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PRINCIPAL INVESTIGATOR: Animesh Barua, Ph.D.

CONTRACTING ORGANIZATION: Rush University Medical Center
Chicago, IL 60612

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14. ABSTRACT: Because of the lack of an effective early detection method, OVCA in most cases are detected at late stages when the rate of 5-year survival is <10%. Although transvaginal ultrasound (TVUS) is the currently favored method, it cannot detect OVCA at early stage due to its limited resolution. Tumor related malignant nuclear transformation is an earlier event in tumor development which leads to the development of anti-Nuclear Matrix Proteins (NMP) antibodies in circulation. Malignant nuclear transformation is followed by tumor associated neo-angiogenesis (TAN). $\alpha_v\beta_3$ -integrins and death receptors (DR)-6 are the two markers of ovarian TAN expressed by neo-angiogenic microvessels. DR-6 are also secreted in serum. If the detection limit of TVUS can be enhanced, $\alpha_v\beta_3$ -integrins expressing microvessels can be an <i>in vivo</i> imaging marker of ovarian TAN and may be used together with serum anti-NMP antibodies and DR-6 to detect OVCA at early stage. Our overall goal is to improve the TVUS detectability of ovarian TAN vessels at early stage by $\alpha_v\beta_3$ -integrins targeted contrast enhanced ultrasound (CE-US) molecular imaging. This goal is being achieved by 3 specific aims. The results so far obtained with the accomplishment of aim 1 and part of aim 2 suggest that CE-US molecular imaging targeting ovarian $\alpha_v\beta_3$ -integrins can detect ovarian TAN at early stage of OVCA in laying hen model of spontaneous OVCA. Changes in OVCA related CE-US imaging indices are associated with the elevation of serum DR-6 levels. These indices are being used to diagnose hens with OVCA at early stage in aim 2.					
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INTRODUCTION:

Ovarian cancer (OVCA) remains as a deadly disease of women with high rate of death among gynecological malignancies. Women with OVCA exhibit specific symptoms only in later stages (Stage III and IV) where the 5-year survival rate is <10%[1]. In contrast, 80-90% OVCA patients when detected in stage I respond to treatment[2]. Unfortunately, symptoms of OVCA at early stage are non-specific and early detection (<25% cases) generally occurs by happenstance[2]. Moreover, the capacity to follow tumor development non-invasively, if it was detected, is non-existent. A valid animal model overcomes the numerous problems associated with clinical studies[3]. Transvaginal ultrasound (TVUS) is the currently favored method for *in vivo* detection of ovarian abnormalities including OVCA. Diagnosis by TVUS scanning is based on the detection of ovarian morphological and vascular abnormalities associated with the disease. However, traditional TVUS cannot detect OVCA related vascular abnormalities specially microvessels at early stage of the disease due to its limited detectability. Malignant nuclear transformation and changes in nuclear matrix proteins (NMPs) as well as ovarian tumor associated neo-angiogenesis (TAN) are the early events in OVCA development. Malignant changes in nucleus are associated with the shedding of NMPs in circulation which elicits autoimmune responses by producing anti-NMP antibodies. $\alpha_v\beta_3$ -integrins and death receptors (DR)-6 are the markers of ovarian TAN and overexpressed by ovarian tumor associated microvessels. In OVCA patients, DR-6 are shed in the circulation. Thus anti-NMP antibodies and DR-6 represents potential circulatory markers of malignant ovarian transformation and ovarian TAN. Our long term goal is to improve the TVUS detectability of ovarian TAN vessels at early stage by molecular targeted ($\alpha_v\beta_3$ -integrins) contrast enhanced ultrasound (CE-US) imaging. Contrast agents are developed to improve the visualization of TAN vessels by TVUS scanning. We are using laying hens because of the difficulty in identifying and accessing patients with early stage OVCA.

BODY: the research accomplishments associated with each task outlined in the approved Statement of Work.

Our overall hypothesis is that early stage OVCA lesions can be detected in the hen OVCA model using $\alpha_v\beta_3$ -integrin targeted CE-US together with anti-NMP antibodies and serum levels of DR-6. This hypothesis is being achieved by accomplishing following three specific aims:

Aim 1: To determine whether CE-US using microbubbles targeted to $\alpha_v\beta_3$ -integrins will identify hens with ovarian TAN at early stage of OVCA;

Aim 2: To examine whether anti-NMP antibodies appear in serum before ovarian TAN becomes detectable;

Aim 3: To determine whether CE-US indices and serum DR-6 levels established in Specific Aim1 will detect ovarian TAN at early stage OVCA in anti-NMP antibody positive hens.

Accomplishments:

Task 1. Molecular targeted contrast enhanced ultrasound (CE-US) imaging of hen ovarian tumor associated neo-angiogenesis (TAN)

1a. Scanning of hens by $\alpha_v\beta_3$ -integrins targeted contrast enhanced molecular imaging

- 1) Gray scale scanning: Pre-contrast and post-contrast examination of hen ovaries and recording of still images and video clips.
- 2) Contrast parameters: Contrast parameters including time of arrival, features of contrast agent binding and wash-out of unbound contrast agents were examined.
- 3) Doppler ultrasound indices: ovarian vasculature of hens was examined by pre- and post-contrast Doppler ultrasound imaging. Resistive (RI) and pulsatility indices (PI) were recorded.
- 4) Blood samples: Collected before the injection of contrast agent, sera were obtained and archived.
- 5) Determination of death receptor (DR-6) levels: Serum DR-6 levels were determined by immunoassay.

1b. Following ultrasound scanning, all animals were euthanized and ovaries were collected and processed for paraffin, frozen and molecular biological studies.

1c. Pathology, molecular biology and biochemical study of hen samples:

1. Paraffin and frozen sections from all ovarian tissues blocks from all hens were made and representative sections were stained by hematoxylin & eosin for the diagnosis of tumor or non-tumor abnormalities.

2. Immunostaining of paraffin or frozen ovarian sections for DR-6 and $\alpha_v\beta_3$ -integrins as well as immunoblotting of ovarian homogenates for the expression of DR-6.
3. Data analysis and establishment of indices for targeted CE-US imaging, RI & PI values and serum DR-6 levels detective of ovarian TAN at early stage OVCA with reference to histopathology and expression of tumor associated angiogenic markers. These indices are being used for the detection of early stage OVCA in subsequent Task (Specific Aim-2).

Task 2. Appearance of anti-nuclear matrix protein (NMP) antibodies in serum and its association with ovarian TAN in prospective study.

2a. Hens with ovaries appearing normal (with normal egg laying rates and low egg laying rates) were scanned using indices established in Task 1 (specific Aim 1).

2b. Serum of all hens selected by CE-US scan (mentioned in 2a) were examined for the presence of anti-NMP antibodies by immunoassay and hens negative for serum anti-NMP antibodies were selected for specific aim 2. These hens are being monitored by CE-US scanning at 15 weeks interval for the detection of ovarian TAN at early stage of OVCA. Sera from all hens are being collected at each scan.

2c. At 1st scan (15 weeks from the start) sera samples of all hens were examined for anti-NMP antibodies.

Detail Report on the accomplishments:

Specific Aim 1: CE-US scanning using microbubbles targeted to $\alpha_v\beta_3$ -integrins and establishment of CE-US indices detective of ovarian TAN at early stage of OVCA.

Animals: A total of 140 White Leghorn hens (3 years old) with low egg laying rates (<125eggs/year) were selected from a flock of laying hens. In the initial study, 20 hens were selected and divided into two groups (10 hens each) and injected with non-targeted or $\alpha_v\beta_3$ -integrins targeted microbubbles (Visistar® Integrin, Targeson Inc., La Jolla, CA) (described as contrast agent throughout the text) and the binding specificity of contrast agent was confirmed and a dose of 10uL/kg body weight was found optimum. In the subsequent study, age matched 100 hens with low egg laying rates and 20 hens with normally egg laying rates and reared under similar environment were selected for CE-US scanning with contrast agent. Blood from all hens were collected prior to CE-US scanning and sera were obtained and stored at -80°C until further use.

CE-US $\alpha_v\beta_3$ -integrins targeted molecular imaging: Sonography and Image Analysis

Pre-contrast scanning: Sonography was performed in a continuous pattern before and after the injection of contrast agent with the mechanical set up reported previously [4]. Briefly, all hens were scanned using an instrument equipped with a 5- to 7.5-MHz endovaginal transducer (MicroMaxx; SonoSite, Inc, Bothell, WA). Each hen was immobilized, the transducer was inserted (transvaginal), and 2-dimensional transvaginal gray scale sonography as well as pulsed Doppler sonography was performed. The resistive index (RI: [systolic velocity – diastolic velocity]/systolic velocity) and the pulsatility index (PI: [systolic velocity – diastolic velocity]/mean) were automatically calculated from at least 2 separate images from the same ovary, and the lower RI and PI values were used for analysis. All images were processed and digitally archived.

Post-contrast Scanning

Post-contrast injection scanning was performed in a similar and continuous manner with identical mechanical settings as described above and the same pre-contrast imaged area was imaged according to the instruction of manufacturer of the contrast agent and earlier report [5]. Within 5-7 min from the arrival, contrast agent was accumulated at the target sites and unbound free microbubbles were washed out. All images were archived digitally in a still format as well as real-time clips (15 minutes for each hen). The effect of contrast agent was evaluated visually during the examination and afterward from reviewing the archived still images and video clips. The time of contrast agent arrival (interval in seconds from administration of the contrast agent to its visual observation [in seconds]) in the normal and tumor ovaries was recorded in real time. After review of the complete clip, the region of interest (ROI) was selected. The average image intensity over a ROI encompassing the tumor was calculated and normalized by the pre-contrast intensity of the same ROI. The pixel intensity of ROI predictive of OVCA was determined. In addition, post-contrast RI and PI were calculated.

Result: Representative ultrasound images of an ovarian tumor at early stage before and after the injection of contrast agent are shown in **Figure 1** (B-mode, showing tumor size and borders. Normal ovaries in healthy hens with low egg laying rates ($n = 20$ selected hens) were found to have large one or two preovulatory and small growing stromal follicles on gray scale sonography. Blood vessels were detected in the ovarian stroma on color Doppler ultrasound imaging. Compared to pre-contrast scans, targeted imaging by contrast agent injection enhanced visualization of solid masses in the ovaries of 15 hens on gray scale and these hens were "predicted to have ovarian tumors" (**Figure 1D**). Overall, compared to pre-contrast, the pixel intensity of the signals from the ovary increased significantly after the injection of contrast agent. The signal intensity for low laying healthy hens was 1651.89 ± 563.79 (mean $\pm$ SD) pixels and it was significantly higher in hens with a small solid ovarian mass predicted to have early stage OVCA ($20,993.04 \pm 1621.44$, range = 17983.38-23289.2, $n = 7$ hens) and late stage OVCA ($25,130.35 \pm 953.73$ pixels) ($n = 8$ hens). The post-contrast RI and PI values for all of these hens with detectable solid ovarian mass were <0.40 and <0.75 , respectively. In addition, 9 hens were observed to have shrunk or regressed ovaries neither with preovulatory and small growing follicles nor with any detectable solid mass. Post-contrast RI and PI values of these hens ranged from 0.40-0.44 and 0.85-0.96, respectively and they were classified as hens with abnormal ovaries.

Ovarian Morphologic and histologic Evaluation

After CE-US molecular targeted imaging, all hens were euthanized and sonographic predictions were confirmed

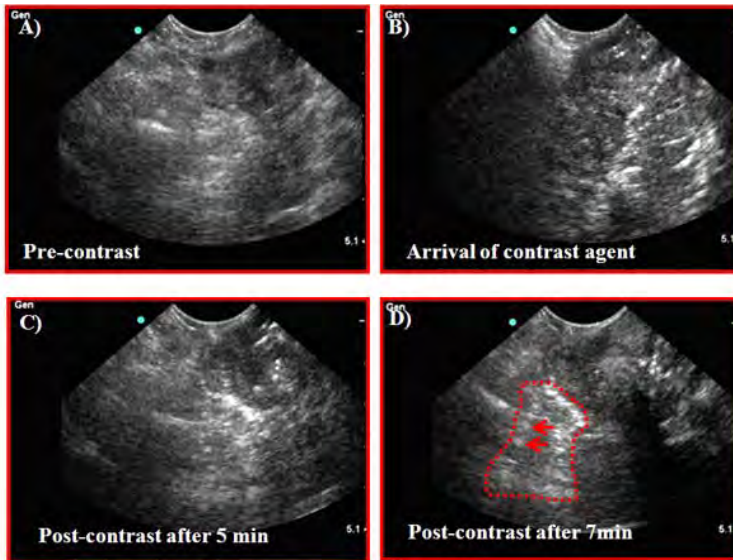


Figure1. Detection of ovarian tumors by $\alpha_v\beta_3$ -integrins targeted contrast enhanced ultrasound (CE-US) molecular imaging. A) Pre-contrast gray scale ultrasound image of a hen ovary. Intensity of the signals from the tissue is very low and the presence of any solid mass is not decisive. B) Gray scale CE-US image of the same ovary at the arrival of contrast agent (CA). Compared to pre-contrast, remarkable increase in signal intensity is seen (appears like a white ring). But the binding of CA is not yet specific. C) Gray scale CE-US image of the same ovary 5 minutes after the injection of CA. With the washing out of unbound CA, bindings of CA are seen to be more specific. D) Gray scale CE-US image of the same ovary 10min after the injection of CA showing specific binding of CA. Regions of interest containing solid tissue mass bounded by targeted CA is indicated as red dotted circle. Arrows indicate the example of specific bindings of CA.



Figure 2. Ovarian tumor in laying hen detected by CE-US $\alpha_v\beta_3$ -integrins targeted molecular imaging. A) Gross appearance of an ovarian tumor in a laying hen with stage I OVCA diagnosed in *figure 1(D)*. Tumor was limited to a part of the ovary (red dotted line). B) Hematoxylin & eosin stained section of the corresponding ovarian tumor indicating the tumor is of serous histology. These observations confirmed the sonographic prediction that $\alpha_v\beta_3$ -integrins targeted CE-US imaging can detect OVCA at early stage.

by gross examination of hens at necropsy. Ovarian tumors, their stages, and types were confirmed by routine histologic examination with hematoxylin-eosin staining (**Figure 2A-B**). As observed on sonography, late-stage OVCA ($n = 8$ hens: 4 serous, 3 endometrioid, 1 mucinous) was associated with moderate to profuse ascites and metastasis to distant organs. Early-stage

ovarian tumor ($n = 7$ hens) was limited to the ovary (3 serous, 3 endometrioid, and 1 mucinous) with little or no ascites. Seven of 9 hens (2 serous, 3 endometrioid, 2 mucinous) initially classified as hens with abnormal ovarian morphologic characteristics without any grossly detectable solid ovarian masses during gray scale sonography had microscopic malignant ovarian lesions (hematoxylin-eosin staining) in 1 or more areas of the

ovary and these hens were added to hens with early stage OVCA group. Thus the total number hens with early stage OVCA was $(7 + 7) = 14$.

Detection of Tumor associated neo-angiogenic (TAN) markers:

Sample preparation: Ovarian tissues from all hens including normal and tumor hens were processed for paraffin, frozen and molecular biological studies.

Detection of tissue expression of neo-angiogenic markers: Paraffin and frozen sections were immunostained for the detection of DR-6+ and $\alpha_v\beta_3$ -integrins+ microvessels and the frequencies of the immunopositive vessels

were counted and analyzed. Differences in the frequency of immunopositive microvessels between normal and hens with OVCA were considered significant when the $P < 0.05$.

Result: Neo-angiogenic immunopositive DR-6 or $\alpha_v\beta_3$ -integrins expressing microvessels appeared to be leaky without any well organized continuous smooth muscle layer surrounding them.

Tissue expression of DR-6: In normal ovaries, very few immunopositive DR-6 expressing vessels were seen in the follicular theca and

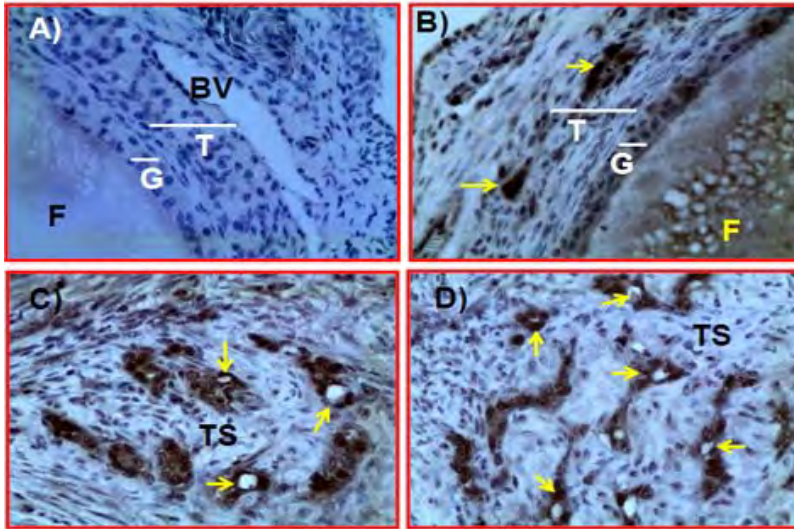


Figure 3. Immunohistochemical detection of DR-6 expressing microvessels by anti-chicken DR-6 antibodies (primary antibodies) in hen ovaries with or without tumor. A) Section of a normal ovarian stroma immunostained by omitting primary antibodies used as control. No immunopositive vessel is seen. B) Serial section from the same normal ovary immunostained with primary antibodies. Very few DR-6+ vessels are seen. C) Ovarian section of a hen with early stage ovarian cancer (OVCA). Compared to normal ovary, many DR-6+ microvessels are seen in the tumor stroma. D) Section of tumor ovary from a hen with late stage OVCA. Many DR-6+ microvessels are localized in the tumor stroma. BV= blood vessel, F= follicle, G= granulosa layer, T = theca layer, TS = tumor stroma. Arrows indicate the examples of DR-6+ microvessels. 40X.

the ovarian stroma. Compared to normal, many DR-6+ microvessels were localized in hens with OVCA (**Figure 3**). The population of DR-6+ microvessels was significantly ($P < 0.05$) higher in hens with early stage OVCA (mean + SD= $5.38 + 1.64$ in $20,000\mu m^2$ of tumor tissue) than that of normal hens ($2.32 + 1.12$ in $20,000\mu m^2$ of ovarian stromal tissue) and increased further in hens with late stage of OVCA ($10.24 + 2.10$ in $20,000\mu m^2$ of tumor tissue) (**Figure 5**).

Tissue expression of $\alpha_v\beta_3$ -integrins: Similar to DR-6, microvessels expressing $\alpha_v\beta_3$ -integrins were localized at the tumor vicinity (spaces between tumor glands) (**Figure 4**). Occasionally ovarian tumor epithelia also expressed $\alpha_v\beta_3$ -integrins. Compared to normal hens (**Figure 4A**), the frequencies of $\alpha_v\beta_3$ - integrin expressing microvessels were significantly ($P < 0.05$) greater in hens with early stage OVCA (**Figure 4B**) and increased further in hens with late stage of OVCA. The population of $\alpha_v\beta_3$ -integrins expressing microvessels in hens with early stage OVCA was significantly

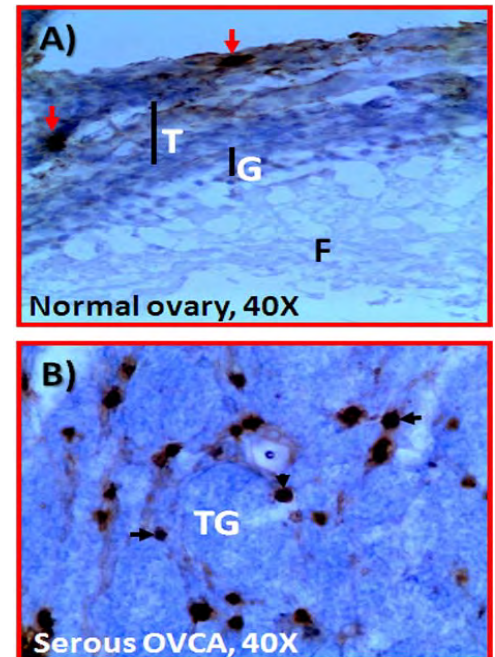


Figure 4: Immunohistochemical detection of $\alpha_v\beta_3$ -integrins expressing microvessels in hen ovaries predicted to be normal or to have ovarian tumor by molecular targeted CE-US imaging. A) Section of a normal hen ovary showing expression of few $\alpha_v\beta_3$ -integrins stained vessels in the follicular theca and stroma beneath the ovarian surface. B) Ovarian section of a hen suspected to have ovarian tumor by molecular targeted CE-US imaging. In contrast to (A) many $\alpha_v\beta_3$ -integrins expressing microvessels are seen in the tumor stroma. G, granulosa layer; T, theca layer of stromal follicle (F); TG, tumor gland. Arrows indicate the examples of $\alpha_v\beta_3$ -integrins expressing microvessels.

($P < 0.05$) higher (mean + SD = 5.72 ± 1.61 in $20,000\mu\text{m}^2$ of tumor tissue) than that of normal hens (2.7 ± 0.84 in $20,000\mu\text{m}^2$ of ovarian stromal tissue) and increased further in hens with late stage OVCA (9.97 ± 1.64 in $20,000\mu\text{m}^2$ of tumor tissue) (Figure 5). These results confirm the observations of molecular targeted CE-US imaging that ovarian tumor associated microvessels express $\alpha_v\beta_3$ -integrins which can be detected *in vivo* and may be used as imaging marker for the detection of OVCA at early stage.

Serum Levels of DR-6: DR-6 levels in the sera of normal or OVCA hens were determined by immunoassay using anti-chicken DR-6

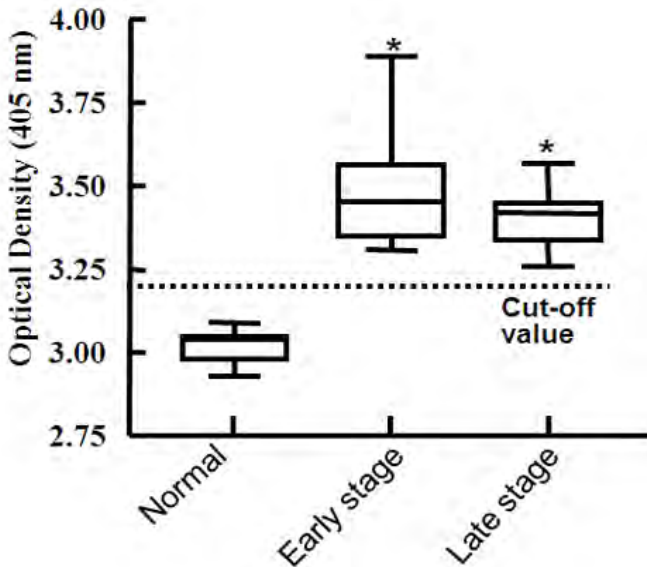


Figure 6. Serum levels of death receptor (DR)-6 proteins in laying hens with or without (normal) ovarian cancer (OVCA) determined by immunoassay. Optical density (OD) values are displayed as a box-and-whiskers plot with median, range (whiskers), 25th to 75th percentile (box). DR-6 proteins in the sera of normal or OVCA hens were detected using rabbit anti-chicken DR-6 antibodies. Sera ($n = 22$; age range 2.5 to 3.0 years) from hens with ovarian cancer were compared with normal hens (healthy hens of the same age, $n = 50$ randomly selected from CE-US scanned hens). OD values of DR-6 greater than the cut off value (mean OD values + 3SD of normal hens) were considered as the diagnostic level of OVCA with reference to histopathology. All hens with OVCA pathology were found to have higher OD values for serum DR-6 levels than the diagnostic level of OVCA. * indicates differences ($P < 0.05$) than normal hens.

hens with normal ovary (4.44 vs 2 , $P < 0.01$, Mann-Whitney Exact test) and increased further as the disease progressed to late stage (8.11 vs 2 , $P < 0.01$). Moreover, TANI was inversely correlated to Doppler indices from OVCA hens (the higher the TANI, the lower the RI and PI) indicating that establishment of ovarian TAN is associated with increase in blood flow to the tumor vicinity. Taken together, the early stage OVCA diagnostic indices established with reference to histopathology and ovarian expression of TAN markers are:

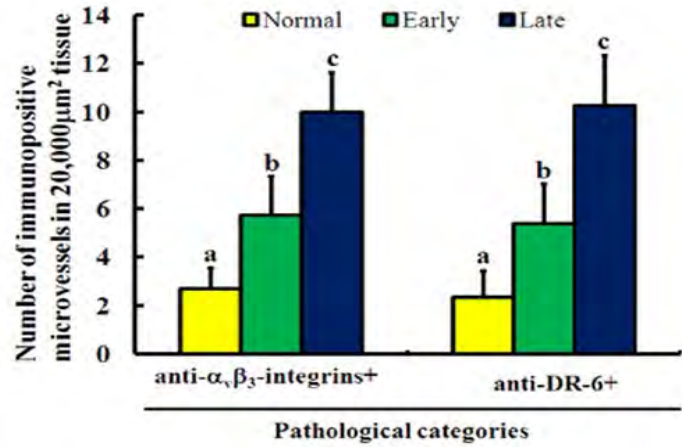


Figure 5. Changes in the frequency of tumor associated neo-angiogenic (TAN) markers (immunopositive ovarian $\alpha_v\beta_3$ -integrins and DR-6 microvessels) relative to ovarian tumor development and progression in hens. Tissue expression of both the marker increased significantly in hens with early and late stages of OVCA. Detail information is included in the text. Each bar with different letter indicates significant differences ($P < 0.05$) among different pathological groups.

antibodies and hens with optical density (OD) values for serum DR-6 level greater than a cut-off value (mean OD of normal hens + 3SD with reference to histopathology) was considered to have OVCA. The mean serum OD value for normal hens was (mean + SD = 3.02 ± 0.06). The OD values for serum DR-6 levels in hens with early stage and late stage OVCA were greater than the mean OD + 3SD of normal hens. Hens with OD values for serum DR-6 levels greater than the cut-off value (mean + 3SD = $3.02 \pm 0.06 \times 3 = 3.20$) were considered to have ovarian tumor associated neo-angiogenesis (TAN) (Figure 6). Immunoreactivity of DR-6 proteins was confirmed by immunoblotting using selected sera samples.

Tumor associated angiogenic indices (TANI): Trends in the tumor associated changes in the frequencies of $\alpha_v\beta_3$ -integrin and DR-6 expressing vessels were found similar in OVCA hens irrespective of tumor stages. A tumor associated neo-angiogenic index (TANI) diagnostic of OVCA with reference to histopathology was calculated as: $TANI = Ti / Ni + T_d / N_d$; where N_i & T_i and N_d & T_d are the number of $\alpha_v\beta_3$ -integrins and DR-6 positive vessels in normal and tumor hens, respectively. The value for neo-angiogenic index for normal ovary was considered as 2 (1 for $\alpha_v\beta_3$ -integrins and 1 for DR-6 expressing vessels).

TANI for early-stage OVCA was significantly higher than in

- Pixel intensities diagnostic of early stage OVCA from molecular targeted CE-US imaging: 18000 pixels or greater
- Doppler indices diagnostic of early stage OVCA from molecular targeted CE-US imaging: RI = 0.44 or lower and PI = 0.96 or lower
- OD values for serum levels of DR-6 diagnostic of early stage OVCA = 3.20 or greater

Aim 2: Detection of Anti-NMP antibodies in the sera of laying hens and prospective monitoring of hens with anti-NMP antibodies to determine that anti-NMP antibodies appear in serum before ovarian TAN becomes detectable.

Aim 2 is being accomplished in a longitudinal design.

A total of 40 laying hens with normally appearing ovaries (20 with low laying rates and without ovarian TAN and 20 with normal egg laying rates) were selected from a flock of laying hens by molecular targeted CE-US imaging using indices established in Aim 1 (mentioned above). Sera of all selected hens were examined for the presence of anti-NMP antibodies by immunoassay and hens negative for serum anti-NMP antibodies were selected for specific aim 2. Anti-NMP antibodies in the sera were determined by immunoassay using nuclear matrix protein extracts from normal or tumor ovaries as reported previously [6]. These hens are being monitored at 15 weeks intervals. Sera from all hens are being collected at each scan. At 1st scan (15 weeks from the start) sera samples of all hens were examined for anti-NMP antibodies.

Results: At first interval (after 15 weeks from the start of monitoring), 3 out of 20 hens in low egg laying group were found positive for sera anti-NMP antibodies (**Figure 7**). However, no abnormality in the ovarian morphology was detected in these hens at CE-US targeted imaging after 15 weeks. All hens will be monitored for 45 weeks.

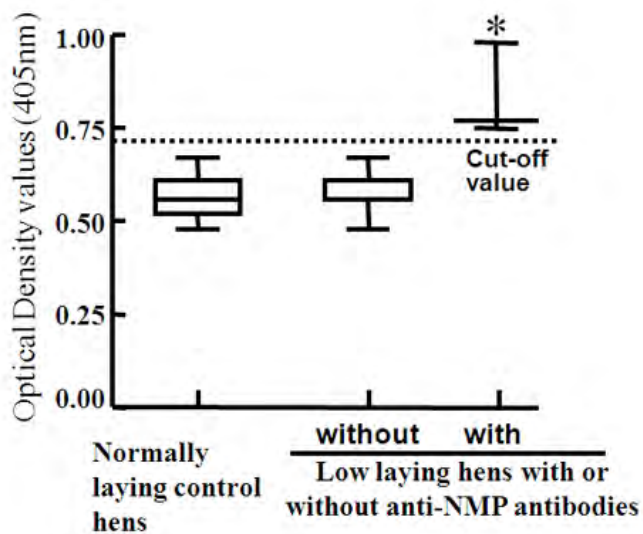


Figure 7. Prevalence of anti-NMP antibodies in the sera of laying hens determined by immunoassay. Optical density (OD) values are displayed as a box-and-whiskers plot with median, range (whiskers), 25th to 75th percentile (box). Presence of anti-NMP antibodies in sera were examined in two assays using NMP antigens either from normal ovary or from ovarian tumors. Sera from hens of same age group (2.5 to 3.0 years old) with normal egg laying rates (n = 20, normal) and hens with low egg laying rates (n = 20) were used. Hens with OD values greater than the cut-off value (mean OD of normal + 3SD, dotted line) were considered positive for serum anti-NMP antibodies. At 1st CE-US targeted scan (after 15 weeks from the start), 3 out of 20 hens were found positive while 17 hens remain negative for serum anti-NMP antibodies. Differences in sera immunoreactivity was not observed between the antigens from normal or ovarian tumors.

KEY RESEARCH ACCOMPLISHMENTS:

- Established the enhancement of detectability of OVCA at early stage by traditional ultrasound imaging using by contrast enhanced $\alpha_v\beta_3$ -integrins targeted molecular imaging.
- Confirmed the binding of contrast agents to the tissue marker of ovarian tumor associated neo-angiogenesis (microvessels expressing $\alpha_v\beta_3$ -integrins) related to the early stage of OVCA.
- Ultrasound prediction of tumor associated overexpression of ovarian $\alpha_v\beta_3$ -integrins confirmed by immunohistochemical detection.

- For the first time, OVCA diagnostic level of serum DR-6, a novel marker of tumor associated neo-angiogenesis at early stage was determined by an immunoassay.
- For the first time, by immunohistochemical detection, this study has shown that ovarian tumor as a source of serum DR-6 in this animal model.
- Establishment of the hypothesis that anti-NMP antibodies are associated with the early ovarian tumor formation is being underway.

REPORTABLE OUTCOMES:

Presentation: Abstract published and presented: (appended)

1. **A. Barua**, A. Yellapa, P. Bitterman, J. M. Bahr, S. Sharma, D. B. Hales, J. L. Luborsky, J. S. Abramowicz (2011): Use of contrast-enhanced ultrasound imaging with microbubbles targeted to $\alpha_v\beta_3$ integrins to enhance detection of early-stage ovarian tumors, ASCO Annual Meeting. Chicago, IL, USA; June 3-7, 2011.

http://www.asco.org/ascov2/Meetings/Abstracts?&vmview=abst_detail_view&confID=102&abstractID=84636

2. Aparna Yellapa, Jacques S. Abramowicz, Pincas Bitterman, Janice M. Bahr, Michael J. Bradaric, Seby L. Edassery, Sameer Sharma, **Animesh Barua**. Interleukin (IL-16) and tumor associated neo-angiogenesis detects ovarian cancer at early stage. AACR 102th Annual Meeting, Orlando, FL, April 2-6, 2011.

<http://www.abstractsonline.com/Plan/ViewAbstract.aspx?sKey=0732f136-3e77-422d-886b-29769face7da&cKey=fc49e373-cefe-430d-ae3f-058a41a93bf4&mKey=%7b507D311A-B6EC-436A-BD67-6D14ED39622C%7d>

Manuscript: One manuscript is under preparation.

CONCLUSION:

The results so far obtained with the accomplishment of Aim 1 and part of Aim 2 suggest that contrast enhanced molecular imaging targeting ovarian $\alpha_v\beta_3$ -integrins can detect ovarian tumor associated neo-angiogenesis at early stage of ovarian cancer in laying hen model of spontaneous ovarian cancer. Changes in early stage ovarian cancer related CE-US imaging indices were associated with the elevation of serum levels of death receptor (DR-6). CE-DUS imaging indices together with the serum levels of DR-6 detective of ovarian tumor associated neo-angiogenesis are being used to diagnose hens with ovarian cancer at early stage in Aim 2.

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APPENDICES:

Abstracts published and presented

Page 12:

- 1) **A. Barua**, A. Yellapa, P. Bitterman, J. M. Bahr, S. Sharma, D. B. Hales, J. L. Luborsky, J. S. Abramowicz (2011): Use of contrast-enhanced ultrasound imaging with microbubbles targeted to $\alpha_v\beta_3$ integrins to enhance detection of early-stage ovarian tumors, ASCO Annual Meeting. Chicago, IL, USA; June 3-7, 2011.

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- 2) Aparna Yellapa, Jacques S. Abramowicz, Pincas Bitterman, Janice M. Bahr, Michael J. Bradaric, Seby L. Edassery, Sameer Sharma, **Animesh Barua**. Interleukin (IL-16) and tumor associated neo-angiogenesis detects ovarian cancer at early stage. AACR 102th Annual Meeting, Orlando, FL, April 2-6, 2011.

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Use of contrast-enhanced ultrasound imaging with microbubbles targeted to $\alpha v \beta 3$ integrins to enhance detection of early-stage ovarian tumors.

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Citation:
J Clin Oncol 29: 2011 (suppl; abstr 5076)

Author(s):

A. Barua, A. Yellapa, P. Bitterman, J. M. Bahr, S. Sharma, D. B. Hales, J. L. Luborsky, J. S. Abramowicz;
Rush University Medical Center, Chicago, IL; University of Illinois at Urbana-Champaign, Urbana, IL;
Southern University of Illinois at Carbondale, Carbondale, IL

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Abstract:

Background: Lack of an early detection test makes ovarian cancer (OVCA) a lethal gynecological malignancy. Tumor associated neo-angiogenesis (TAN) is an early event in tumor development and represents a potential target for early detection. Traditional transvaginal ultrasound (TVUS) cannot detect TAN at early stage. The goal of this pilot study was to examine the enhancement of TVUS using $\alpha v \beta 3$ integrins targeted microbubble ultrasound contrast agent (UCA) in laying hens, a preclinical spontaneous model of human OVCA. **Methods:** 3-4 years old hens (n=50) were selected randomly and scanned continuously by TVUS before, during and after UCA (Visister-integrins, Targeson Inc. CA) injection at brachial veins. UCA was visualized using a low mechanical index contrast imaging pulse sequence. All pre- and post-contrast injection images were archived and analyzed off-line. Gross diagnosis was recorded at euthanasia and tissues were processed for histology. Tumors and their types were confirmed by routine histology. TAN markers (SMA and $\alpha v \beta 3$ integrins) were detected by immunohistochemistry. TAN vessels were counted and correlation with ultrasound prediction was examined. **Results:** $\alpha v \beta 3$ -integrins targeted UCA enhanced the visualization of ovarian tumors significantly in laying hens. At gray scale, UCA bounded areas appeared as a ring on the ovarian surfaces of 8 of 50 hens and were suspected for ovarian tumors. Tumors were confirmed in all hens predicted to have OVCA. In 7 hens tumors were early stage and in one hen the tumor metastasized to the oviduct. A microscopic lesion was found in one hen which was not detected by ultrasound imaging. Thus 9 of 50 hens had ovarian tumors and UCA detected approximately 88% at early stages. The frequency of immunopositive TAN vessels (SMA and $\alpha v \beta 3$ integrins positive) were significantly higher in OVCA hens than normal hens ($P < 0.05$) and was positively correlated (0.86, $P < 0.05$) with ultrasound prediction. **Conclusions:** Our results demonstrate that UCA targeting $\alpha v \beta 3$ integrin enhanced TVUS detection of early stage ovarian tumors in a pre-clinical model and may form the foundation for a clinical study. Support: Department of Defense (#09-3303), Sramek Foundation.

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1. Use of contrast-enhanced ultrasound imaging with microbubbles targeted to $\alpha v \beta 3$ integrins to enhance detection of early-stage ovarian tumors.

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Abstract
Body:

Background: The lack of an early detection test is one of the reasons of high mortality rate of women due to epithelial ovarian cancer (OVCA). Understanding the pathogenesis and progression of OVCA is essential to establish an effective early detection test. Tumor associated -immuno-chemotaxis (TAI) and -neoangiogenesis (TAN) are the two earlier events in tumor development. CD8+ T cells in tumor vicinity produce immune-chemotactic IL-16 cytokines which stimulates the production of pro-angiogenic cytokines responsible for TAN. Ovarian TAI and TAN represent potential target for an effective early detection test. Access to patients at early stage OVCA is very difficult and laying hens develop spontaneous OVCA with histopathology similar to humans.

Objectives: The goal of the present study was to determine the feasibility of markers of ovarian TAI and TAN in detecting early stage OVCA in laying hens.

Materials and Methods: 3 years old laying hens (normal, low or stopped egg-laying) were scanned by Ultrasound, sera were collected, hens were euthanized and ovarian tissues were processed for paraffin and frozen sections, and mRNA extraction. Ovarian tumor stages were confirmed at gross and routine histology. Samples were divided into 4 groups namely normal (control), early stage OVCA [microscopic or tumors limited to the ovaries], late stage OVCA, with non-tumor ovarian abnormalities (atrophied ovaries). Sera were tested for IL-16 levels by ELISA and confirmed by 2D-Western blot (WB). Ovarian expression of TAN markerS (VEGF) and, neoangiogenic microvessel density (MVD) as well as IL-16 mRNA was determined.

Results: The population of CD8+ T cells and the serum IL-16 levels were significantly higher in tumor hens (P < 0.05) than normal hens. Significant increase in IL-16 levels in hens with microscopic tumor (undetectable at gross examination) suggesting that serum IL-16 may be a potential indicator of ovarian tumors at very early stage. Differences in serum IL-16 levels were not observed between hens with non-tumor ovarian pathology and normal hens suggesting that increased serum IL-16 levels in OVCA hens are tumor specific. Two immunoreactive bands (12 & 50kDa) for IL-16 were identified in the ovary while only one band (50 kDa) was detected in sera suggesting that IL-16 may be the active in tetramerized form. IL-16 protein and mRNA expression as well as the frequency of MVD were significantly higher in hens with OVCA than healthy hens. VEGF expressing microvessels were localized in the ovarian stroma preceding the tumor indicating that ovarian TAN precedes tumor progression.

Conclusion: The results of the study suggest that changes in serum levels of IL-16 are positively

correlated with tumor initiation and progression. Thus serum IL-16 level together with marker of ovarian TAN may constitute a feasible test for the early detection of OVCA. Support: Prevent Cancer Foundation, Sramak Foundation and DOD (OC#093303).

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