Combined detection of CEA+ extracellular vesicles and soluble CEA retrospectively predicts prognosis of subjects with refractory colorectal tumors

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1 Abstract

Carcinoembryonic antigen (CEA) is a biomarker commonly used postoperatively to guide surveillance of colorectal cancer (CRC). Perioperative monitoring of CEA is recommended by current guidelines, although its utility as a prognostic factor has not been confirmed. Improved CEA assays may enable earlier determination of treatment failure, or earlier detection of recurrence. CEA is secreted from CRC cells in soluble form, but we have identified an alternative secretion of CEA in association with extracellular vesicles (EVs). We hypothesized that soluble CEA and EV-associated CEA may act as independent, but complementary, biomarkers providing unique information about a tumor and improving sensitivity compared to measuring soluble CEA alone. We developed sensitive, multi-marker electrochemiluminescence immunoassays for measuring intact EVs presenting CEA, CD73, or both in commercially sourced human plasma, and tested three independent sample sets including two longitudinal refractory CRC sample sets. We observed that plasma levels of CEA+EVs, CD73+EVs, and CEA+CD73+EVs were significantly elevated in late-stage CRC samples compared to controls ($p<0.001$). Additionally, 10-25% of stage IV samples in each sample set had elevated CEA+EV and CEA+CD73+EV levels but not elevated soluble CEA. In light of these observations, we combined the two assays into a single classifier for late-stage CRC samples and controls, yielding improved receiver operator characteristic (ROC) with higher area under the curve than soluble CEA alone. Increased levels of CEA+EVs and CEA+CD73+EVs at the onset of treatment correlated with poor overall survival in both longitudinal sample sets, and correlated with progression-free survival in one sample set. Multivariate analysis showed that the most significant prognostic model included both soluble CEA and CEA+EV levels. This retrospective study suggests that assaying both soluble and EV-associated forms of CEA in plasma may improve the value of CEA measurement in CRC and may help overcome the currently limited accuracy of CEA as a prognosis factor for CRC.

2 Methods

A. High-throughput plasma EV purification using Captocore 400 core-shell resin (Cytiva)

Principle

- **Porous shells** exclude extracellular vesicles and other macromolecular structures with molecular weight (MW) > 400kDa
- **Ligand-activated core** (octylamine) retains molecules below the MW cutoff
- **High throughput**: Operator can process 8 plates per day (768 samples). This would take several weeks using gravity SEC columns.

Procedure at a glance

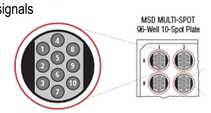
- 100 µL plasma per sample is diluted and added to washed resin in deep well plates
- Resin / Samples incubated with mixing for 1h
- Centrifugal processing in 96W fritted microplates to retain resin with bound protein and elute purified EVs

B. MSD® ECL-based assays measure the concentration of soluble proteins and intact EVs in complex biological samples

Electrochemiluminescence (ECL) Technology

MSD's ECL detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® microplates.

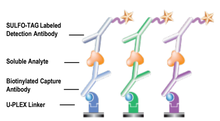
- **High sensitivity:** Multiple excitation cycles amplify signals to enhance light levels.
- **Broad dynamic range:** High and low abundance analytes can be measured without multiple sample dilutions.
- **Low background:** Electronic stimulation of detection label is decoupled from the output signal (light) resulting in extremely low background signals



U-PLEX® Multiplexed Immunoassays

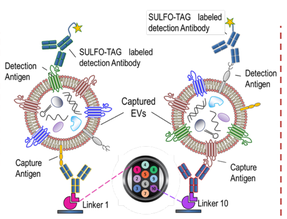
Biotinylated capture antibodies are each coupled to one of ten unique U-PLEX Linkers, which self-assemble onto unique array elements (or "spots") on the U-PLEX plate. Multiplexing conserves valuable samples by allowing up to ten determinations per well.

Assaying soluble analytes: e.g. CEA, CD73, Albumin



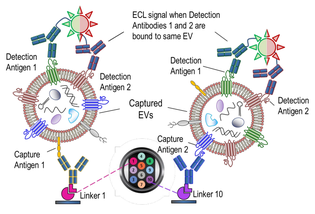
Soluble analytes are captured and detected using sandwich immunoassay format with SULFO-TAG ECL label

Assaying intact EVs: e.g. CEA⁺ EVs, CD73⁺ EVs



Intact EVs are captured by antibodies targeting distinct surface antigens, e.g. CD73 or CEA. Bound EVs are detected using SULFO-TAG labeled detection antibodies that recognize specific detection antigen(s) on the EVs; usually this is a cocktail of common EV proteins: CD81, CD9, and CD63.

Assaying more specific population of EVs: e.g. CEA⁺CD73⁺ EVs



Bound EVs are detected using a pair of detection antibodies targeting two distinct detection antigens. Ultrasensitive assay format only generates signal when both detection antigens are present on the same EV. At least one of the two detection antigens is typically a common EV protein: CD81, CD9, or CD63.

3 Samples tested

All plasma samples used in this study were collected in EDTA tubes and were double spun immediately after collection to remove platelets. For all EV assays, the samples were purified using the high-throughput approach described in the methods. All samples were assayed for soluble CEA, CEA⁺ EVs, CD73⁺ EVs, and CEA⁺ CD73⁺ EVs .

1. Discovery Cohort 1: 23 remnant plasma samples purchased from iSpecimen. This sample set included plasma from 8 healthy individuals and 15 Stage IV CRC individuals.

2. Discovery Cohort 2: 37 Plasma samples from the Vanderbilt University Medical Center (VUMC) Cooperative Human Tissue Network (CHTN). This sample set included plasma from 14 healthy individuals and 23 Stage IV CRC individuals.

3: Validation Cohort 1: 105 plasma samples from the VUMC CHTN. Sample set included plasma from 23 healthy individuals, 17 Stage I CRC individuals, 18 Stage II CRC individuals, 23 Stage III CRC individuals, and 24 Stage IV CRC individuals.

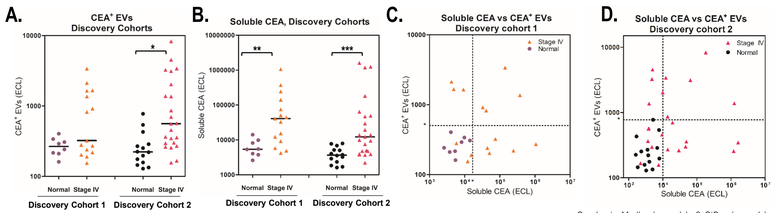
4: Validation Cohort 2: 206 plasma samples from 58 individuals affected with Stage IV refractory CRC enrolled in the XTRAP longitudinal study[1] from the Duke Cancer Institute.

5: Validation Cohort 3: 131 plasma samples from 30 individuals affected with Stage IV refractory CRC enrolled in the CaboMab longitudinal study[2] from the Duke Cancer Institute.

4 CEA⁺ EVs and soluble CEA are elevated in distinct sets of CRC samples

A. Observation in two discovery cohorts

In the two discovery cohorts, CEA⁺EV (**A**) and soluble CEA levels (**B**) are markedly increased in samples from stage IV CRC compared to normal samples. In both cohorts, a subset of stage IV CRC samples (3/15) (**C**), and (4/23) (**D**) showed elevated CEA⁺EVs but low soluble CEA.

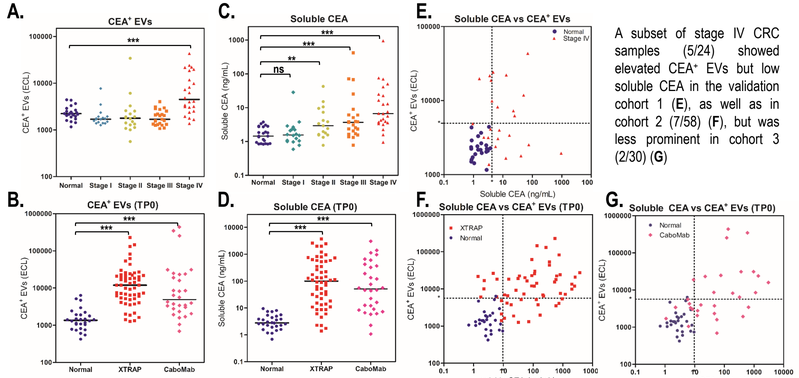


*Mann Whitney test, 2tailed, *:p<0.05, **:p<0.01, ***:p<0.001*

Quadrants: Median (normals) +3xStDev (normals)

B. Confirmation of the observations in three validation cohorts

Elevated CEA⁺ EV (**A**) and (**B**),and soluble CEA (**C**) and (**D**) levels were confirmed in stage IV CRC individuals in the validation cohort 1, as well as in validation cohorts 2 and 3, respectively. Only the timepoint before treatment (TP0) is illustrated for the two longitudinal cohorts.



A subset of stage IV CRC samples (5/24) showed elevated CEA⁺ EVs but low soluble CEA in the validation cohort 1 (**E**), as well as in cohort 2 (7/58) (**F**), but was less prominent in cohort 3 (2/30) (**G**)

*Mann Whitney test, 2tailed, *:p<0.05, **:p<0.01, ***:p<0.001*

Quadrants: Median (normals) +3xStDev (normals)

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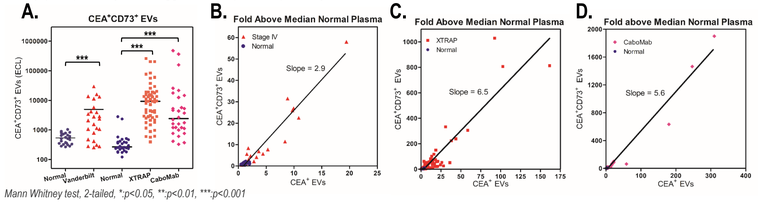
References:

1. Strickler et al., BMC Cancer, 2019
2. Strickler et al., The Oncologist, 2021

5 CEA⁺CD73⁺ EV assay increases specificity of detection of CRC-derived EVs

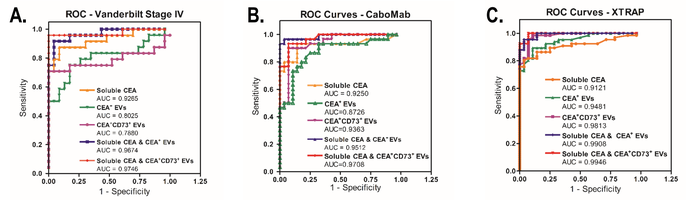
CD73⁺ EVs were significantly elevated in all three validation cohorts, with a high correlation with CEA⁺ EVs (data not shown). CEA⁺CD73⁺ double-positive EVs were significantly elevated in all three validation cohorts (**A**). A comparison of the CEA⁺ and CEA⁺CD73⁺ EV assays shows a better separation of CRC samples from normal samples for the double-positive EV assay, of ~3-fold, 6.5-fold and 5.6-fold in the validation cohort 1 (**B**), 2 (**C**) and 3 (**D**), respectively. This suggests an improved specificity for CEA⁺CD73⁺ EV relative to CEA⁺EVs.



*Mann Whitney test, 2-tailed, *:p<0.05, **:p<0.01, ***:p<0.001*

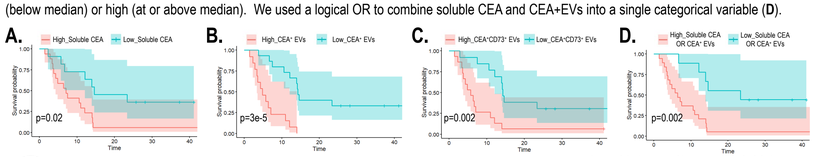
6 Detection of CEA⁺ EVs in addition to soluble CEA improves Stage IV CRC sample identification

The linear combination of soluble CEA with CEA⁺EVs or CEA⁺CD73⁺EVs improved specificity and sensitivity for separation of stage IV CRC from normal samples in validation cohorts 1 (**A**), 2 (**B**) and 3 (**C**), as shown in the receiving operating characteristics (ROC) curves.



7 Combined detection of CEA+ EVs and soluble CEA predicts prognosis of subjects with refractory CRC tumors

Cox proportional hazards regression was used to investigate the association between CEA⁺EVs and/or soluble CEA measured at the first timepoint of the studies (before treatment) and the observed overall survival time (OS) and progression free survival time (PFS). For the XTRAP Cohort, univariate Cox analysis showed significant negative association between OS and both CEA⁺ EVs (HR=1.3, p=0.01), and CEA⁺CD73⁺ EVs (HR=1.2, p=0.0055) but not soluble CEA. For CaboMab cohort, univariate Cox analysis showed significant negative association between OS and CEA⁺ EVs (HR=1.5, p=5.6E-05), CEA⁺CD73⁺ EVs (HR=1.3, p=0.00045) and soluble CEA (HR=1.3, p=0.00075) and between PFS and CEA⁺ EVs (HR=1.4, p=0.0023), CEA⁺CD73⁺ EVs (HR=1.3, p=0.0022). The significant covariates were combined in various multivariate Cox models, and for the CaboMab cohort, the best model combined soluble CEA and CEA⁺ EVs (Concordance = 0.78, p=3E-05). Kaplan-Meier curves for OS in CaboMab cohort are shown for significant variables identified by Cox analysis: soluble CEA (**A**), CEA⁺ EVs (**B**), CEA⁺CD73⁺ EVs (**C**) with continuous variables categorized as low (below median) or high (at or above median). We used a logical OR to combine soluble CEA and CEA+EVs into a single categorical variable (**D**).



Log rank test was used to compare survival between groups

8 Conclusion

In five independent stage IV CRC cohorts, we showed that CEA⁺EVs were detectable in a subset of individuals including some (~10-20%) that did not exhibit elevated plasma levels of soluble CEA. We also demonstrated that an ultrasensitive assay for CEA⁺CD73⁺ double positive EVs may represent an improved method for quantifying colorectal tumor-derived EVs by improving specificity relative to CEA⁺ EVs. The combination of CEA⁺ EV assays with existing methods for soluble CEA measurement showed improved discrimination between advanced CRC and control samples. Elevated levels of both EVs and soluble forms of CEA at the onset of treatment correlated with poor survival for both longitudinal sample sets, and multivariate analysis showed that the most significant prognosis model includes the combination of soluble and CEA⁺ EVs. This retrospective study suggests that assaying both soluble and EV-associated forms of CEA in plasma may improve the value of CEA measurement in CRC and may help overcome the current limited accuracy of CEA as a prognosis factor for CRC.

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