Targeted microbubbles for characterization of sentinel lymph nodes

Melanoma or Skin Cancer

- Melanoma is a malignancy that develops from the pigment-containing cells called melanocytes
- In last 30 years melanoma cases have tripled. In 2015, 73,870 cases of melanoma and 9,980 deaths due to this cancer was estimated in the USA
- For localized melanoma (84% of all cases) 5 year survival rate is 98% ...
- The presence or absence of melanoma cells in lymph nodes is the prognostic factor in the determination of the extent of the proliferation of melanoma cells in the body

Sentinel Lymph Node

- SLN characterization is important to check for presence or absence of melanoma cells in nodes
- Melanoma spreads to the lymph nodes nearest to the area of primary melanoma tumor
- SLN’s are first of those lymph nodes to receive drainage from the primary tumor, and therefore is the most important prognostic factor to determine the spread of melanoma cells, if any of them have spread

Objective

- To determine the in vitro binding of dual targeted microbubble to tumor vasculature in metastatic lymph nodes
- And to characterize sentinel lymph node using dual targeted molecular contrast enhanced ultrasound imaging

Swine model

- A trial of 6 Sinclair swine (Sinclair Bio-resources, Columbia, MO) with naturally occurring melanoma tumors were evaluated in this study
- Weight range: 3.0 kg to 7.0 kg
- Swine model is used because:
  - The melanoma tumor has same histopathological resemblance to that of a human melanoma
  - Swine that have melanoma tumors have a 70% incidence of metastasis-to-drain into the regional SLNs

Ultrasound Contrast Agents

- Ultrasound Contrast Agents: Shell make up and gas core:
  - Microparticle shells are composed of albumen, galactose, lipid or polymers
  - Gas core can comprise of heavy gases like perfluorocarbon or nitrogen etc.
- Diameter 1-4μm
- The following UCAs are FDA approved:
  - Optison (GE Healthcare)
  - Levovist (Schering)
  - Imagent (Alliance Pharmaceuticals)
  - Definity (Lantheus Medical Imaging)
Targeted UCA

- Same general features as an ultrasound contrast agent, however there outer shell is attached with ligands that bind to specific receptors exposed by cell type, interest, cancer cells etc.
- These UCA's have lipid monolayer shell with a perfluorocarbon gas core
- Ligands are attached to the outer surface to bind to specific receptors, ligands attached using: 
  - Carboximid, maleimide, biotin-streptavidin.
- Antibodies used for targeting are not humanized, if these UCA's administered to humans would initiate immune response.

Table: Ultrasound Contrast Agents Used

<table>
<thead>
<tr>
<th></th>
<th>SonoVue</th>
<th>Dual Targeted</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufacturer</td>
<td>GE Healthcare, Oslo Norway</td>
<td>Targason, San Diego, CA</td>
<td>Targason, San Diego, CA</td>
</tr>
<tr>
<td>Diameter</td>
<td>2.4µm-3.0µm</td>
<td>2.0µm to 3.0µm</td>
<td>2.0µm to 3.0µm</td>
</tr>
<tr>
<td>Dose</td>
<td>0.25µl</td>
<td>1.27 µl</td>
<td>1.27 µl</td>
</tr>
<tr>
<td>Shell Make-up</td>
<td>perfluorocarbon, suspended in oxygen</td>
<td>perfluorocarbon, suspended in oxygen</td>
<td>perfluorocarbon, suspended in oxygen</td>
</tr>
<tr>
<td>Administration</td>
<td>IV</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td>Use</td>
<td>Retrospective, lymph node specific, detection of sentinel lymph node</td>
<td>Characterize metastatic lymph nodes</td>
<td>To help in the detection accuracy of dual targeted agents</td>
</tr>
</tbody>
</table>

Specific Molecular Markers of Angiogenesis

- Angiogenesis is the formation of new blood vessel formation from the existing vessels, and is a critical determinant of tumor growth, invasion and metastatic potential
- Earlier studies have shown several specific molecular markers of angiogenesis, including vascular endothelial growth factor (VEGF), αβ3 integrin and p-selectin are overexpressed on tumor vascular endothelial cells
- To construct most appropriate targeted UCA's total of 12 abnormal lymph nodes were removed and were submitted for biological examination, to determine metastatic involvement in lymph node
- Evaluation of expression of VEGFR-2, αβ3 integrin and p-selectin using microscopy (×40 Nikon, Labophot-2)
- It was found that VEGFR-2 expression was 10% lower than αβ3 integrin and p-selectin. Hence αβ3 integrin and p-selectin were used to create dual targeted microbubbles for this study

Cell Culture Study for Validation of Microbubble Attachment on Lymph Nodes

- Two solution 0.5 ml of dual targeted microbubble(P-selectin and αβ3 integrin) and IGG (Control) microbubble each was made in a 50ml sterile solution.
- To test in-vivo binding specificity 5 metastatic nodes and 5 benign nodes are 5.0 µm sectioned cut onto a glass slide.
- After a 4 minute static exposure the slides were dipped in 50 ml of PBS solution, to remove any unattached microbubble on the slide.
- Slides were allowed to dry and mounted with cover slip before imaging.
- 4 images are determined by [Nikon, Labophot-2] microscope (×40) in phase contrast and normal mode.
- Phase contrast: the background light is shifted ~90° relative to the background light, eliminating the phase difference between the background and the scattered light, leading to an increased intensity between foreground and background.
- The mean number of microbubbles is quantified using sub-routines created in sub-routines created in ImagePro Plus.

Characterization of SLN

Study for validation of microbubble attachment in a tissue

Clinical study to test dual targeted contrast agents retention in lymphatic nodes

Study for validation of microbubble attachment on tissues

Dual-targeted microbubble attachment

IgG(Control) microbubble attachment

Antigen Removal
To further test the specificity of the dual targeted microbubbles in vitro blocking was performed where antigen retrieved slides which were exposed in a solution of 50 µg of biotinylated (αvβ3-integrin and p-selectin antibodies) mixed in 10 ml of saline solution.

Once the slides with metastatic nodes and benign nodes were exposed with the antibody solution and dipped in the PBS solution to get rid of any unattached microbubble, slides were imaged in phase contrast and normal modes.

The mean number of microbubble attached were quantified

Sub-routine was created based on:

- Image
- Color Thresholding
- Object Count
- Result - Quantified

Determination of microbubble criteria

- Image quantified for microbubble based on two criterias
  - Diameter (µm)
  - Roundness (Defined as: perimeter^2/(4*pi*area))

- Range of roundness is quantified for a diameter range of 2.75 µm to 8.00 µm. For this range the minimum roundness was 1.65

- Roundness value checked for diameter of count more than 8 µm. Min value of roundness was 5.02

- Figure a) shows microbubble in phase contrast mode and b) shows microbubble in normal mode.

Microbubble Criteria

- These ranges were selected to avoid any overlap in the values of intracellular structures such as cell nuclei and other structures, which would cause error in microbubble quantification.

- The image shows the count of the number of microbubbles where there was not tissue in the background.

Metastatic tissue imaging: Phase contrast Vs Normal mode

Figure shows a contrast phase image of a metastatic lymph node with green depicting the count of the structure which might be microbubbles.
Bubble Attachment Study

Identification of SLN using Sonazoid

- 0.25 ml of a peritumoral injection of Sonazoid was administered near a melanoma tumor in a melanoma swine model using a peritumoral UCA injection.
- Sonazoid is used because it is a reticuloendothelial system specific UCA.
- Sonazoid microbubbles are destroyed for an hour using a high ultrasound pulse (MF=1.5).
- This is done to destroy any retained Sonazoid in SLN before administration of dual targeted contrast agent.

Administration of dual targeted microbubbles

- A high power destructive pulse is used to destroy microbubbles.
- Dual targeted UCA is injected, after a 4 minute delay, lymph node scan is done.
- Post destruction pulse scan is stored.
- Resultant scan exist of the attached MB’s.

Dual targeted contrast enhanced ultrasound

- 11 Sentinel lymph node and 13 non-sentinel lymph node where characterized using dual targeted microbubbles.
- S3000 scanner (Siemens Medical Solutions, Mountain View, CA) with a linear array 9L4 probe was used to perform contrast enhanced ultrasound.
- Contrast enhanced ultrasound exams were conducted for dual targeted and IgG (control) injection in metastatic and benign nodes.
- CEUS data was acquired using Cadence™ Pulse Sequencing (Siemens). It is a low-power multipulse technique where three pulses with varied phase and amplitude are transmitted and the resulting echoes are summed. CPS imaging results in considerable tissue suppression, allowing for better detection of the contrast microbubbles.

CEUS Data Analysis

- Offline processing was performed in ImageJ (NIH, Bethesda, MD).
- The CEUS data was divided into two parts:
  - Pre-destruction clips.
  - Post-destruction clips.
- Average video intensity for pre destruction clips and post destruction clips was performed.
- T-test was used to analyze the data between dual targeted and IgG control for metastatic lymph nodes, benign lymph nodes.

Clinical study to test dual targeted contrast agents retention in lymphatic nodes

- SLN’s are detected in the melanoma swine model using a peritumoral UCA injection.
- Dual targeted UCA injection is used to determine metastatic involvement in nodes.
- Dual targeted microbubble retention is compared with IgG (Control) microbubble retention, to determine dual targeted contrast agent binding affinity in tumor vasculature in metastatic lymph nodes.
- SLN’s are resected and were sent to pathology for histopathological analysis.
Results

- Bubble attachment studies showed that there was a significant difference in the amount of microbubbles attached in metastatic node for dual targeted vs IgG (Control). (p = 0.001)
- Antigen blocking showed that ανβ3-integrin and p-selectin were expressed, since there was no microbubble attached to the slides.
- A total of 11 sentinel and 13 non-sentinel LNs was imaged.
- The mean signal intensity in metastatic node for dual-targeted contrast agent VS IgG (17.2 ± 15.8 vs 1.5 ± 1.3; p = 0.036).
- The mean signal intensity in benign nodes for dual-targeted and IgG-control microbubbles was not significantly different (1.5 ± 0.6 vs 1.6 ± 0.8; p = 0.87).

Limitations

- In microbubble attachment study there was difficulty in the differentiation of slides which had metastatic involvement greater than 65%. Since the background was dark, there can be some microbubbles that were not quantified.
- Another limitation of this study is the shelf life of the dual targeted microbubble; once open they are only good for 6 hours making it really difficult to collect a more number of images to quantify microbubble attachment.
- There is only one farm in the USA that breeds the swine in this study; it was really difficult to get hold of the swine with naturally occurring melanoma in them.
Conclusion

- Microbubble attachment study shows that there is a significant number of dual targeted microbubble attached in a metastatic lymph node; when quantified in phase contrast.
- In vitro studies show that there is a significant difference in the video intensity of the retained dual targeted microbubbles in a metastatic lymph node.
- Similarly there was no to less significant number of microbubbles attached in the benign nodes, suggesting in the successful ability to determine difference in the metastatic and benign lymph nodes non-invasively.