Colocalization of ovarian cancer-associated biomarkers in tumors, cancer cells and extracellular vesicles

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Introduction

We have previously shown that ovarian cancer can be detected with high specificity and sensitivity by interrogating colocalized, membrane-associated, cell-surface biomarkers on single tumor-associated extracellular vesicles (EVs). Our blood-based Mercy Halo Ovarian Cancer Test (OC Test) is composed of five cancer-associated biomarkers (BST2, FOLR1, MUC-1, MUC-16, and sialylated Thomsen-nouveau antigen (sTn)), previously shown to be upregulated in ovarian cancer relative to healthy tissues. The test employs these five biomarkers in three combinations to capture and detect EVs from human plasma to distinguish high-grade serous ovarian carcinoma (HGSC) from both benign ovarian tumors and normal samples. Using super-resolution microscopy on ovarian cancer cell line EVs and multiplex immunohistochemistry of ovarian benign and tumor tissue, our study aimed to show that the ovarian cancer biomarkers demonstrate expression and colocalization on the surface of EVs derived from cancer cell lines, and coexpression and colocalization on cancer cells within ovarian tumor biopsies that correlate with assay signal from human plasma or serum.

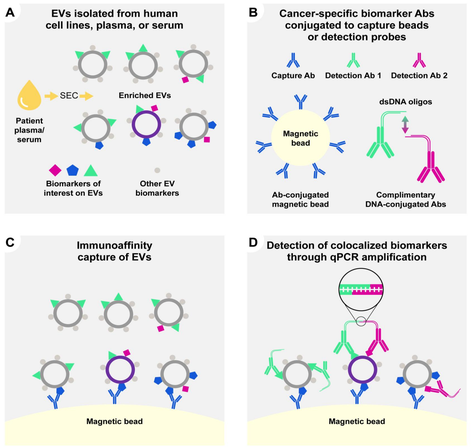
Methods

Samples:
58 HGSC (17 Stage I, 30 Stage II, and 10 Stage III) and 17 benign ovarian tumor K_2EDTA plasma samples and paired FFPE tissues were sourced from the Ovarian Cancer Research Program (OVCARE, Vancouver BC, Canada).

Mercy Halo Ovarian Cancer Test:
OC Tests were run on each plasma sample.

A. EVs were enriched from human plasma by SEC.
B. Antibodies to cancer-associated, cell-surface biomarkers used for EV capture and detection were conjugated to magnetic beads (capture) or complementary DNA-conjugated oligonucleotides (detection).
C. SEC-enriched EVs were captured using antibody-functionalized magnetic beads.
D. Immunocaptured EVs were then incubated with two additional cancer-specific biomarker detection antibodies. The dsDNA oligonucleotides contain single-stranded overhangs which ligate only when in proximity on the same EV to a complementary probe-antibody, generating a template for PCR.

The abundance of EVs containing the specific set of biomarkers was measured using the qPCR cycle threshold values (Ct) for each combination. The Ct values from each combination were used by the test algorithm to calculate the final OC Test score.



Tissue Immunohistochemistry:
A tumor microarray from the matched FFPE tissue samples was stained with an Opal multiplex-IHC (mIHC) assay comprised of antibodies to BST2, MUC1, sTn, pan-cytokeratin and DAPI. Machine learning based image analysis was utilized to determine colocalization of the biomarkers at the cellular level within tumor and non-tumor tissue.

Super-Resolution Microscopy:
Super-resolution microscopy was employed to visualize stained biomarkers on the cell surface of COV413A ovarian cancer cell line EVs. SEC-purified EVs were captured on the surface of a glass slide using either S4 (PtdSer capture) or testraspanin antibody cocktail (TetTrio) according to the manufacturer's protocol (Oxford Nanoimaging). Biomarkers on individual EVs were visualized with biomarker-specific antibodies conjugated to Alexa Fluor® 488, Alexa Fluor® 555, or CF® 647 and detected using an EV Profiler Kit and Nanoimager (Oxford Nanoimaging) following the instructions provided by the manufacturer. All analysis was done using the AutoEV module in the CODI software in consultation with Oxford Nanoimaging.

RNA Sequencing:
RNA sequencing analysis was carried out to assess expression levels of the biomarkers in each FFPE tissue sample. Four, 10-micron FFPE scrolls adjacent to the H&E stained section were cut and shipped to Novogene (Sacramento, CA USA) for RNA sequencing. ST6GALNAC1 RNA expression was used as a surrogate for the glycosylation marker, sTn.

References

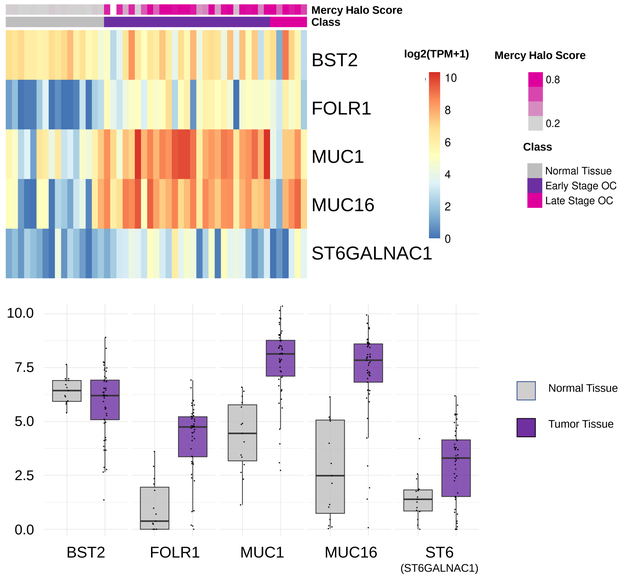
Colocalization of Cancer Associated Biomarkers on Single Extracellular Vesicles for Early Cancer Detection
Daniel P. Salem, Laura T. Bortolin, Anthony D. Couvillon, Dan Gusenleitner, Jonian Grosha, Ibukunoluwapo O. Zabroski, Kelly M. Biette, Sanchari Banerjee, Christopher R. Sedlak, Delaney M. Byrne, Bilal F. Hamzeh, MacKenzie S. King, Lauren T. Cuoco, Peter A. Duff, Emily S. Winn-Deen, Eric K. Huang, Randy Schekman, Joseph C. Sedlak
https://www.biorxiv.org/content/10.1101/2023.02.07.527360v2

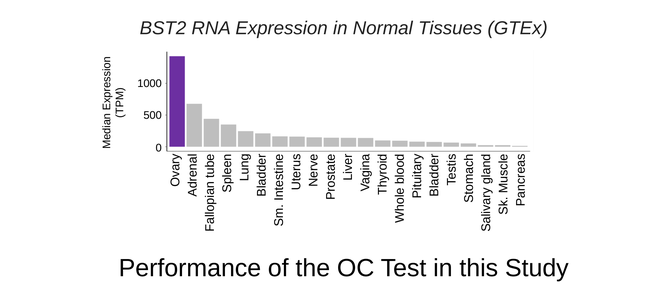
OC Test Signal Reflects Cellular Biomarker RNA Expression

Sample distribution for RNA sequencing data analysis

Sample Type	Initial Sample Number	Passed ≥20% Tumor Requirement	Passed RNA and Library Preparation	Passed Sequencing QC (Coverage and Q Score)	Passed ≥50% Tumor Metric for Final Data Analysis
HGSC, Stage I	17	16	16	16	10
HGSC, Stage II	30	27	24	24	17
HGSC, Stage III	11	9	8	8	6
Benign	17	1	1	1	0
Reclassified as Normal	N/A	16	15	15	15
Total	**75**	**69**	**64**	**64**	**48**

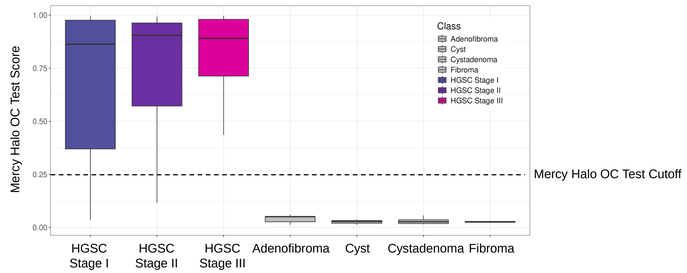
RNA Expression of OC Test Genes in Normal and Tumor Tissues



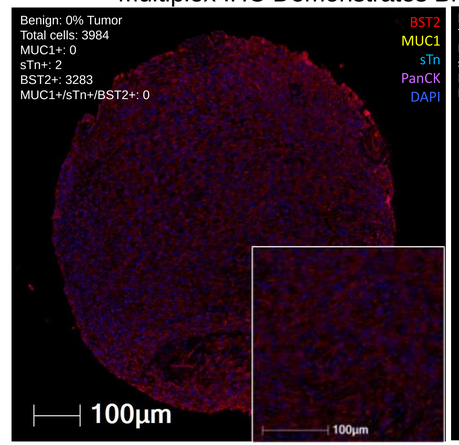


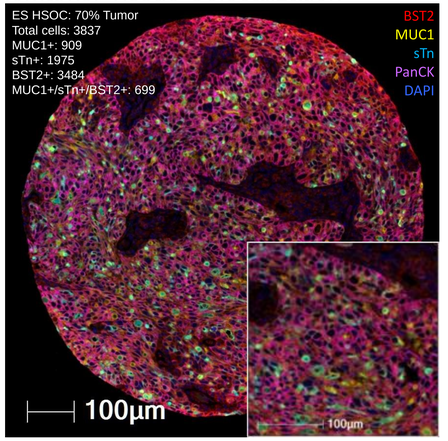
BST2 RNA Expression in Normal Tissues (GTEx)

Performance of the OC Test in this Study



Multiplex IHC Demonstrates Biomarker Tissue Colocalization



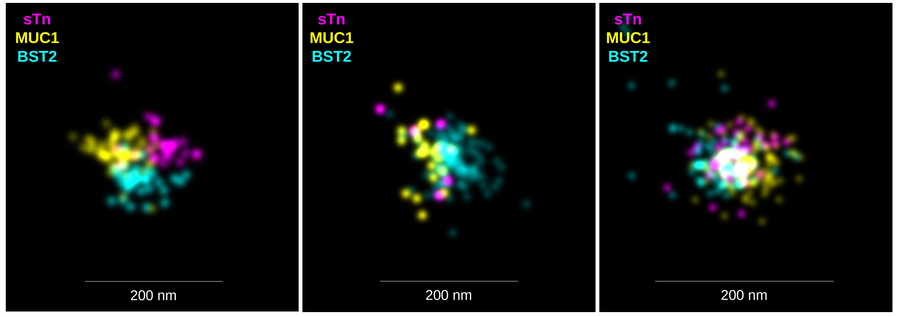


Summary of TMA Cellular Segmentation Data (Indica Labs HALO™ AI)

Class	n	MUC1/BST2/sTn Raw Ct value	OC Test Score (0-1)	MUC1+	BST2+	sTn+	MUC1/BST2+/sTn+
Benign	13	33.2	0.032	1 %	57 %	2 %	0 %
ES HGSC	25	24.9	0.709	23 %	59 %	42 %	8 %

Mercy Halo Biomarkers are Detected on Individual, Intact EVs

Nanoimaging of COV413A-derived EVs by STORM



Results Summary

Super-resolution microscopy demonstrated colocalization of OC Test combination protein biomarkers on EVs derived from ovarian cancer cells. Machine learning-based image analysis of tumor and benign tissue microarrays stained for BST2/MUC1/sTn using mIHC, showed strong evidence for colocalization of these biomarkers on single cells within ovarian tumor tissue, particularly in early-stage tumors, but not on benign tissue. RNA sequencing analysis supports the hypothesis that *FOLR1, MUC1* and *MUC16* RNA expression in tissue is also reflected in the proteins/EVs measured by the MH OC Test and that *ST6GALNAC1* RNA expression can be used as a surrogate for the glycosylation marker, sTn.

Discussion

The results presented here demonstrate that the OC Test biomarkers are coexpressed and colocalized on tumor tissue, EVs from ovarian cancer cell lines, and patient plasma. RNA and protein expression levels show good correlation with OC Test results. *BST2* expression is an interesting exception to the concept of only using biomarkers that are over-expressed in tumor tissue compared to normal tissue or benign tumors, as its expression patterns in ovarian tissue are quite similar between these groups. According to the Human Protein Atlas, *BST2* expression at both the RNA and protein level is substantially higher in normal ovary than in any other organ. Thus, the role it plays in the OC Test may be primarily to provide tissue specificity.

These results provide further evidence that colocalized biomarkers on EVs can be utilized for early detection of ovarian and potentially other types of cancer.