Abstract # 1500

Development of a Quantitative Immunofluorescence Imaging Assay to Assess β-catenin Translocation and Multiplex Biomarkers for Epithelial-Mesenchymal Transition (EMT) in Tumor Tissues

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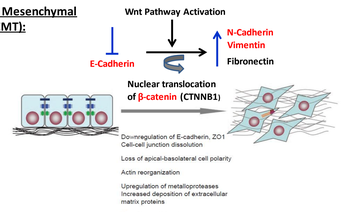
Background

Wnt/β-catenin signaling and cadherin-mediated cell adhesion are two processes that promote cell proliferation and epithelial-mesenchymal transition (EMT) of carcinomas. Therapeutic inhibitors of Wnt-induced tumor activation prevent stabilization and nuclear translocation of β-catenin and putatively lead to inhibition of tumor metastasis by upregulation of E-Cadherin and downregulation of N-Cadherin and Vimentin.

Epithelial to Mesenchymal Transition (EMT):

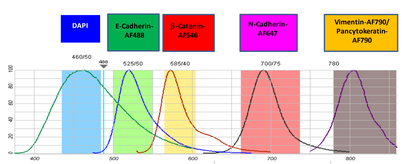


Methods

A multiplex quantitative immunofluorescence assay (qIFA) was developed to assess changes in levels of EMT biomarkers (E-Cadherin, N-Cadherin, Vimentin/Pancytokeratin) as well as monitor intracellular translocation of β-catenin in tumor tissues. A panel of highly specific monoclonal antibodies against EMT targets, directly conjugated to various fluorophores, was optimized for multiplex staining of FFPE tissues using colorectal (CRC) and triple-negative breast (TNBC) cancer cell lines and xenograft tissues as tumor controls for target expression. Rat kidney was used as a positive control for endogenous biomarker expression in tissues. Image acquisition was performed with confocal and epifluorescence microscopy. Image segmentation and quantitative multiplex analysis were performed using NIS Elements and Definiens Tissue Studio software to obtain total mean integrated signal per biomarker within a tissue region of interest.

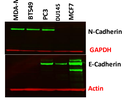
EMT Multiplex IFA Assay Panel

Target Mab	Source	Host, Clonality	Clone ID	Species Reactivity	Target Specificity	Chamber Slides (ICF) 10% NBF; Triton-X	FFPE (IHF) 10% NBF HIER Citrate
E-Cadherin-AF488	BD Biosciences	Ms, Mab	Clone 36	Hu, Rat, Ms, Dg	✓ (Western, IFA)	✓	✓
N-Cadherin-AF647	BD Biosciences	Ms, Mab	Clone 32	Hu, Rat, Ms, Chk	✓ (Western, IFA)	✓	✓
β-Catenin-AF546 (CTNNB1)	Epitomics	Rb, Mab	E247D	Hu, Rat, Ms	✓ (Western, IFA)	✓	✓
Vimentin-AF790	Abcam	Ms, Mab	V9	Hu, Dg, Rat, Ch	✓ (Western, IFA)	✓	✓
Pancytokeratin-AF790	Santa Cruz	Ms, Mab	C11	Hu, Ms, Rat	✓ (IFA)	✓	✓

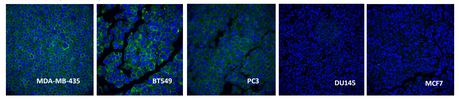


E-Cadherin and N-Cadherin Antibody Specificity Validation:

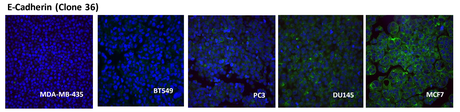
Validation of monoclonal antibody specificity on epithelial and mesenchymal tumor cell models by Immunofluorescence and Western Analysis



N-Cadherin (Clone 32)



E-Cadherin (Clone 36)

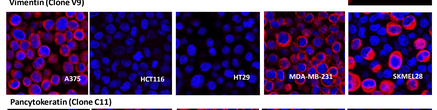


Results

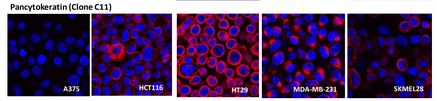
Vimentin and Pancytokeratin Antibody Validation

Validation of monoclonal antibody specificity on epithelial or mesenchymal tumor cell models by Immunofluorescence Confocal Microscopy and Western Analysis

Vimentin (Clone V9)



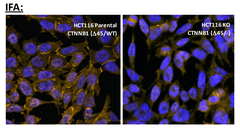
Pancytokeratin (Clone C11)



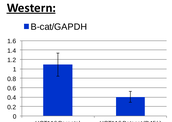
β-catenin Antibody Validation in CTNNB1 Knockout Cell Line

Validation of β–catenin antibody specificity on parental vs. CTNNB1 heterozygous KO HCT116 cell lines by confocal immunofluorescence and Western analysis

IFA:

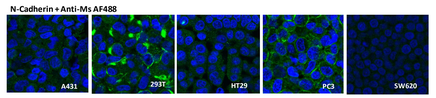


Western:

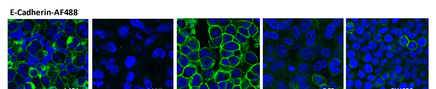


Staining Validation of Various EMT Targets on Control FFPE Tumor Cell Models by Confocal Microscopy

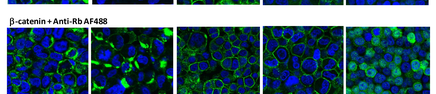
N-Cadherin + Anti-Ms AF488



E-Cadherin-AF488



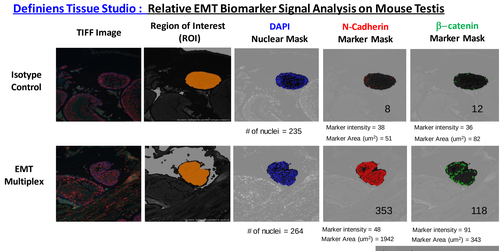
β-catenin + Anti-Rb AF488



Summary of EMT Multiplex IFA Staining on CRC, TNBC, and Melanoma FFPE Tumor Cell Models

Cell Line	E-Cadherin	CTNNB1	N-Cadherin	Vimentin	Tumor Type
SW620	+ (Patchy)	+++ Membrane, Cytoplasm Nucleus	-	+ (Patchy)	CRC
Colo201	+++	+++ Membrane, Cytoplasm	-	-	CRC
Colo205	+++	+++ Membrane, Cytoplasm	-	-	CRC
DLD-1	++	+++ Membrane, Cytoplasm	-	-	CRC
HT29	++	++ Membrane	-	-	CRC
HCT116	-	+ Membrane	-	-	CRC
MDA-MB-231	-	+	-	+++	TNBC
BT549	-	+	+++	+++	TNBC
A375	-	++	++	++	Melanoma
MDA-MB-435	-	++ Membrane	+++	++	Melanoma
SKMEL28	+	+ Membrane	+	++	Melanoma

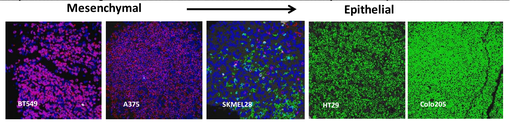
Definiens Tissue Studio : Relative EMT Biomarker Signal Analysis on Mouse Testis



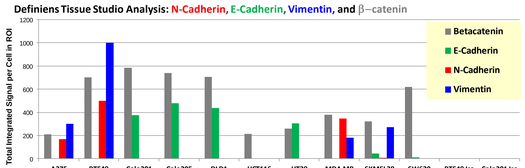
Total integrated Signal per cell in ROI = (Marker intensity) X (Marker area) * / (# of nuclei)

*Average marker intensity and area were thresholded based on isotype control-stained adjacent tissue section

Multiplex IFA Staining Validation of EMT Biomarkers in Mesenchymal and Epithelial Tumor Models

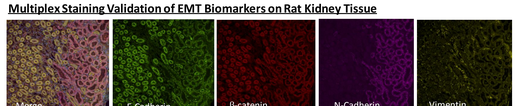


Definiens Tissue Studio Analysis: N-Cadherin, E-Cadherin, Vimentin, and β—catenin

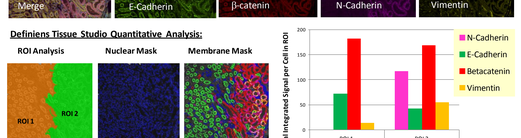


Colorectal (CRC), melanoma or triple-negative breast cancer (TNBC) tumor pellets were stained with the validated panel of EMT multiplex IFA antibodies and images were analyzed by Definiens Tissue Studio software to quantify biomarker levels of N-Cadherin (red), E-Cadherin (green), Vimentin (blue), and β–catenin (gray). Bar graphs show the total integrated signal per cell in the region of interest (ROI), which reflects the variations in EMT biomarkers evident in particular mesenchymal to epithelial phenotypes.

Multiplex Staining Validation of EMT Biomarkers on Rat Kidney Tissue

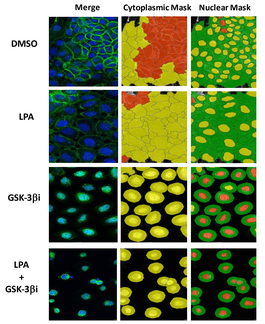


Definiens Tissue Studio Quantitative Analysis:

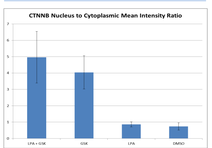


Rat kidney tissue was stained with the validated panel of EMT multiplex IFA or isotype control antibodies and images were analyzed by Definiens Tissue Studio software to determine relative levels of N-Cadherin (pink), E-Cadherin (green), β–catenin (red) and Vimentin (blue) biomarkers. Rat kidneys endogenously express each biomarker (tissue expression varies by location and relative levels). Rat kidney tissue could be used as an assay calibrator for reagent lots and for validation of the multiplex staining method. The upper panel shows expression of each biomarker within different epifluorescent channels. Bar graphs show the total integrated signal per cell in the ROI, corrected against the background obtained from an isotype control-stained adjacent tissue section.

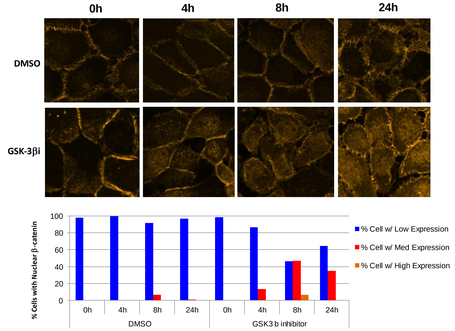
Fitness For Purpose Assay Validation: β–catenin Nuclear Translocation *In Vitro*



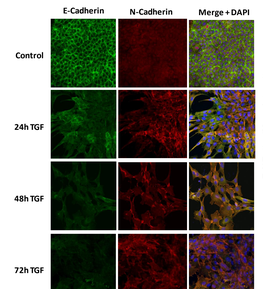
Fitness for purpose evaluation of the biomarkers was performed in vitro to demonstrate nuclear translocation of β-catenin.
A431 squamous epithelial cell lines treated with lysophosphatidic acid (LPA) induced adherens junction (AJ) dissociation of membrane E-Cadherin and β-catenin, while co-treatment with specific inhibitors to glycogen synthase kinase-3β (GSK-3β) induced a 5-fold increase in the nuclear- to-cytoplasmic mean intensity ratio of β-catenin after 24h.

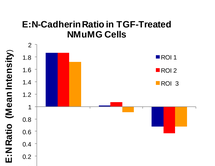


Time course for β–catenin Nuclear Translocation *In Vitro*



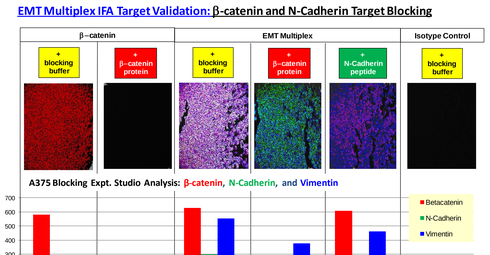
Fitness For Purpose Assay Validation: E-Cadherin to N-Cadherin Membrane Intensity Ratio Quantitation





TGF-β-treatment of the NMuMG mouse epithelial cell line induced a significant decrease in E- to N-Cadherin membrane intensity ratio after 24 and 72h in vitro, determined from images segmented and analyzed with NIS Elements software.

EMT Multiplex IFA Target Validation: β-catenin and N-Cadherin Target Blocking

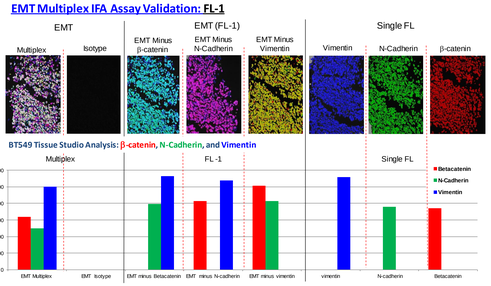


Recombinant Protein and Peptide Blockers of EMT Antibodies

EMT Target	Protein or Peptide	Source	Soluble/ Purity	His Tag removed	Molecular Weight	Molar Excess For Blocking	Total µg
N-Cadherin	42-mer Peptide	NEP	95%	N/A	4.7 k	200 fold	49.1 µg
β—catenin	Rec. FL Protein	ANL	Yes/SDS	Yes	87.8 kD	10 fold	37.1 µg
E-Cadherin	Rec. C-terminus (a.a. 731-882)	ANL	Yes	Yes	17.4 kD	20 fold	18.7 µg
Vimentin	Rec. FL Protein	Abcam	9M Urea Dialyzed into PBS	Yes	54.0 kD	10 fold	28.8 µg

Target validation was performed by incubating each antibody with molar-fold excess concentrations of the recombinant protein or peptide corresponding to each target at 4 ºC overnight then staining tissue sections with the EMT multiplex antibody mixture. Complete blocking of target binding was observed when the signal from each EMT antibody was blocked by the overexpressed target either alone or in combination with the multiplex of EMT antibodies, demonstrating specificity of each Mab to its tissue target.

EMT Multiplex IFA Assay Validation: FL-1



Fluorescence Minus One experiment compares the fluorescence signals from each channel when all 4 antibodies are mixed together (EMT multiplex) or any one of the antibodies is left out (FL-1), versus when only one antibody is used for staining tissue sections (single FL). This experiment determines whether any of the antibodies interact within the multiplex mixture during staining (antibody reactivity) as well as determines any potential fluorescence overlap or interference that may arise based on the multiplex IFA platform (signal interference).

Conclusions

We have developed a quantitative imaging assay that integrates multiplex changes in EMT biomarkers to assay the pharmacodynamic activities of potential targeted agents currently in development at the National Cancer Institute (NCI). This assay could also be applied for retrospective analysis of clinical samples to document drug-induced changes in tumor phenotypes. Funded by NCI Contract No. HHSN261200800001E.