. Tumor intraepithelial T-regs were counted manually in the three densest high-power fields (hpf). Affymetrix U1333A microarray analysis was performed on frozen tissue samples from a subset of 47 advanced cancers with the highest and lowest numbers of infiltrating T-regs. A genomic signature of high T-reg infiltration was developed using binary regression and differences in pathway expression were assessed using Ingenuity Pathway Analysis (IPA) software.

Results: High T-reg infiltration was more common in high-grade cancers (mean T-regs/hpf: 1.4 vs 9.6 in borderline/grade 1 vs grade 2/3 tumors, $P < 0.0001$) and in advanced-stage cancers (mean T-regs/hpf: 4.1 vs 10.0 in stage I/II vs stage III/IV tumors, $P = 0.0036$). In advanced-stage cases, there was no association between high T-reg infiltration and survival, whereas, as has previously been reported, a high level of tumor-infiltrating CD8 CTLs (median cutoff: >2.1 intraepithelial CTLs/hpf) was associated with favorable survival (median survival: 48.7 months vs 34.6 months, $P = 0.01$). The microarray-based genomic signature for high tumor-infiltrating T-regs had 77% predictive accuracy for high versus low T-regs using leave-one-out cross-validation. ANOVA of microarray data with correction for multiple comparisons revealed the antigen presentation pathway as the most differentially expressed canonical pathway ($P = 6.36E - 06$) between cancers with high and low T-reg tumor infiltration.

Conclusions: High numbers of tumor-infiltrating T-regs in ovarian cancers are associated with higher grade and advanced stage, but not with survival. This suggests that these immunosuppressive cells may facilitate metastasis of some ovarian cancers by downregulating immune mechanisms that normally inhibit this process. Increased T-reg tumor infiltration is characterized by a genomic signature enriched with several differentially expressed immunologic pathway genes. Therapeutic strategies that reduce tumor-infiltrating T-regs are under investigation and may prove useful in a subset of ovarian cancers with high numbers of these cells.

BAD phosphorylation status: A critical determinant of ovarian cancer platinum sensitivity

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Objectives: The development of chemoresistance dramatically influences survival for patients with ovarian cancer (OC). We previously described the phenotypic and genotypic evolution of OC platinum resistance using a combination of serial cell line cisplatin (CIS) treatments paralleled by measures of genome-wide expression change. We now seek to define the biologic pathways associated with that chemoresistance development and further, to characterize and interrogate one specific apoptotic pathway: BCL2 antagonist of cell death (BAD).

Methods: Functional pathway analysis of genomic data previously obtained from cells during serial CIS treatments was performed. With the use of immunofluorescence, BAD protein (phosphorylated [Ser 75, 99, 118] and nonphosphorylated) was quantified in the series of OC CIS-treated cell lines. Phospho-BAD (P-BAD) and nonphosphorylated BAD (NP-BAD) levels were measured in 18 primary OCs (9 platinum sensitive and 9 platinum resistant). AKT-mediated BAD phosphorylation was inhibited using triciribine (TCN, an inhibitor of AKT activation) and the effect on CIS sensitivity and P-BAD status was evaluated. Finally, in an effort to confirm the central role of P-BAD status in OC CIS sensitivity, OC cells were subject to BAD small interfering RNA gene expression knockdown and targeted mutagenesis of the three BAD phosphorylation sites.

Results: Functional pathway analysis identified apoptosis (BAD pathway), transcription, cell cycle control, G-protein signaling and cyto-/chemokine-mediated signaling as the most significant cell processes ($P < 0.001$) associated with evolution of CIS resistance. P-BAD expression increased (Pearson's correlation, $R = 0.74$ to 0.91) and NP-BAD expression decreased ($R = -0.71$ to -0.94) with induced evolution of CIS resistance. CIS-resistant patient samples demonstrated 3-fold higher P-BAD expression ($P < 0.05$) and 4.9-fold lower NP-BAD expression ($P = 0.12$) compared with CIS-sensitive tumors. TCN inhibition of AKT activation resulted in almost complete inhibition of BAD phosphorylation, with an accompanying 2- to 8-fold increase in CIS sensitivity. Importantly, BAD small interfering RNA gene knockdown and targeted mutagenesis of BAD phosphorylation sites also increased CIS sensitivity.

Conclusions: BAD phosphorylation status plays a critical role in determination of OC cell platinum sensitivity. AKT inhibition likely increases platinum sensitivity via inhibition of BAD phosphorylation. The BAD survival/apoptosis pathway represents a compelling target for future OC therapeutics.