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Role of Cyclin E as an Early Event in Ovarian Carcinogenesis

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Cyclin E amplification and overexpression characterizes a subset of epithelial ovarian cancers. We hypothesized that this subset of tumors may demonstrate an enhanced response to targeted therapy with the proteasome inhibitor, bortezomib. Cyclin E also exists in multiple low molecular weight (LMW) isoforms in cancer cells which demonstrate greater neoplastic activity. A natural dietary phytochemical called Indole-3-Carbinol (I3C) disrupts cyclin E processing through the inhibition of the enzyme that cleaves cyclin E into its LMW isoforms. When combining I3C with bortezomib, we found I3C to synergistically sensitize ovarian cancer cells to bortezomib. This finding has translational potential as bortezomib as a single-agent was found to have minimal activity in a phase II treatment trial of recurrent ovarian cancer. This finding could re-introduce bortezomib to the therapeutic armamentarium against ovarian cancer if the in vitro results replicate in mice and humans.

15. SUBJECT TERMS
Cyclin E, P27, SKP2, RB, bortezomib, Indole-3-Carbinol, mouse model, ovarian cancer initiation

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INTRODUCTION

We are interested in defining the genetic changes that initiate and drive the aggressive behavior of epithelial ovarian malignancies. In a pilot study looking at the genetic changes occurring across the whole genome of high-grade papillary serous ovarian cancers, we identified *cyclin E* as an interesting candidate gene. We found high-copy number amplification of the *cyclin E* gene locus to be the single most notable recurrent genetic event. Furthermore, epidemiological evidence links the subset of cyclin E overexpressing epithelial ovarian cancers to an increased number of lifetime ovulatory cycles and the "incessant ovulation" theory of ovarian cancer causality. Experimental systems have shown deregulation of cyclin E levels to result in chromosomal instability, a hallmark feature of epithelial ovarian cancers. This led us to hypothesize that cyclin E deregulation is an important initial event in ovarian carcinogenesis. We proposed three specific aims: (1) to characterize the genetic events induced along with cyclin E amplification and overexpression; (2) to determine the role of cyclin E and its collaborating genetic events in ovarian cancer initiation; and (3) to define the subset of ovarian cancers with impaired cyclin E inhibition and to determine whether these tumors demonstrate an enhanced response to targeted therapy. Here, we report research accomplishments from the first year of the study.

BODY

Specific Aim 1: To characterize the genetic events induced along with cyclin E amplification and overexpression.

Task 1: DNA analysis for genetic events occurring with cyclin E gene amplification using anatomical samples.

Upon screening 72 ovarian cancer cases on a frozen tumor tissue microarray for *CCNE1* gene amplification using fluorescence in-situ hybridization (FISH), we identified 11 cases with a cluster pattern of cyclin E. These samples have also undergone gene expression profiling on an Agilent Human 1A VS Chip. Together, these studies have found a high correlation between cyclin E gene amplification and cyclin E overexpression. We planned to have these 11 samples arrayed with the Affymetrix 250K *Nsp* oligonucleotide microarray to determine the genetic events that occur commonly among ovarian cancer samples with *CCNE1* gene amplification. However, we have put this task on hold for now as our collaborator has generated Agilent array CGH data on 128 ovarian cancer samples from our tumor bank. Of these, 20 tumors demonstrated focal amplification of the *CCNE1* gene locus. We will be working together in the next year to determine the genetic copy number variation events that occur in this subset of 20 tumors.

We plan to take this analysis a step further with FISH analysis of formalin-fixed, paraffin-embedded tissue from the 132 papillary serous ovarian cancers in the gene expression dataset that were not analyzed by array CGH. This will allow us to

correlate *CCNE1* gene amplification and cyclin E expression patterns. We will be able to further refine our analysis by limiting the correlations to those tumors with *CCNE1* gene amplification. Cases with normal *CCNE1* gene copy number but high levels of cyclin E gene expression can be analyzed in a separate analysis. Differences in gene expression patterns between the two subsets of cyclin-E overexpressing tumors can also be analyzed.

Bacterial artificial chromosome (BAC) clone RPCI11.C-345J21 (Invitrogen, Carlsbad, CA) will be used as the FISH probe. Briefly, slide sections will be deparaffinized, treated with a protease solution, denatured, and hybridized overnight with the fluorescently-labeled FISH probe. The sections will then be washed and analyzed by fluorescence microscopy to detect cyclin E amplification. The results obtained from FISH experiments will be analyzed along with the corresponding gene expression profiling data from these same samples. Such analyses will allow for correlative studies between cyclin E amplification and other genes which are concurrently up-regulated or down-regulated.

Task 2: RNA analysis for gene pathways activated with cyclin E overexpression, using anatomical samples.

We have completed RNA isolation and gene expression profiling from 132 papillary serous ovarian cancer samples. We have performed an analysis to identify the genes that are upregulated with cyclin E overexpression. We found the majority of the genes are cell cycle genes functionally related to cyclin E and cell cycle progression. This is unlike Her2 in breast cancer, where the genes correlating with HER2 are located on the same amplicon. However, we found some correlated genes adjacent to *CCNE1*, including *C19orf1*, *C19orf12* and *ZNF587*.

Specific Aim 2: To determine the role of cyclin E and its collaborating genetic events in ovarian cancer initiation

Task 3: Mouse model to test ability of cyclin E and its collaborating genetic events to induce oncogenic activation

To test the cancer initiating potential of cyclin E overexpression, we will use a mouse model which allows for introduction of collaborating genetic events to lead to transformation of mouse primary ovarian surface epithelial cells. Full-length cyclin E and two truncated cyclin E isoforms will be expressed by a retroviral vector which is able to introduce a gene of interest into a specific cell type or tissue.

Lower molecular weight isoforms of cyclin E have been described by Dr. K. Keyomarsi's group in Texas [1]. As many as five low molecular weight (LMW) isoforms of cyclin E exist in cancer tissues, while only the 50-kDa cyclin E form is expressed in normal tissues. The LMW isoforms have been described to have greater malignant potential.

Cyclin E overexpression: We are creating the reagents that will allow for introduction of the full-length cyclin E gene and two truncated cyclin E isoforms into our mouse model. OVCAR5 cells were transfected with 2 µg pRC-CMV-cyclin E, pcDNA3-cyclin E FL, pcDNA3-cyclin E trunc 1, or pcDNA3-cyclin E trunc 2 using the BioT transfection reagent (Bioland Scientific, La Palma, CA). Whole cell lysates were collected and 25 µg of protein was analyzed by Western blot. Cyclin E protein expression was detected using an anti-cyclin E mouse monoclonal antibody (clone HE12, Santa Cruz Biotechnology, Santa Cruz, CA), followed by a fluorescently-conjugated secondary antibody for visualization using the LI-COR Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NB). The pRC-CMV-cyclin E construct was kindly provided by B. Weinberg and the pcDNA3-cyclin E constructs were provided by K. Keyomarsi (University of Texas).

Whole cell lysates were collected and the protein analyzed by Western blot (Fig. 1). The pRC-CMV-cyclin E construct expressed full-length cyclin E (50 kDa) in addition to the lower molecular weight isoforms (45 kDa, 35 kDa). The pcDNA3-cyclin E FL also expressed all isoforms, especially the 50 kDa protein. The pcDNA3-cyclin E trunk 1 and trunk 2 expressed the 45 and 35 kDa isoforms, respectively. The cyclin E expression cassettes from these constructs will be recombined into the RCAS retroviral vector for introduction of the viral vector into our mouse model.

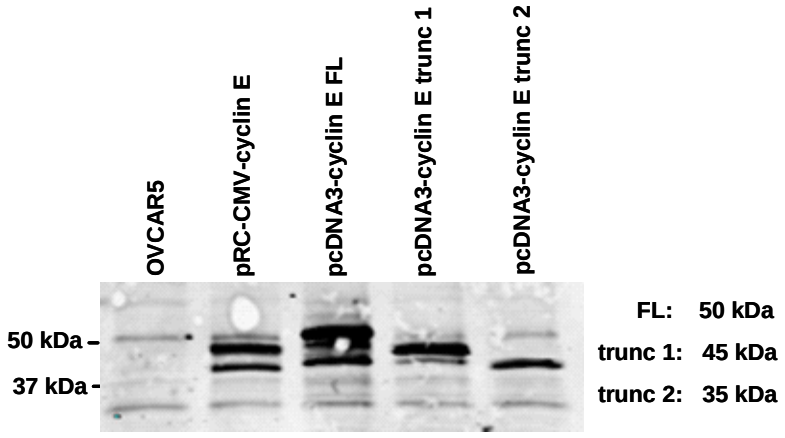


Figure 1. Western blot analysis of cyclin E expression. OVCAR5 cells were left untreated (lane 1) or transfected with 2 µg pRC-CMV-cyclin E, pcDNA3-cyclin E FL, pcDNA3-cyclin E trunc 1, or pcDNA3-cyclin E trunc 2 (lanes 2-5). Whole cell lysates were collected and analyzed by Western blot.

Recombination Reaction with RCAS Vector: To test the cancer initiating potential of cyclin E overexpression using a mouse model, we will express full-length and truncated cyclin E isoforms from a retroviral vector called RCAS (replication-competent ASLV long terminal repeat with splice acceptor). The Gateway Technology system (Invitrogen, Carlsbad, CA) will be used for recombination of cyclin E into the RCAS vector (contains *att*R recombination sites). First, the cyclin E

expression cassettes from the constructs mentioned above will be cloned into a donor vector, generating an entry clone with *att*L recombination sites. The LR reaction, which facilitates recombination of an *att*L substrate (entry clone) with an *att*R substrate (destination clone) in the presence of the LR clonase enzyme, will be performed for generating RCAS-cyclin E (Fig. 2). Cyclin E will replace the lethal *ccd*B gene present in the parental destination vector.

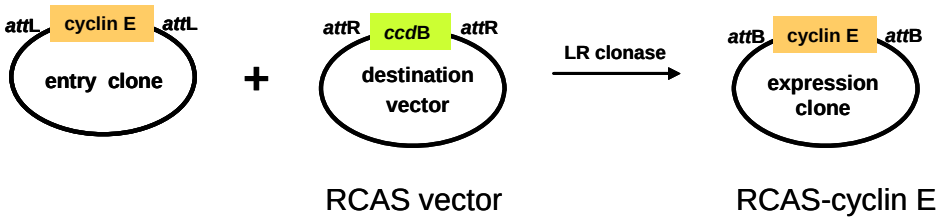


Figure 2. LR recombination with cyclin E entry clone and RCAS destination vector. Full-length cyclin E and truncated low molecular weight isoforms will be cloned into a donor vector generating an entry clone with *att*L sites. The entry clone will recombine with the destination vector (RCAS) containing *att*R sites, replacing the lethal *ccd*B gene. The final expression clone is the RCAS vector expressing cyclin E (flanked by *att*B sites).

Mouse Model: We are in process of crossing transgenic mice that express Keratin5-TVA (chicken retroviral keratin receptor that is expressed on the ovary) with conditional P53 mutant mice. K5-TVA mice have been crossed with 129S4-Trp53^{tm2Tyj} (P53 LSL R172H) mice, which carry a conditional point-mutant allele of the p53 gene that can be activated by Cre-mediated recombination. This line contains a LoxP site and a transcriptional / translational STOP sequence in intron 1 (making it functionally equivalent to a null mutation) and an R172H missense mutation in exon 5. The strain was maintained on a 129S4/SvJae background. Activation with Cre-recombinase leads to deletion of the transcriptional termination sequence (Lox-Stop-Lox) and expression of the oncogenic P53 protein. The genotyping strategy is illustrated in figure 3.

Primers:
T036 : 5'-agc tag cca cca tgg ctt gag taa gtc tgc a -3'
T035 : 5'-ctt gga gac ata gcc aca ctg -3'
T037 : 5'-tta cac atc cag cct ctg tgg -3'

Product Sizes:
T036/T035: 279 bp (Mut LSL)
T037/T035: 166 bp (Wild Type)

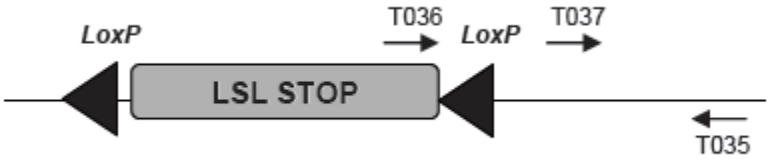


Figure 3. Genotyping strategy of P53 LSL R172H mice. The noted primers allow us to detect the wild type allele with T037 and T035, which amplify intron 1, and the mutant allele with T036 and T035, which amplifies the LSL element.

We are genotyping mice with one-step PCR procedures using mouse tail tissues to isolate DNA from crude lysates. Mice that carry the K5-TVA transgene and the conditional mutant P53 allele will be selected for further experiments. The RCAS-Cyclin E vector and Ad-Cre will be introduced into the OSE of K5-TVA p53 LSL R172H mice to determine the oncogenic potential of cyclin E overexpression in the setting of P53 deficiency. Experiments will be performed with the full length cyclin E construct as well as with the truncated cyclin E low molecular weight isoforms.

Specific Aim 3: To define the subset of ovarian cancers with impaired cyclin E inhibition and to determine whether these tumors demonstrate an enhanced response to targeted therapy

Task 4: Immunohistochemistry (IHC) for cyclin E, SKP2, P27 using anatomic samples

We will perform immunohistochemistry for cyclin E, SKP2 and P27 in patient ovarian cancer samples. This task will be performed in the upcoming year.

Task 5: DNA mutation analysis for FBXW7 mutations, using anatomical samples

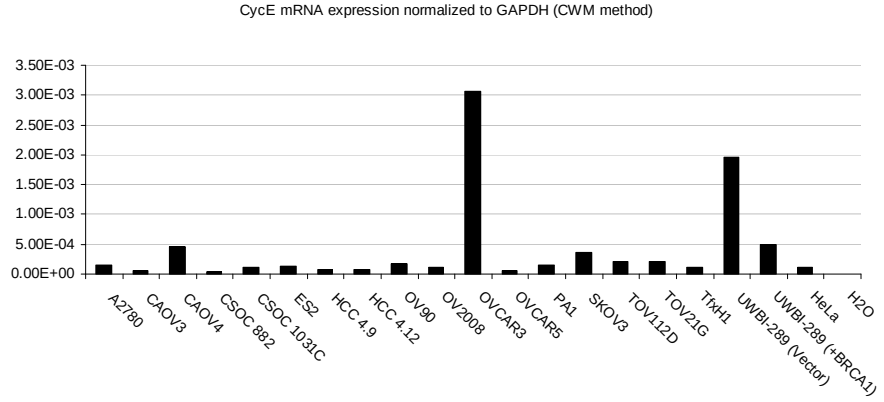
In our pilot study, we identified thirteen samples with loss of heterozygosity at the *FBXW7* gene locus. The SCF-Fbw7 ubiquitin ligase system ensures tight control of cyclin E levels. Disruption of the Fbw7 tumor suppressor leads to genetic instability through deregulated cyclin E activity. The *FBXW7* gene has been found to be mutated in ovarian cancer cell lines, implicating its potential role in the pathogenesis of this malignancy. We planned to screen for mutations in the *FBXW7* gene in these 13 ovarian cancer samples. We did a search of publically available data from Sanger and the cancer genome atlas (TCGA). In the Sanger data, one *FBXW7* mutation (c.1417delA) was found in a single cell line (T-24) among a panel of 21 ovarian cancer cell lines. Sanger data also included sequencing in 183 clinical tumors (including breast, CNS, kidney, colon, lung, pancreas, pleura, salivary gland, skin, upper GI tract, and urinary tract) and found mutations in *FBXW7* in 2 (1%) of samples. No clinical ovarian cancer samples were included in the Sanger data, but the TCGA used NextGen sequencing and found no *FBXW7* mutations in 60 to 80 ovarian cancer samples. Based on this publically available data, *FBXW7* appears to be very infrequently mutated in multiple tumor types, including ovarian cancer. Therefore, we have not performed this task and have focused our attention on other areas that are likely to be of higher yield.

Task 6: In vitro proliferation assays, using 6 serous ovarian cancer cell lines with various cyclin E and SKP2 expression, and assessing for therapeutic response to the proteasome inhibitor, bortezomib

The majority of our work over the last year in specific aim 3 was spent addressing this task. The goal of this task was to determine whether targeted therapies could be used to specifically inhibit ovarian cancers that overexpress cyclin E.

To accomplish this task, we assayed a panel of ovarian cancer cell lines to determine endogenous levels of cyclin E and SKP2. We found OVCAR3 cells to express high levels of both proteins and OVCAR5 cell to express low levels of both proteins (figure 4). We used these two cell lines in further experiments. Furthermore, we found OVCAR3 cells to have a genetic amplification of the *CCNE1* gene locus on chromosome 19, similar to the amplifications seen in clinical ovarian cancer cell lines (figure 5). This led us to conclude that OVCAR3 cells are a good model for studying therapeutic responses in the setting of cyclin E amplification and overexpression.

4A.



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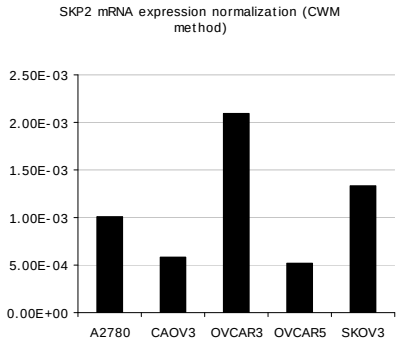

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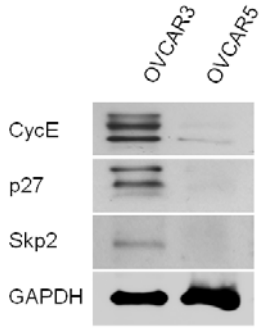

Figure 4. Cyclin E and SKP2 expression in panel of ovarian cancer cell lines. 4A. Real time PCR demonstrating differing cyclin E expression levels in various ovarian cancer cell lines. **4B.** Real time PCR demonstrating differing SKP2 levels in various ovarian cancer cell lines. **4C.** Western blot demonstrating cyclin E, P27, SKP2 levels in OVCAR3 and OVCAR5.

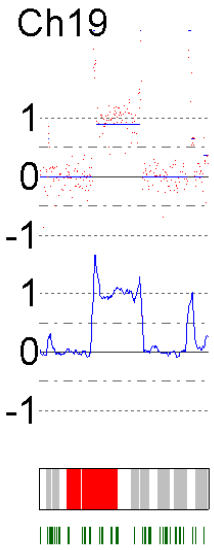

Figure 5. Amplification at the cyclin E gene locus in the OVCAR3 ovarian cancer cell line. Blue lines projecting above the "0" copy number line represents areas of genetic amplification. The broad area of copy number amplification contains the *CCNE1* gene.

Tumors with cyclin E deregulation were hypothesized to be attractive targets for therapy with SKP2 inhibitors. SKP2 is a ubiquitin ligase that targets P27kip1 for degradation. P27 is a powerful negative regulator of the cell cycle, preventing activation of cyclin E-cdk2 or cyclin D-cdk4 complexes and cell cycle progression at the G1 to S boundary. Therefore, inhibition of SKP2 could lead to upregulation of P27 levels and inhibition of aberrant cyclin E activity and inhibition of progression through the cell cycle. Recently, the proteasome inhibitor bortezomib (Velcade) was shown to inhibit the growth of colorectal tumor cell lines through upregulation of P27 and induction of apoptosis.

We tested the sensitivity of OVCAR3 and OVCAR5 ovarian cancer cell lines to the effects of bortezomib. Consistent with our hypothesis, we discovered that the cyclin E overexpressing OVCAR3 cells were indeed more sensitive to bortezomib (figure 6).

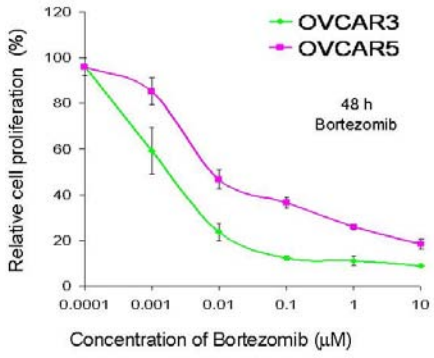

Figure 6. OVCAR3 (high cyclin E levels) cells are more sensitive to bortezomib than OVCAR5 cells (low cyclin E levels). Relative cell proliferation to various concentrations of bortezomib was measured by the MTT assay at 48 hours.

However, given the fact that OVCAR3 and OVCAR5 cells differ in many ways other than cyclin E levels, we set out to create a more informative model system. We transfected OVCAR5 cells to overexpress cyclin E or an empty control vector. We treated these cells with bortezomib and found no difference in their response (figure

7). Similar negative data were obtained with stable transfection in OVCAR5 cells, as well as with overexpression of cyclin E in other ovarian cancer cell lines such as SKPV3 and A2780 (data not shown). This led us to conclude that the differential effects demonstrated between OVCAR3 and OVCAR5 were not due to cyclin E levels.

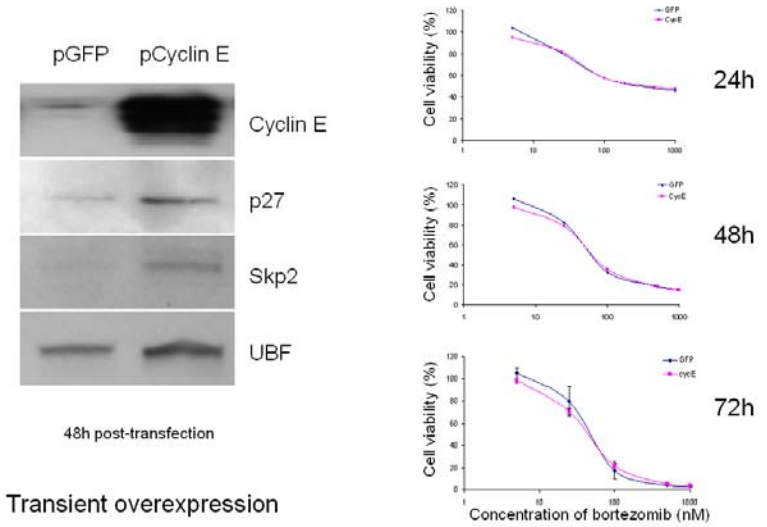

Figure 7. No difference in sensitivity to bortezomib between OVCAR5 cells transfected with empty vector (pGFP) or cyclin E (pCyclin E). Data are shown for transient overexpression of cyclin E.

In light of this data disproving our original hypothesis, we searched the literature for alternative agents that might be capable of differentially targeting cyclin E overexpressing cells. We found a report of a natural dietary phytochemical, indole-3-carbinole (I3C), that works as a natural elastase inhibitor and disrupts cyclin E activity [2]. The low molecular weight (LMW) isoforms of cyclin E are tumor-specific and cause increased cell proliferation, elevated kinase activity and increased clonogenicity. These LMW cyclin E isoforms are generated via proteolysis of the normal 50 kDa cyclin E form by the elastase enzyme, which itself can be selectively inhibited by I3C. I3C exhibits potent anti-carcinogenic properties and has been shown to shift the accumulation of cyclin E from the LMW to the 50 kDa isoform and to induce a G1 cell cycle arrest.

Considering the specific inhibitory properties of I3C and bortezomib in the processing and expression of cyclin E, we investigated the hypothesis that ovarian cancer overexpressing cyclin E may demonstrate an enhanced response to targeted combination therapy with I3C and bortezomib. We found synergistic cytotoxicity of I3C and bortezomib in both OVCAR3 and OVCAR5 cells, with greater sensitivity of each individual drug in OVCAR3 cells and greater synergistic effect of the drug combination in OVCAR5 cells (figure 8).

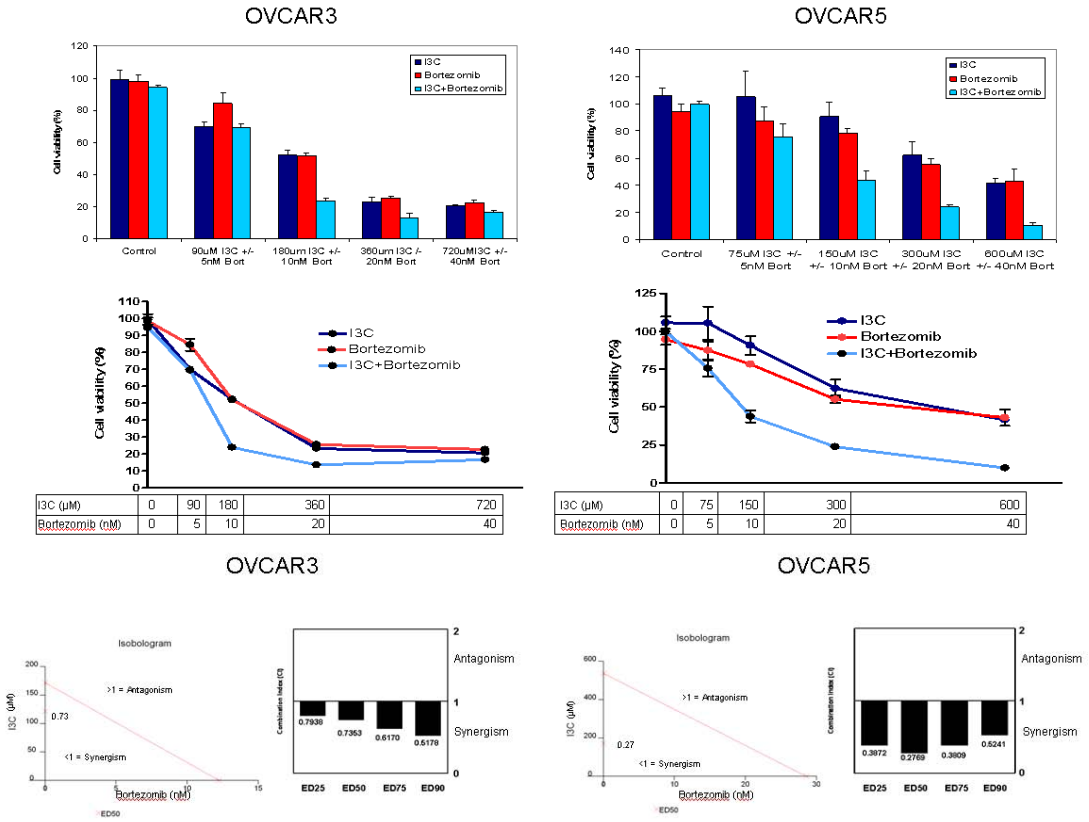

Figure 8. OVCAR3 cells are more sensitive to the effects of I3C and bortezomib alone and OVCAR5 cells are more sensitive to the combination treatment. Cell viability data are generated from Cell Titer-Glo Luminescent Cell Viability Assay (upper panel). Synergy data are determined by isobologram analysis using CalcuSyn software (lower panel).

We found that I3C and bortezomib have varying effects on the cell cycle in the two different cell lines (figure 9). I3C induces an S phase accumulation in OVCAR3 and a G1 arrest in OVCAR5 cells. Bortezomib induces a G2/M arrest in both cell lines, but this is more pronounced in the OVCAR5 cells. The combination of the two drugs causes a G2/M arrest and the accumulation of a sub-G1 population of cells that are undergoing apoptosis.

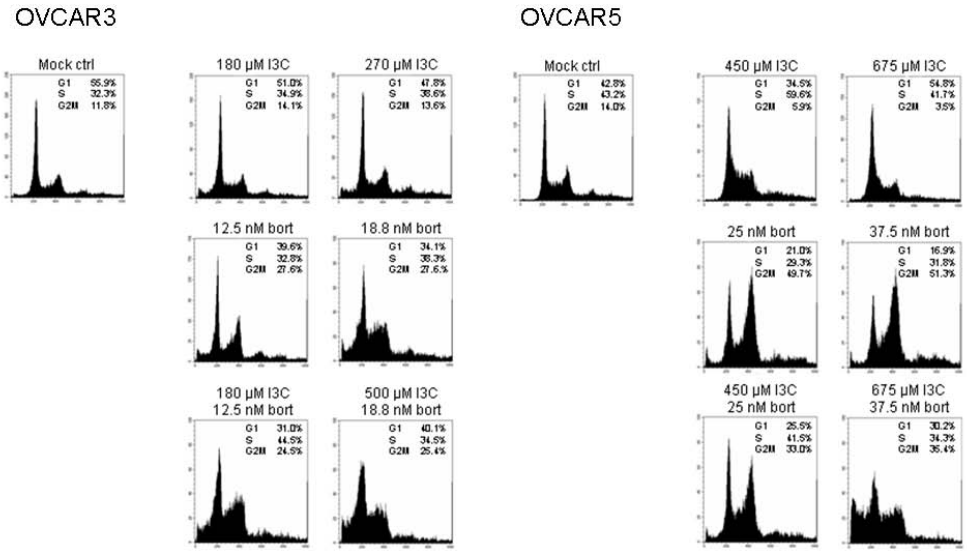

Figure 9. I3C and bortezomib alone and in combination alter the cell cycle and enhance apoptosis in OVCAR3 and OVCAR5 cells. For cell cycle analysis, cells were treated with the indicated concentrations of drugs and harvested 24 hours post treatment. Samples were fixed with 70% ethanol and labeled with propidium iodide (PI). Samples were analyzed for PI incorporation with a Becton Dickinson FACScan using ModFit LT software. The results were generated from multiple independent experiments performed in triplicate.

The combination of the two agents appeared to have a greater impact in inducting apoptosis in the OVCAR5 cells (figure 10).

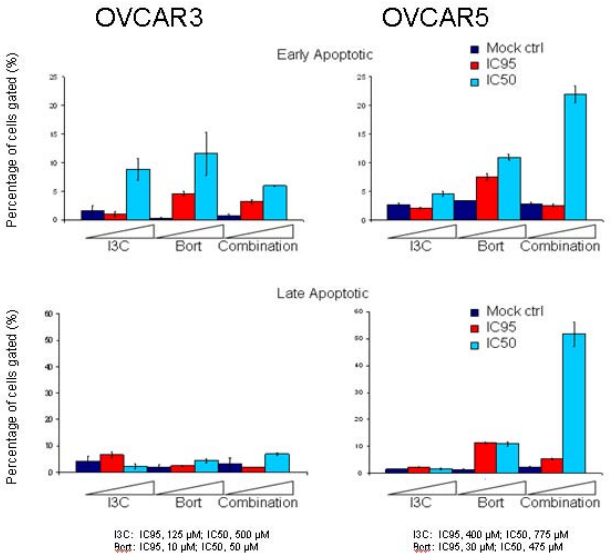

Figure 10. I3C and bortezomib induce apoptosis. The greatest effect is seen in OVCAR5 cells with a combination of drugs. FACS analysis of Annexin V and propidium iodide (PI) stained cells were used to discriminate between early and late apoptotic cells.

We performed western blotting analysis for proteins from various cellular pathways to interrogate the mechanisms for the observed data. We found a decrease in

phospho-Rb levels with increasing drug concentration in both cell lines for single and combination treatment, with the effect being more pronounced in the OVCAR5 cells (figure 11). This data suggest an inhibitory effect of the drugs on progression through the cell cycle at the G1/S phase. P27kip1 levels are not altered and do not appear to be responsible for the observed effects. Western blotting data for additional proteins representing additional pathways are currently underway.

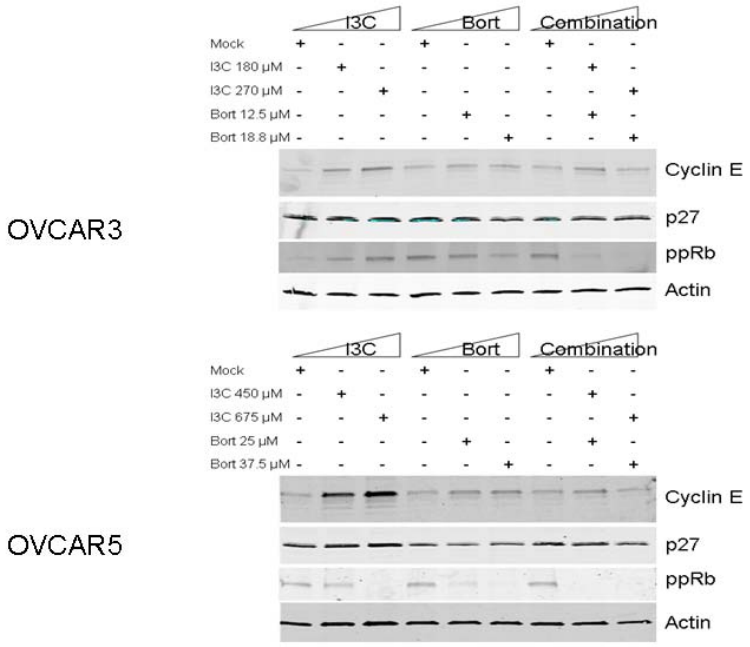

Figure 11. Decreased phosphor-Rb levels with increasing doses of I3C and bortezomib alone and in combination. Western blotting was used for protein expression analysis of OVCAR3 and OVCAR5 cells treated with the indicated agents for 24 hours.

This data were prepared and accepted for poster presentation at the 2010 AACR (American Association for Cancer Research meeting in Washington D.C.; see appendix) and a manuscript is currently in preparation.

This data have particular relevance in light of recent phase II data demonstrating limited single-agent activity of bortezomib in recurrent ovarian cancer [3]. The finding that I3C, a natural dietary phytochemical found in cruciferous vegetables, synergistically sensitizes ovarian cancer cells to the cytotoxic effects of bortezomib may lead to a novel therapeutic combination.

Task 7: siRNA experiments against cyclin E and SKP2 using ovarian cancer cell lines that overexpress both proteins

Small interfering RNAs (siRNAs) against cyclin E and SKP2 were purchased from Sigma Aldrich and introduced into the OVCAR3 cell line (which expressed high endogenous levels of cyclin E and SKP2). We were able to achieve partial knock-down of protein expression levels of the two targets (figure 12). However, we found the cells with cyclin E knock-down to grow poorly and to be a poor experimental

system for further manipulation, such as treatment with drugs. Furthermore, we were initially interested in SKP2 as a target for inhibition in tumors that overexpress cyclin E. Based on the negative data generated in task 6 (no difference in cell proliferation in cells expressing different levels of cyclin E when treated with the proteasome inhibitor bortezomib) and the generation of only partial knock-down of SKP2 levels, we did not pursue further experiments with these cells.

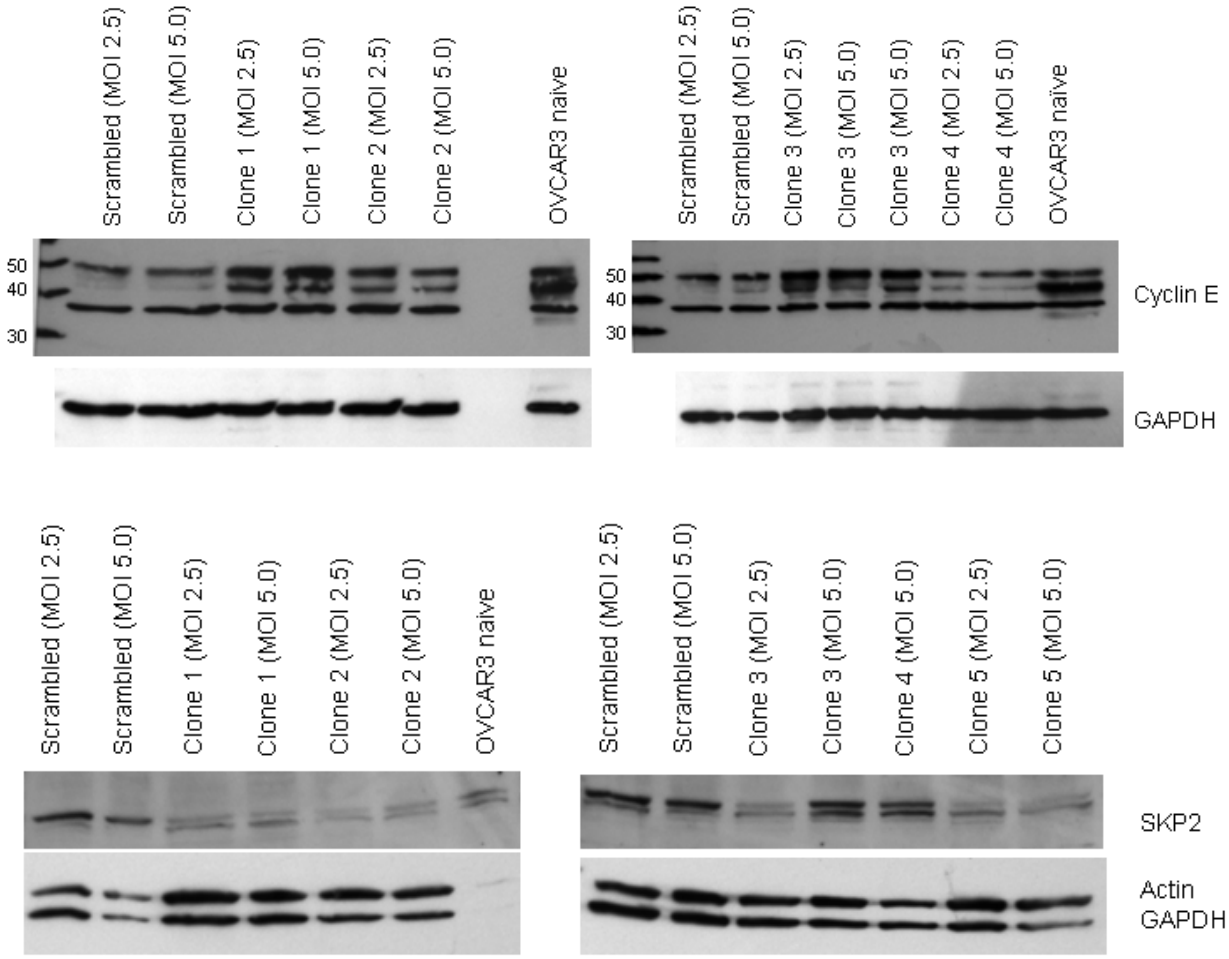

Figure 12. Small interfering RNA inhibition of cyclin E (upper panel) and SKP2 in OVCAR3 ovarian cancer cells. Western blotting data demonstrate partial knock-down of the two target proteins.

Task 8: in vivo tumor xenograft experiments

In vivo tumor xenograft experiments have not yet begun.

Task 9: Data analysis and manuscript generation and grant preparation

Data analysis has been on-going throughout the year and has driven the experimental processes, with re-evaluation of hypotheses and generation of additional experiments to address the evolving data. Data from specific aim 3 were presented in poster format at the 2010 AACR meeting in Washington D.C. (see appendix). A manuscript is currently being generated with this data and will be submitted for publication in the next month. Furthermore, a review article on ovarian cancer biomarkers was published during this year by myself and a mentor on this award, Beth Karlan (see appendix) [4]. Dr. Karlan provided me with the opportunity to write this review to further support my academic and career development activities related to ovarian cancer research.

KEY RESEARCH ACCOMPLISHMENTS

- We found synergistic cytotoxicity of I3C and bortezomib in ovarian cancer cell lines with differing levels of cyclin E expression. These findings provide a potential novel therapeutic option in the treatment of ovarian cancer expressing high or low levels of cyclin E. This may have particular clinical relevance in light of recent phase II clinical data showing limited activity of bortezomib as a single-agent in recurrent ovarian or peritoneal carcinomas.
- We have generated a cross between K5-TVA transgenic mice and P53 conditional mutant mice that will be the basis for our on-going work in specific aim 2.
- We examined gene expression profiles among cyclin E overexpressing ovarian cancers and found the co-expressed genes to be drivers of the cell cycle rather than neighboring genes on the *CCNE1* gene locus.

REPORTABLE OUTCOMES

- Taylor-Harding B, Agadjanian H, Nassanian H, Berenson JR, Miller C, Karlan BY, Orsulic S, **Walsh CS**. The natural dietary phytochemical Indole-3-Carbinol (I3C) sensitizes ovarian cancer cells to the proteasome inhibitor bortezomib. Abstract presented as poster at the American Association for Cancer Research meeting in Washington D.C., April 2010
- **Walsh CS**, Karlan BY. Molecular signatures of ovarian cancer: from detection to prognosis. Mol Diagn Ther 14(1):13-22, 2010

CONCLUSION

At the genetic level, ovarian cancer is characterized by a large degree of genetic instability. High copy-number amplification at the *CCNE1* (cyclin E) gene locus is the single most notable recurrent change, occurring in about 20% of tumors. We have hypothesized that *CCNE1* gene amplification is an initiating event in the carcinogenic

process of a subset of epithelial ovarian cancers. We have further hypothesized that this subset of tumors can be treated with specific targeted therapies, based on the biology of cyclin E overexpression. In the first year of this award, we have made progress towards testing our hypothesis of cyclin E-induced ovarian cancer initiation in a mouse model. We have successfully crossed the K5-TVA mice with P53 conditional mutant mice to generate the model for introduction of genetic changes to ovarian surface epithelial cells. We are in the process of constructing the vectors to introduce full-length cyclin E, truncated low molecular weight cyclin E, and other collaborating genetic events to the mouse model. In testing for a targeted response of cyclin E-overexpressing cells, we have demonstrated that the proteasome inhibitor bortezomib does not affect ovarian cancer cells through a cyclin E-mediated pathway. However, based on the biology of low molecular weight cyclin E isoforms, we found a natural dietary phytochemical called Indole-3-Carbinol (I3C) that disrupts cyclin E processing through the inhibition of the elastase enzyme. When combining I3C with bortezomib, we found I3C to synergistically sensitize ovarian cancer cells to bortezomib. This finding has translational potential as bortezomib as a single-agent was found to have minimal activity in a phase II treatment trial of recurrent ovarian cancer. This finding could re-introduce bortezomib to the therapeutic armamentarium against ovarian cancer if the in vitro results replicate in mice and humans.

In the upcoming two years of the grant, we look forward to examining the potential ovarian cancer initiating effects of cyclin E overexpression in ovarian surface epithelial cells with our mouse model. We will also examine and describe the genetic events that occur commonly among cyclin E overexpressing clinical ovarian cancer samples and look for clues to the biology underlying these tumors. We will further our work in looking for targeted therapies to treat this subset of epithelial ovarian cancers.

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The Natural Dietary Phytochemical Indole-3-Carbinol (I3C) Sensitizes Ovarian Cancer Cells to the Proteasome Inhibitor Bortezomib through Inhibition of Cyclin E Activity

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Background

Epithelial ovarian cancer (EOC) remains the leading cause of gynecologic cancer mortality. Cyclin E deregulation is an important initial event in a subset of EOCs associated with poor outcome. The proteasome inhibitor bortezomib has been shown to inhibit the growth of both ovarian and colorectal tumor cell lines through upregulation of p27, indicating its potential therapeutic role in the subset of ovarian cancers that overexpress cyclin E. As many as five low molecular weight (LMW) isoforms of cyclin E exist in cancer tissues, while only the 50-kDa cyclin E form is expressed in normal tissues. These LMW isoforms are generated via proteolysis of the normal 50-kDa cyclin E form by elastase. Proteolytic activity of elastase can be selectively inhibited by indole-3-carbinol (I3C), a natural component of Brassica vegetables and potent anticarcinogen. Considering the specific inhibitory properties of I3C and bortezomib in the processing and potential expression of cyclin E, respectively, we hypothesize that ovarian cancers overexpressing cyclin E may demonstrate an enhanced response to targeted combination therapy with I3C and bortezomib.

Methods

Viability of OVCAR3 and OVCAR5 human ovarian cancer cells ± I3C and ± bortezomib for 48 h was assessed by MTT assays.

Synergy between I3C and bortezomib was determined using isobologram analysis using Calcusyn software; combination indices (CI)<1.0 are considered synergistic.

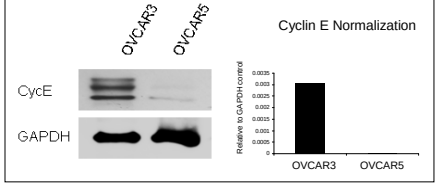
FACS analysis of Annexin V and propidium iodide (PI) stained cells was used to discriminate between early and late apoptosis in OVCAR3 and OVCAR5 cells treated with ± I3C and ± bortezomib for 24 h.

Western blotting was used for protein expression analysis of OVCAR3 and OVCAR5 cells treated with ± I3C and ± bortezomib for 24 h.

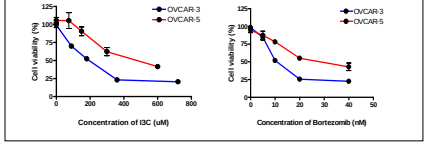
Real-time PCR was used for gene expression analysis.

Results

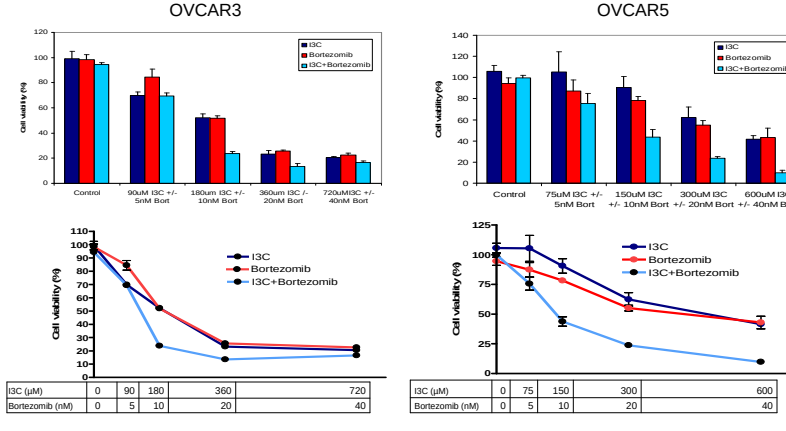
OVCAR3 Cells Express High Levels of Cyclin E Compared to OVCAR5 Cells



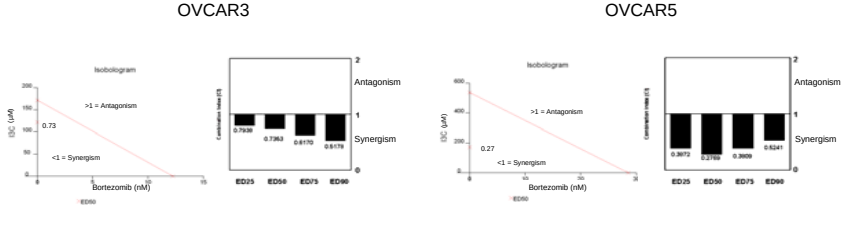
OVCAR3 Cells are More Chemosensitive to Individual Treatment with I3C and Bortezomib Compared to OVCAR5 Cells



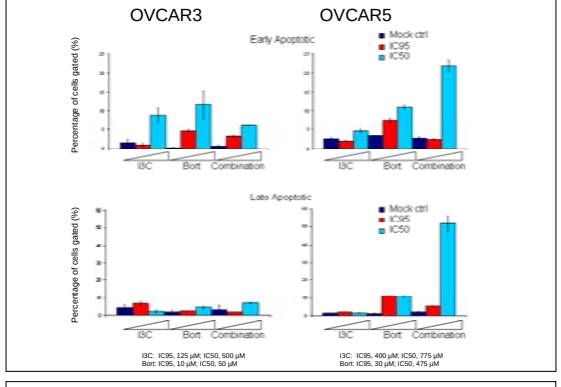
Synergistic Cytotoxicity of I3C and Bortezomib in Both OVCAR3 and OVCAR5 Cells



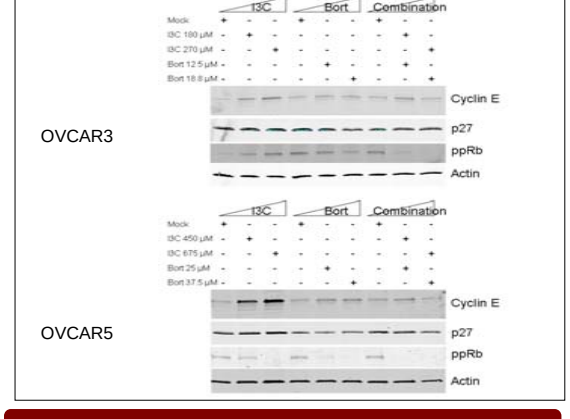
Greater Synergy of I3C and Bortezomib in OVCAR5 Cells Compared to OVCAR3 Cells



I3C and Bortezomib Enhance Apoptosis

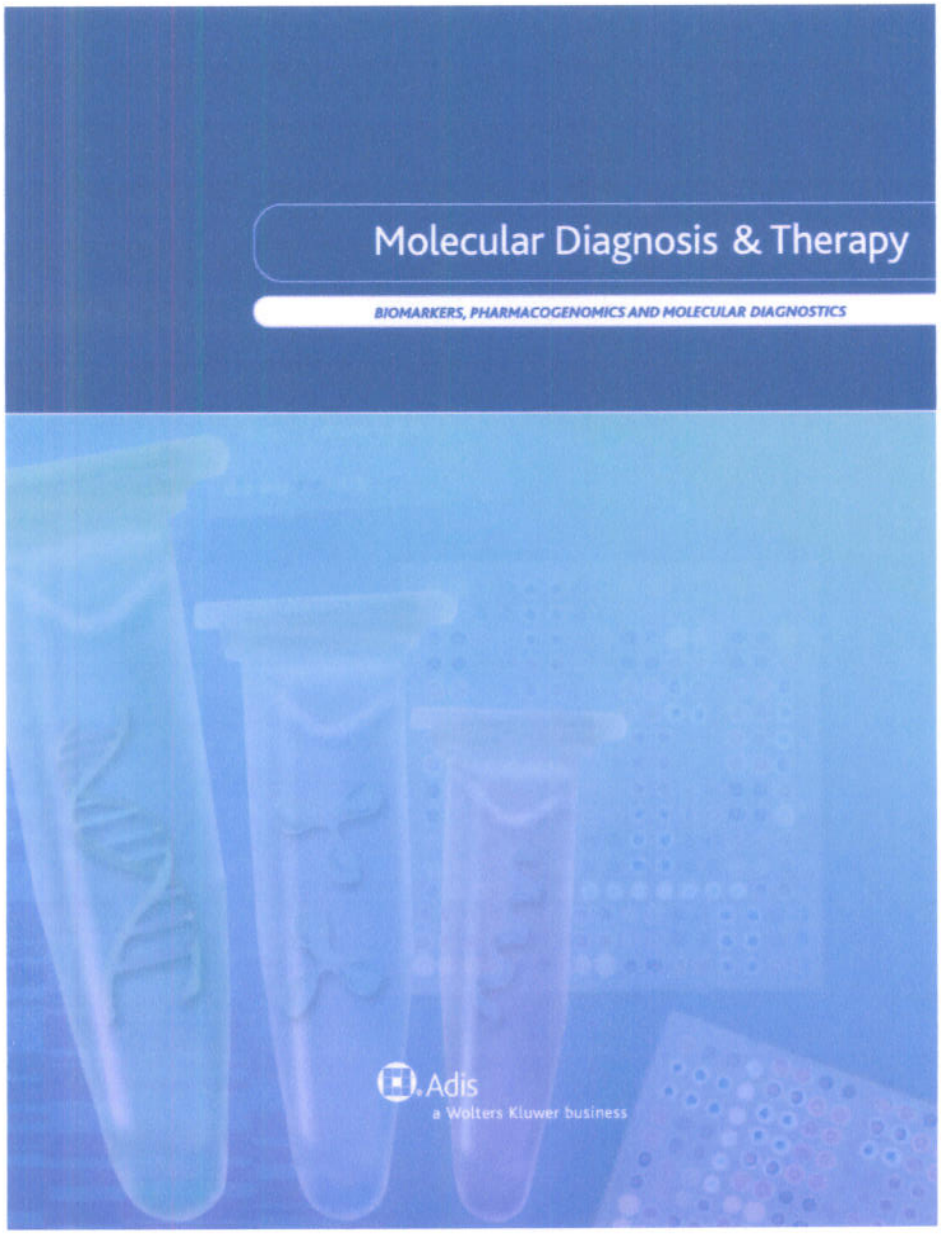


I3C and Bortezomib Alter Cell Cycle Protein Expression



Translational Potential

Our data demonstrate synergistic cytotoxicity of I3C and bortezomib, through premature apoptosis and impairment of cell cycle progression. Our findings support a possible therapeutic role for I3C and bortezomib in the treatment of ovarian cancers expressing high or low levels of cyclin E.



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Molecular Signatures of Ovarian Cancer

From Detection to Prognosis

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Contents

Abstract

The search for an effective screening test for the early detection of ovarian cancer has been intensive. Transvaginal ultrasound and the serum biomarker cancer antigen 125 (CA125) have been used clinically for decades in high-risk populations despite the lack of evidence demonstrating efficacy. More recently, new technologies have identified novel biomarker panels that attempt to improve on the performance of currently available modalities. Some of these tests report superior performance characteristics (sensitivity, specificity, positive predictive value) when compared with CA125 testing alone. Based on early encouraging studies, two commercial ovarian cancer screening products were recently marketed to the public and medical community. They were both withdrawn after concerns were raised by the US FDA and the scientific community regarding their validation and efficacy. There is no clear and established pipeline for the development and approval of these types of tests, and the FDA is working to fill in a large regulatory gap. In order to minimize the potential for public harm, an ovarian cancer screening test will need to be appropriately tested before being made available to the general population. In this review, we discuss the current state of biomarker development for the early detection of ovarian cancer and explore the continuing challenges to realizing this goal.

An effective means for the early detection of ovarian cancer is a much sought-after goal, yet remains an unmet need. The vast majority of ovarian cancers are diagnosed by clinical symptoms at advanced stages, when the chance of surviving beyond 5 years is approximately 30%. Only one-third of ovarian cancers are diagnosed at an early stage, when the 5-year survival is 90%.[1] This disparity in survival statistics has provided strong motivation to find a means for earlier diagnosis and detection before symptoms develop. Current efforts are heavily focused on biomarkers, including single markers, marker patterns over time, and marker panels. In this review, we will discuss the current state and challenges toward finding an effective screening test for ovarian cancer.

1. Challenges to Early Ovarian Cancer Detection

Ovarian cancer is notoriously difficult to diagnose. The ovaries are tucked away in the pelvis and are relatively

inaccessible; in addition, they can give rise to a broad spectrum of pathology with great genetic and molecular heterogeneity. Epithelial ovarian cancers comprise the histologic subtypes that are responsible for the majority of ovarian cancer deaths. The papillary serous histologic subtype represents 70% of epithelial ovarian cancers and is one of the most lethal.[2] The quest toward the discovery of an effective screening test for this challenging clinical condition has been marked with numerous difficulties. Among the greatest of the challenges are (i) the lack of information about a detectable preclinical stage; (ii) the low prevalence of the disease; and (iii) the inability to easily biopsy the ovary.

The single most important criterion to allow for effective early detection of a disease through screening is the presence of a detectable preclinical stage of sufficient duration during the development of the disease.[3] Until recently, very little was known about the natural biology underlying the development of papillary serous ovarian cancers. The length of time from a localized tumor to widely disseminated disease had not been defined, and prior screening studies demonstrating the development of advanced-stage cancers within 6–12 months of a negative screen suggested that this time interval was relatively short.[4-6]

However, a new study now suggests that serous cancers spend >4 years as *in situ* or early-stage cancers and approximately 1 year as advanced-stage cancers before they become clinically apparent.[7] These estimates were derived from a model of growth and progression, based on data from occult serous tumors found at the time of prophylactic bilateral salpingo-oophorectomy in high-risk *BRCA1* (breast cancer 1, early onset) mutation carriers. This model further estimates that occult serous tumors have a diameter of <3 mm and spend >90% of the duration of the window of opportunity for early detection at a diameter of <9 mm. By the time a tumor has reached 3 cm in diameter, >50% have already metastasized to stage III or IV. Therefore, to have an impact on mortality reduction, the authors suggest that a screening test in an average-risk population would need to detect a 4 mm tumor to achieve 80% sensitivity.[7] These data are encouraging because they suggest that a preclinical stage of sufficient duration exists for one of the most deadly types of ovarian cancer. However, our currently available tests are not yet sensitive enough for detection of these subcentimeter lesions.

The second major challenge to the development of a widely applicable screening test is the low prevalence of ovarian cancer in the general population.[8] Ovarian cancer remains a relatively uncommon disease, affecting approximately 1 in 2500 postmenopausal women in the US. In this low-prevalence setting, a screening test would need to achieve near-perfect specificity in order to minimize the potential harm resulting from false-positive results.[9] For example, a test with a sensitivity of 99%, or a false-positive rate of 1%, would subject 25 of 2500 healthy women to the worry, anxiety, and risks of additional follow-up procedures resulting from a positive screen that falsely suggests the presence of ovarian cancer. Even a hypothetical test with an extremely high specificity of 99.6% and a sensitivity of 75% would achieve a positive predictive value (PPV) of only 10% and would result in the diagnostic evaluation and work-up of ten women for every one with ovarian cancer. This seemingly high trade-off has been suggested to be an acceptable goal for this low-prevalence condition.[10]

The third major challenge to ovarian cancer screening is the inaccessibility of the ovaries to further diagnostic evaluation. The ovaries are not readily biopsied, and any positive result on an ovarian cancer screening test, either true or false, subjects that individual to invasive exploratory surgery. Furthermore, if we achieve the goal of developing a screening test that can detect subcentimeter ovarian tumors, the majority of which cannot be seen with gross evaluation at the time of surgery, the only rational evaluation would be bilateral salpingo-oophorectomy followed by meticulous pathologic evaluation of the specimen. The potential risks of such an invasive evaluation in healthy women as the result of a poor-performance screening test cannot be overstated.

2. Cancer Antigen 125

Cancer antigen 125 (CA125) was the first ovarian cancer biomarker to be described.[11,12] Although CA125 has demonstrated utility for monitoring established disease and response to treatment,[13] it performs poorly as a screening tool. Half of all early ovarian cancers, the presumed targets of early detection, would not be detected through the use of this serum biomarker.[14] In addition to the poor sensitivity of 50% for early-stage ovarian cancer, the specificity of CA125 is limited by the fact that many benign conditions cause false elevations of its levels.[15]

Despite the fact that CA125 has limited sensitivity and specificity as an early detection serum biomarker, it has been combined with transvaginal ultrasound in two large, randomized controlled trials of ovarian cancer screening.[16-18] The PLCO (Prostate, Lung, Colorectal, and Ovarian) Cancer Screening Trial is being conducted in the US by the National Institutes of Health (NIH).[19] The objective in the ovarian

cancer arm of the screening trial is to determine whether screening with both serum CA125 and transvaginal ultrasonography in healthy women aged 55–74 years reduces mortality from ovarian cancer. The trial is designed to detect a 30% reduction in mortality over 16 years of follow-up.

Mortality data from this trial will not be available for many more years, but the performance characteristics of serum CA125 and transvaginal ultrasound have been reported. In the prevalence screen (T0) of 28 826 women, a total of 1706 (5.9%) had abnormal results: 1338 (4.7%) had an abnormal ultrasound study; 402 (1.4%) had an abnormal CA125 level (≥35 units/mL); and 34 (0.1%) had abnormal results on both tests.[16] This baseline screen resulted in 570 oophorectomies being performed for 29 cancers (20 invasive, 9 low malignant potential) and 541 benign conditions, demonstrating the poor specificity of these tests. The PPV for invasive cancer was estimated at 1% for ultrasound, 3.7% for CA125, and 23.5% for the combination of the tests. However, only 9 of 29 (31%) of the invasive or borderline cancers were associated with abnormalities of both tests. Additionally, the majority of the invasive cancers (83%) detected by screening were stage III and IV.[16]

Over 3 additional years of annual screening (T1–T3), 89 invasive ovarian or peritoneal cancers were diagnosed.[17] Among these, only 60 (68%) were detected by screening. An additional 19 (21%) were detected in the interval between screenings, and 10 (11%) were detected in women that had never been screened. The PPV remained low, ranging from 1.0% to 1.3% over the 3 years, and the overall ratio of surgeries to screen-detected cancers remained high at 19 to 1.[17]

The high rate of surgery for benign conditions was largely due to false-positive screens on transvaginal ultrasound.[17] The ratio of surgeries to cancer was 44 to 1 at baseline T0, and then incrementally improved to 23 to 1 in the subsequent screening rounds, T1–T3. The ratio of surgeries to cancer was a more favorable 4.5 to 1 after a positive CA125 screen. However, the majority of cancers detected after a positive CA125 were late stage (89% of 27 cases were stage III/IV), while the majority of cancers detected after a positive transvaginal ultrasound screen were early stage (71% of 14 cases were stage I/II).[17]

The multicenter UKCTOCS (United Kingdom Collaborative Trial of Ovarian Cancer Screening) is the other ongoing large randomized controlled trial designed to assess the impact of ovarian cancer screening on mortality.[18] From 2001 to 2005, >200 000 postmenopausal women aged 50–74 years were randomized to a control arm or a screening arm. The screening arm was divided into two different strategies; an ultrasound-based screening approach (USS) or a multimodal screening approach (MMS). In the USS arm, subjects underwent screening with transvaginal ultrasound alone (and no serum CA125 measurement). In the MMS screening arm, serum CA125 levels were measured and assessed with a risk of ovarian cancer (ROC) algorithm. Instead of relying on a single threshold cut-off or static CA125 value, the ROC algorithm considers patient age, the absolute CA125 level, and the rate of CA125 level change to assign a level of risk.[20] Patients classified as low risk undergo repeat CA125 testing in 1 year. Individuals with a persistently intermediate-risk classification (which triggers a repeat CA125 in 12 weeks) or a high-risk classification are triaged to further evaluation with ultrasound. The performance of the ROC algorithm was first reported to have a sensitivity of 83%, a specificity of 99.7%, and PPV of 16% in the initial retrospective analysis.[20] In a subsequent prospective pilot study applying the algorithm to >6500 women, its performance maintained a specificity of 99.8% and PPV of 19%.[21]

The prevalence screen from the UKCTOCS found 59 invasive ovarian and tubal cancers (34 in MMS, 25 in USS) and 28 borderline tumors (8 in MMS, 20 in USS).[18] In contrast to the PLCO study, almost half of the invasive cancers (48%, or 28 of 59) were detected while at an early stage (I/II), with no difference in stage distribution seen between the two screening groups. Of the tumors, 44% (20 of 45) detected in the USS group were of low malignant potential. The high prevalence of benign adnexal masses and borderline tumors detected in the USS group led to a higher rate of repeat testing and surgeries and lower specificity in this screening arm. The rate of surgery to invasive cancer was 35 to 1 for the USS strategy compared with 2.9 to 1 for the MMS strategy, making the rate of surgery almost 9-fold higher in the USS arm.[18]

The performance characteristics of CA125 and transvaginal ultrasound in these two large randomized controlled trials are summarized in table I. Transvaginal ultrasound screening comes at a high cost of many invasive surgeries for benign or borderline tumors but may detect a higher rate of early-stage disease. This has also been demonstrated in prior ultrasound screening studies, which reported that 59–65% of cancers were detected at an early stage but with a similar high rate of false-positive screening results.[4,22,23] Both studies were consistent in demonstrating improved specificity of the serum biomarker over ultrasound imaging. However, the use of a static CA125 value has poor predictive ability in detecting early-stage disease, while the use of longitudinal assessments such as the ROC algorithm demonstrates better utility in picking up early-stage disease.

Whether any of these strategies has an impact on ovarian cancer mortality remains to be determined. In the PLCO trial,

Table I. Performance characteristics of serum cancer antigen 125 (CA125) and transvaginal ultrasound in two large randomized trials of ovarian cancer screening in the average-risk postmenopausal population

Performance characteristics	CA125			Transvaginal ultrasound		
	PLCO baseline T0[16]	PLCO annual T1–T3[17]	UKCTOCS MSS baseline[18]	PLCO baseline T0[16]	PLCO annual T1–T3[17]	UKCTOCS USS baseline[18]
Positive screen (%)	1.4	1.6–1.8	8.7	4.6	2.9–3.4	12
No. of borderline tumors	1	0, 1, 0	8	9	4, 0, 0	20
No. of invasive cancers	13	9, 13, 11	34	12	10, 6, 5	25
Proportion stage I/II (%)		11	47		71	50
No. of surgeries per 1 invasive cancer	4.5	4.5	2.9	44	23	35.2
Apparent sensitivity (%)			89.5			75
Specificity (%)			99.8			98.2
PPV (%)	3.2	2.1–2.7	35.1	0.9	0.7–1.1	2.8

MSS = multimodality screening strategy; **PLCO** = Prostate, Lung, Colorectal, Ovarian (Cancer Screening Trial); **PPV** = positive predictive value; **T0** = time 0, i.e. prevalence screen at baseline time 0; **T1–3** = time 1–3, i.e. incidence screen at years 1–3; **UKCTOCS** = United Kingdom Collaborative Trial of Ovarian Cancer Screening; **USS** = ultrasound screening strategy.

the stage distribution of the cancers detected by screening was not appreciably different than would be expected from clinical detection and, therefore, it would be surprising if this trial were to find an impact on mortality. The UKCTOCS demonstrated a shift in stage distribution toward earlier stages with screening. If there is a comparable decrease in ovarian cancer-specific mortality among the screened population, this might provide justification for screening in the general population with currently available technology.

3. Other Candidate Serum Biomarkers

The poor sensitivity and specificity of CA125 for preclinical disease has spurred an intensive search for alternatives that could more reliably herald the presence of early-stage cancers. Various serum markers have been evaluated through a candidate approach, either alone or in combination with CA125. Over 30 serum biomarkers have been analyzed, including autotoxin, CA15-3, CA72-4, CA19-9, claudin 3, human epididymis secretory protein 4 (HE4), human kallekreins, lipid-associated sialic acid, lipophosphatidic acid, macrophage colony-stimulating factor, matrix metalloproteinases (MMPs), mesothelin, osteopontin, OVX1, soluble epidermal growth factor receptor (EGFR), and vascular endothelial growth factor (VEGF).[10,24-29] When combined with CA125, some of these markers have demonstrated a slight improvement in sensitivity, compared with CA125 alone, when specificity is fixed at 97% or 98% (table II).

One of the most promising ovarian cancer biomarkers appears to be HE4, the protein product of the *WFDC2* gene.[36] Experiments using gene expression and cDNA microarray technologies found HE4 to be amplified in ovarian carcinomas but not in normal control tissues.[37,38] When compared with CA125, HE4 is better at detecting early-stage disease (improved sensitivity) and better at ruling out benign conditions (improved specificity).[39] HE4 has also been shown to complement CA125 when the two biomarkers are multiplexed together.[40] In postmenopausal women presenting with a pelvic mass, the dual marker combination of HE4 and CA125 can better classify patients into groups with a high or low risk of malignancy, with a sensitivity of 92.3% and a specificity of 75%.[41] In the context of early detection, a two-step screening algorithm that uses HE4 >1.8 ng/mL as step 1 followed by positive CA125, glycodelin, or plasminogen activator urokinase receptor (PLAUR) as step 2 achieves a sensitivity of 73.7% and a specificity of 93.7% for stage I/II disease.[34]

4. Biomarkers and Monitoring for Disease Recurrence

CA125 is widely used in clinical practice to monitor for ovarian cancer recurrence. Rising levels, even when remaining below the upper limit of normal (<35 U/mL), are highly predictive of disease recurrence.[42] A biomarker panel consisting of HE4, MMP7, and glycodelin was found to predict disease recurrence prior to elevation of CA125 in 56% of cases and in an equivalent timeframe to CA125 in 41% of cases, with a lead time ranging from 6 to 69 weeks.[34] In 2008, Allard et al.[43] presented data on the use of HE4 for monitoring patients with epithelial ovarian cancer. Among 80 patients with ovarian cancer, serial HE4 levels correlated with CT imaging

findings of recurrence in 76% of patients, and the addition of HE4 to CA125 led to a further increased correlation with clinical status (84%). These data are not yet published, but led to recent US FDA approval for Fujirebio Diagnostics, Inc. to market HE4 in combination with CA125 for the early detection of disease recurrence. However, the impact of early detection of disease recurrence on overall survival and quality of life has recently been called into question by the findings of a randomized controlled trial reported by Rustin et al.[44] in 2009.

5. Biomarker Discovery

The completion of the Human Genome Project has opened doors to a more global approach to biomarker discovery. Through the mechanisms of alternative splicing and post-translational modification, an estimated 30 000 genes lead to the production of 1.5 million protein products in our bodies, or approximately 50 protein products per gene.[45] High-throughput platforms allow for the profiling of thousands of potential

Table II. Performance of various serum biomarker panels in differentiating serum samples from ovarian cancer patients and various control populations

Studies	Screening method	Sensitivity (%)	Specificity (%)	Population tested
Petricoin et al., 2002[30]	SELDI-TOF	100	95	50 cancers (18 early), 66 benign
Zhang et al., 2004[31]	CA125	65	97	138 cancers,
	APOA1, TTR, inter-α trypsin inhibitor	74	97	63 controls
Skates et al., 2004[26]	CA125	45	98	60 early-stage cancers,
	CA125/CA72-4	67	98	98 controls
	CA125/CA72-4/M-CSF	70	98	
	CA125/CA72-4/M-CSF/CA15-3	65	98	
McIntosh et al., 2004[27]	CA125	78.8	98	53 cancers,
	CA125/mesothelin	86.5	98	43 benign, 220 controls
Gorelik et al., 2005[28]	CA125/IL-6/IL-8/VEGF/EGF	84	95	44 early-stage cancers,
	CA125/ IL-6/G-CSF/VEGF/EGF	86.5	93	37 benign, 45 controls
Mor et al., 2005[32]	Leptin, prolactin, osteopontin, IGF2	95	94	100 cancers, 106 controls
Visintin et al., 2008[33]	Leptin, prolactin, osteopontin, IGF2, MIF, CA125	95.3[a]	99.4[a]	Training set: 113 cancers, 181 controls Test set: 43 cancers, 181 controls
Havrilesky et al., 2008[34]	CA125, HE4, glycodelin, PLAUR, MUC1, PAI-1	80.5[b]	96.5[b]	200 cancers (133 stage I/II), 396 healthy controls
Shah et al., 2009[29]	CA125	78	98	143 cancers,
	HE4	68–82	98	124 benign,
	Mesothelin	31–44	98	344 controls
Amonkar et al., 2009[35]	CA125, CA19-9, EGFR, CRP, myoglobin, APOA1, APOC3, MIP1A, IL-6, IL-18, tenascin C	91.3	88.5	115 cancers, 93 benign, 24 controls, 13 non-ovarian cancers

a Combines training and test sets.

b Stage I/II.

APOA1 = apolipoprotein A1; **APOC3** = apolipoprotein C3; **CA** = cancer antigen; **CRP** = C-reactive protein; **EGF** = epidermal growth factor; **EGFR** = EGF receptor; **G-CSF** = granulocyte colony-stimulating factor; **HE4** = human epididymis protein 4; **IGF2** = insulin-like growth factor 2; **IL** = interleukin; **M-CSF** = macrophage colony-stimulating factor; **MIF** = macrophage migration inhibitory factor; **MIP1A** = macrophage inflammatory protein 1α (CCL3); **MUC1** = mucin 1; **PAI-1** = plasminogen activator inhibitor (also known as SERPINE1); **PLAUR** = plasminogen activator, urokinase receptor; **SELDI-TOF** = surface-enhanced laser desorption/ionization time of flight; **TTR** = transthyretin; **VEGF** = vascular endothelial growth factor.

biomarkers or of the entire serum proteome in a single experiment, enabling scientists to break out of the confines of the candidate biomarker approach, which relies on biological inference.

Proteomic technologies have been applied to the discovery of biomarkers that distinguish the sera of ovarian cancer patients from their healthy counterparts. Two general approaches have been utilized. In the first approach, surface-enhanced laser desorption/ionization time of flight (SELDI-TOF) and mass spectroscopy are used to profile proteins in serum according to the size and net electrical charge of each of the individual proteins. Proteins are bound to a protein array, a laser desorbs and ionizes the proteins from the bound surfaces, and the time of flight of the protein fragments is translated into a spectrum of peaks. The peptides responsible for the discriminatory peaks can be further sequenced to identify the serum proteins.[46] In the second approach, a panel of known markers can be assayed through more traditional techniques, such as antibody microarrays or enzyme-linked immunosorbent assay (ELISA). Multiplex platforms have been developed that allow for the assessment of multiple markers with a very small volume of serum.

In a high-profile 2002 Lancet publication resulting from collaboration between researchers from the NIH, the FDA and a private firm, Correlogic Systems, Inc., a proteomics study using SELDI-TOF and mass spectrometry demonstrated 100% sensitivity and 95% specificity for correctly classifying the sera from 50 women with ovarian cancer and 66 healthy controls.[30] Mass spectrometry revealed discriminatory peaks that could differentiate samples as being from a cancer patient or a control, using proprietary pattern-recognition software. Although these results were based on a small set of stored and frozen serum samples, the findings rippled through the mass media and created a sensation.

However, enthusiasm waned when the initial results could not be replicated.[47,48] Major criticisms of the study emerged, including the possibility of bias related to artifacts in sample collection, storage, and processing; the nature of the clinical samples used; the mass spectrometry instrument; and the bioinformatics analysis.[48,49] Reanalysis of the raw data by a different set of investigators led to the conclusion that the discriminatory peaks between cancer and control sera were doubtful in the setting of substantial, non-biologic experimental bias, including experimental noise due to matrix effects.[47,49] Furthermore, the PPV of 94% claimed in the study was an artificially inflated value that reflected the high prevalence of ovarian cancer in an enriched study population.[50,51] The lack of identification of the peptides associated with the discriminatory peaks was regarded as a further flaw.[52]

The original authors acknowledged the problem of unacceptable week-to-week and machine-to-machine variability with the Cipergen ProteinChip™ Biomarker System-II mass spectrometer, which was the low-resolution platform used in the original 2002 publication.[30] In a follow-up study, the high-resolution hybrid quadrupole time-of-flight mass spectrometer was found to yield a superior classification pattern with 100% sensitivity and 100% specificity in the classification of sera from 68 cancers and 43 healthy controls.[53] This report was published in 2004, but it is unclear where Correlogic plans to go with proteomic peak profiling at this time.

More recent publications from the company demonstrate a shift in strategy toward evaluating the levels of known analytes in the sera of patients with ovarian cancer or benign conditions.[35,54] The analytes cover a broad range of biologic activities and include cancer antigens, hormones, clotting factors, tissue modeling factors, lipoprotein constituents, proteases and protease inhibitors, markers of cardiovascular risk, growth factors, cytokines/chemokines, soluble forms of cell signaling receptors, and inflammatory and acute-phase reactants. Using a bead-based approach, the levels of 204 molecules were measured simultaneously in sera from 147 patients with ovarian cancer and 147 patients with benign ovarian pathology.[54] By generating a receiver operating characteristic curve for each analyte, the area under the curve (AUC) values were compared with that of an uninformative marker (AUC 0.5).

The analyte with the highest AUC value was CA125, with an AUC of 0.906. Analytes with AUC values between 0.756 and 0.701 included C-reactive protein, soluble EGFR, interleukin (IL)-10, IL-8, connective tissue growth factor, haptoglobin, and tissue inhibitor of metalloproteinase 1 (TIMP1). These markers largely represented inflammatory markers and acute-phase reactants that were upregulated in ovarian cancer sera. All 26 informative autoimmune and infectious disease markers were downregulated in ovarian cancer samples, suggesting a possible overall immune compromise in these patients.[54]

The 204-marker panel included 35 that had been proposed as potentially useful markers for ovarian cancer in prior studies. Only 12 of these 35 (apolipoprotein A1 [APOA1], CA125, CA19-9, C-reactive protein [CRP], EGFR, haptoglobin, IL-6, IL-8, ferritin, leptin, tumor necrosis factor-α, and VEGF) were dysregulated in this study.[54] The most discriminatory markers included the well-studied CA125, in addition to markers of inflammation (CRP), cell cycle mediators (EGFR), angiogenic factors (VEGF), and extracellular matrix regulators (TIMP1).[54]

Only five markers had statistically dysregulated levels in early-stage I and II cancers, including CA125, CA19-9, CRP, creatine kinase-MB, and EGFR. This is in contrast to

40 markers that were found to be dysregulated in late stage III and IV cancers. There was no single diagnostic marker that emerged as informative. The results underscore the heterogeneity of ovarian carcinogenesis and the inability of any single marker to capture the diversity of disease.

The 204-analyte panel was further studied in 91 stage I data sets and an equivalent number of controls, resulting in the identification of an 11-analyte panel that appeared to be informative for all stages and common subtypes of ovarian cancer.[35] The panel was composed of CA125, CA19-9, EGFR, CRP, myoglobin, APOA1, apolipoprotein C3 (APOC3), macrophage inflammatory protein 1α (MIP1A; also known as CCL3), IL-6, IL-18, and tenascin C. When applied to a test set of 245 samples, the panel was found to have 91.3% sensitivity and 88.5% specificity.[35] Correlogic has recently completed a blinded, prospective clinical validation study, the results of which are forthcoming.

Other studies using proteomics technologies have defined additional biomarker panels. An approach using the Cipergen ProteinChip™, SELDI-TOF and mass spectrometry identified a panel that includes transthyretin (TTR), β-hemoglobin, APOA1, and transferrin.[55,56] An independent group using the Ciphergen ProteinChip™ identified a three-marker panel containing APOA1, TTR, and inter-α-trypsin inhibitor.[31] This panel reported 74% sensitivity for ovarian cancer detection at a fixed specificity of 97% (table II).

In another well-publicized effort conducted by investigators at Yale University, a panel of four biomarkers was identified through an antibody microarray screening method called cytokine rolling-circle amplification microarray. This panel, including leptin, prolactin, osteopontin, and insulin-like growth factor 2, performed with a sensitivity of 95% and specificity of 95% in differentiating the sera from 100 patients with ovarian cancer and 106 controls.[32] In an attempt to further improve the specificity, two additional markers, macrophage migration inhibitory factor (MIF) and CA125, were added to the panel. A multiplex, bead-based, immunoassay system was used to evaluate the six-marker panel in a training set (113 ovarian cancers, 181 controls) and a test set (43 ovarian cancers, 181 controls). The performance of this panel was reported to have a sensitivity of 95.3% and a specificity of 99.4%.[33] However, this 'final model' provided an overinflated assessment of the test's performance, as observations were combined from the training and test sets.[57] The reported PPV of 99.3% in this study was also falsely elevated. A recalculation to a low-prevalence setting would more accurately represent the PPV at only 6.5%.[58,59] The study was further criticized, as the samples used were not representative of those targeted through screening, with only 13 samples coming from patients with stage I cancers.[58] These criticisms are relevant, as the more favorable results were used as justification to bring an ovarian cancer screening test prematurely to the market.

6. Regulatory Issues

The pace of biomarker discovery and the attempts to rapidly bring non-validated products to the market prematurely have exposed a large regulatory gap. OvaCheck® was an ovarian cancer product that was widely anticipated to come on the market in 2004 when one of the two laboratories licensed to perform the test began distributing marketing materials.[60] The data on this Correlogic Systems, Inc. product was reported in a 2002 Lancet publication,[30] but the results were never replicated or validated, causing the FDA to step in. In February 2004, the FDA sent a letter to the CEO of Correlogic Systems, Inc. indicating that the agency was aware the company was "contemplating or has begun the commercial distribution" of the test. In March 2004, the FDA sent letters to Quest Diagnostics Inc. and Laboratory Corporation of America (LabCorp), the two laboratories licensed to conduct the tests, stating that "because the nature of this test is not clear from the materials we have reviewed, we are uncertain if your ovarian cancer offering will be subject to regulation only by the Clinical Laboratory Improvement Amendments of 1988 (CLIA), or whether it may also require premarket review by FDA under the Federal Food, Drug, and Cosmetic Act."[60] Prior to this notification, it had been assumed that the product could be defined as a laboratory-developed 'home brew' test that would be overseen by the less stringent Centers for Medicare and Medicaid's CLIA rules. The FDA subsequently released a draft guidance that considers *in vitro* diagnostic multivariate index assays as medical devices that fall under its regulatory guidance and that require premarket approval.

Despite this new guidance by the FDA, in June 2009, LabCorp began marketing OvaSure™ based on the results of the six-marker panel published by the Yale University group in 2008.[33] The FDA responded by sending a letter to LabCorp in August 2009, stating that "we believe you are offering a high-risk test that has not received adequate clinical validation and may harm the public health." OvaSure™ was then withdrawn from the market.

7. Biomarker Development and Clinical Use

Currently, there are no approved biomarkers for the early detection of ovarian cancer, nor are there clear pipelines for the transfer of a test to the marketplace. It has been suggested that a

comprehensive biomarker pipeline should contain six essential components: (i) candidate discovery; (ii) qualification; (iii) verification; (iv) research assay optimization; (v) biomarker validation; and (vi) commercialization.[61] Candidate biomarkers identified in the discovery phase undergo 'qualification' to confirm the differential expression in diseased and normal samples and 'verification' to confirm sensitivity and to begin to assess specificity when studied in a broader range of samples that capture the heterogeneity of the population to be tested. A high-throughput assay that can be applied to many samples is developed in the 'research assay optimization' phase and tested in the target population in the 'validation' phase. Finally, the assay is refined to meet the rigorous standards required for clinical tests in the 'commercialization' stage.[61] The test should ultimately be assessed in three different populations: a retrospective collection of stored specimens that includes prediagnostic samples from women with early-stage disease; a prospective screening study; and a cancer control study to ultimately determine if the test reduces the population burden of disease.[62]

To date, CA125 is the only marker that is being tested in the general postmenopausal screening population. Publications testing other biomarkers and panels have been limited to retrospective populations highly enriched for ovarian cancer. The performance of these panels in a more general population with a low prevalence of disease has not yet been determined. The specificity and PPV of these tests in the general population will be of paramount importance in limiting the morbidity arising from false-positive results. Further, the randomized controlled trial remains the gold-standard method for determining the effect of a screening test on cancer-specific mortality. New approaches that will allow for assessment of the costs and benefits of screening in a more efficient and timely fashion are needed.[62]

Three additional points require consideration in the translation of current biomarker discovery into clinical use. The first consideration is the control group. Some studies have developed assays based on differential expression of various serum components between ovarian cancer patients and those with benign ovarian pathology, while other studies have used normal healthy subjects as the controls. Studies that have used sera from both types of controls have found different performance characteristics of the test, depending on whether the control population had benign pathology or normal ovaries.[63] This has a bearing on the appropriate clinical application of the assay. An assay that was developed using benign ovarian pathology as the control population would be better suited to assess the risk of malignancy in the setting of a confirmed pelvic mass. In contrast, an assay that was developed with a normal control population would be more appropriate to offer as a screening study to the general population.

The second issue to consider is case selection in these studies. These serum biomarker panels were developed using clinically diagnosed ovarian cancers as the cases, many of which were late stage. These samples are not representative of those that would be the targets of screening. To find a test that is truly appropriate for early detection, biomarker discovery would be more appropriately performed using the serum samples collected from patients *prior to* the clinical discovery of disease. This is where banked samples from the participants of prospective studies, such as the PLCO trial or the UKCTOCS, will become invaluable.

Finally, current biomarker panels are largely composed of markers that reflect a patient's systemic reaction to cancer rather than those that capture the unique proteins secreted by the tumor.[49] Tumor-specific markers circulate at concentrations that are orders of magnitude lower than the proteins that can be measured by current mass spectrometry technology.[7,49,64] With our current technology, proteins secreted from millimeter-sized tumors could be detected only if secreted at high rates and with zero background, which is an unrealistic condition.[65] The signal from proteins secreted by subcentimeter-sized tumors are drowned out by the much more abundant proteins secreted in response to inflammation, infection, and malnutrition.[49] Cancer-specific antigens, such as CA125 or prostate-specific antigen (PSA), are detected only when tumors typically reach a size in the many-centimeter range.[65]

8. Conclusion

Ovarian cancer is responsible for the highest fatality rate among the gynecologic malignancies, and there is great interest in defining a screening test that would allow for early detection. As ovarian cancers are complex and heterogeneous, no single biomarker will be able to detect all histologic subtypes or stages. Biomarker panels have reported excellent performance characteristics (sensitivity, specificity, PPV) but have not yet undergone appropriate validation. As a positive result leads to invasive diagnostic testing and potential for harm, the specificity of an ovarian cancer screening test needs to be close to 100% to limit the number of false-positive results. Appropriate validation of a test is critical, as a test could result in more harm than good when applied to a healthy population. Current biomarker panels measure acute-phase reactants and markers of inflammation rather than protein products secreted by tumors. The identification of unique cancer proteins or products will require advances in current technology. The identification

of biomarkers that herald the presence of subcentimeter lesions will require a change in case selection at the biomarker discovery phase. More work is required before we will realize the goals of early detection of ovarian cancer: to discover lesions when they are localized and curable; to prevent mortality; and to reduce morbidity and cost.[62]

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