

Evaluation of Immune Checkpoint Marker Co-expression Profiles in the Tumor Microenvironment Kai Wilkens¹, Jeff Kim³, Na Li², Xiao-Jun Ma³, Emily Park³

Background

Recent clinical successes with immune checkpoint blockades have provided promising immune-based therapeutic approaches for controlling malignancy. While therapeutic antibodies against CTLA-4 and PD-1/PD-L1 have resulted in potent and durable clinical responses in many patients, there still remains an urgent need to develop biomarker based assays to identify patients who may benefit from these approaches while avoiding toxicity and without compromising efficacy. While gene and protein expression of immune cell and checkpoint markers can be obtained through traditional techniques, insight regarding spatial and cell-specific expression within the tumor microenvironment is lacking, particularly in regards to secreted proteins with key immune functions. RNAscope® is an advanced *in situ* hybridization assay that allows for the visualization of single-cell gene expression, targeting mRNA sequences directly in tissues. The probes designed for this approach permit the detection of secreted markers, including cytokines and chemokines

Design

In this investigation of 60 archived formalin-fixed paraffin embedded (FFPE) non-small cell lung cancer (NSCLC) biopsies, expression profiles of immune checkpoint markers and immune functional molecules were evaluated in the tissue environment by RNAscope® ISH assay (Figure 1). Each of the tumors displayed distinct co-expression patterns of immune checkpoint markers as well as varying levels of cytokines (including IFNy and TGF β 1) and chemokines, and immune cell markers.

Tissue microarray (TMA) preparation and RNAscope analysis: FFPE tissues of 60 Lung carcinoma samples were assembled as TMAs as shown in Figure 2. Serial sections of slides were subjected to gene expression analysis by applying manual RNAscope 2.5 HD duplex assay (Figure 1).

Immunohistochamistry (IHC) analysis of PD-L1: FFPE tissue sections of TMAs were assays for the expression of PD-L1 protein using two antibody clones by NeoGenomics Laboratories.

Imaging and quantitation: Images were acquired using a Leica Biosystems Aperio AT2 Digital Pathology Slide Scanner and quantified using quantified by the HALO[™] imaging analysis algorithm (Indica Labs).

Figure 1. RNAscope[®] technology and workflow



Figure 2. Human NSCLC TMA schematic and HALO analysis

Dialing the algorithm

A CONTRACTOR

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Results

Figure 3. PD-L1 expression profiles in 56 NSCLC tumor and 4 normal lung tissues: comparison of RNAscope ISH and IHC



immune lineage, checkpoint-related, chemokine/cytokine, or tumor markers.

signify tissues that were folded over or missing more than 50%.

			101	202	201	252	205	1 4 2	252	1 \ 4	102	104	101	150	102	204	102	1 ^ 7	102	202	252	1 D 1	100	1 5 5	204	104	202	202	1.0.4	1 DE	202	
		CD45	101	202	14.05	20.00	200	1A3	213	1A4	14.00	22.27	27.06	22.25	57.49	17.11	25.54	1AZ	12.86	12.90	252	12.10	17.59	162	204	11.02	20.42	205	21.10	2.91	20.60	-
	ne	CD45	17.83	17.88	21.02	29.00	26.02	18.60	16.53	47.20	25.08	18 71	11 75	10.08	23.24	12.76	13 17	7.02	45.60	8.84	8.14	19.19	10.23	10.00	18.16	10.34	12.62	1 70	10.77	9.63	29.00	
	m		67.26	66.06	70.80	69.19	21.65	61.01	10.55	72.25	56.22	26.90	60.16	12.00	23.24	11.66	15.17	50.74	17.71	52.26	28.09	19.04	26.21	6.40	27.45	25 21	51.46	22.24	26.44	17.66	57.09	2
	<u>=</u>	EOVP3	1/ 89	11 23	5 15	28.24	1 29	13.66	48.55	14.45	16.10	16.24	5.68	16.81	12.17	0.94	7.28	7 20	45.11	2.88	1 90	8.67	13.09	8.29	27.43	8.08	2 75	6 35	10.45	3 22	0.40	2
		TOXID	14.05	11.25	5.15	20.24	1.25	15.00	J.22	14.45	10.10	10.24	5.00	10.01	12.40	0.94	7.20	7.20	15.02	2.00	1.50	0.07	15.05	0.25	20.14	0.00	2.75	0.55	10.45	5.22	0.40	
		PDI 1A	52.83	51.04	36.93	36.27	31.23	21.98	21.97	21.82	20.80	20.74	20.52	20.45	19.18	19.10	17.97	17.94	17.54	15.64	14.64	14.25	13.76	13.31	13.22	13.12	12.65	12.63	12.47	12.05	11.19	1
		PD1	6.84	2.49	5.64	9.12	x	4.43	4.80	5.63	11.11	5.42	1.99	8.01	10.70	6.97	9.44	5.00	9.22	2.19	11.75	2.74	4.59	3.03	x	x	7.30	2.22	10.61	1.90	6.65	
		PDL2	11.94	2.98	5.97	15.00	15.73	6.25	5.13	5.29	9.17	3.46	4,91	9,94	13.28	5.90	9.63	5.10	7.32	4.00	9,90	3.33	5.20	3.94	6.23	1.72	10.72	1.63	9.58	2.36	4.80	
		LAG3	47.59	12.71	19.76	29.61	21.89	27.96	26.38	29.81	33.52	6.54	16.11	31.88	18.69	10.32	19.53	10.50	28.95	16.24	5.97	44.44	13.49	21.73	20.87	17.04	14.46	X	14.35	13.74	41.44	
	¥	HAVCR2	19.11	9.45	15.22	25.62	22.83	18.34	13.48	22.69	16.01	х	14.90	14.19	22.77	х	22.05	12.03	10.43	16.66	10.13	11.49	x	10.98	8.17	x	12.76	10.28	20.87	4.17	14.44	
	30İr	CTLA4	x	4.31	8.60	15.75	12.16	16.78	11.75	9.10	17.66	9.64	5.38	37.12	25.58	10.11	21.78	9.87	25.46	6.57	9.62	9.29	x	8.70	7.19	6.56	12.12	6.03	19.88	2.85	11.89	
	sckl	ICOS	20.10	10.82	23.44	17.68	25.07	17.89	14.90	19.02	23.76	34.02	4.36	24.58	21.12	12.30	16.13	9.19	20.66	10.40	5.39	17.34	12.40	14.20	13.43	8.08	11.77	10.24	10.31	6.93	23.94	
	Ċ	IDO1	11.41	3.05	7.03	3.84	17.07	3.24	4.00	8.18	10.08	2.91	1.29	5.57	4.96	4.64	2.53	1.91	10.54	2.69	5.91	3.33	4.49	2.19	6.24	2.65	6.61	1.56	6.33	1.19	5.40	:
		TNFRSF9	15.99	5.43	10.25	10.95	10.99	14.59	3.50	10.97	17.15	3.71	3.95	8.36	8.72	3.69	4.24	5.27	11.95	5.75	4.42	7.25	5.10	4.85	5.17	2.38	х	2.08	6.28	1.97	10.61	:
		TNFRSF18	22.49	7.65	23.85	13.27	13.19	17.32	8.48	х	20.98	5.71	6.67	13.22	х	6.56	5.55	17.80	17.49	20.17	5.86	6.35	8.90	5.74	7.51	6.59	17.52	3.43	9.71	6.66	19.29	1
		VTCN1	5.41	5.41	36.10	4.39	12.88	4.28	2.28	4.24	8.89	7.94	1.07	8.46	9.62	х	6.41	4.83	9.67	32.69	8.94	х	5.07	1.56	6.29	х	20.04	3.35	13.81	х	29.86	
		CD80	х	12.59	18.57	21.31	31.81	22.69	15.81	28.75	29.46	42.36	9.02	28.82	29.87	21.29	25.40	35.55	25.41	15.27	7.85	10.48	16.30	25.96	17.06	16.98	13.31	14.16	16.46	20.01	19.58	9
												-	-			-																
	a	IFNγ	15.18	2.05	8.78	4.99	11.09	8.77	11.04	11.87	18.43	13.60	4.64	18.05	9.35	6.10	6.39	х	15.23	6.45	3.65	12.99	8.89	9.20	6.01	8.53	8.21	2.19	8.96	5.46	8.15	
	Ę.	TGFβ1	x	37.68	51.04	31.96	38.73	х	29.31	х	x	х	35.45	33.41	60.68	12.61	37.77	42.29	34.85	42.82	7.89	15.94	18.00	39.72	20.63	29.57	23.80	25.03	24.07	52.63	37.27	1
	ê	IL10	5.06	2.71	6.95	2.91	14.68	4.95	2.71	5.14	7.96	3.55	1.18	9.41	9.86	х	7.71	6.82	12.28	1.89	х	2.19	4.69	2.50	4.97	1.58	8.15	1.90	10.07	2.80	3.62	
	he	IL15	22.77	6.68	21.07	11.58	27.16	18.10	16.65	23.09	18.37	11.47	18.28	13.14	14.08	16.61	7.55	13.76	17.33	4.19	7.96	2.26	20.09	6.02	6.38	6.57	13.10	3.06	11.68	1.52	5.78	-
	e/C	CCL2	10.99	1.25	5.66	2.26	8.11	3.92	1.63	5.92	9.94	2.33	1.04	4.21	х	3.09	2.03	х	8.95	1.60	3.03	2.89	х	1.81	4.04	х	6.69	1.34	3.75	0.64	4.56	(
	kin	CCR2	10.41	3.32	3.78	1.03	8.13	4.00	2.03	7.83	8.59	6.83	10.73	4.22	х	3.86	3.15	x	8.21	2.01	4.39	0.42	х	3.30	4.31	х	7.59	2.87	3.19	0.60	1.29	
	χ	CCR4	17.61	9.35	12.47	5.37	15.18	10.25	5.71	9.46	11.93	х	55.35	7.92	9.79	6.97	8.63	4.71	х	14.66	14.51	1.32	13.44	5.65	5.64	х	14.79	4.79	4.03	1.14	9.94	8
	0	CSF1	35.06	30.79	37.87	33.93	49.03	50.82	31.84	47.31	40.45	30.11	47.97	33.68	33.35	29.91	29.16	38.72	26.50	39.08	19.95	31.05	38.14	28.60	х	24.76	28.74	10.23	29.95	12.48	33.31	2
_																																
	her	EGFR	47.74	13.44	43.04	16.59	27.52	30.04	31.52	x	11.43	x	44.88	10.78	х	36.76	4.28	38.76	13.00	45.78	15.92	10.85	26.79	66.48	17.46	x	39.40	13.04	×	80.42	33.91	1
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Results

-Expression profiles from serial sections in the selected tumor region









FFPE tissues by RNAscope[®].

Conclusions

- characterization of specific target RNA detection within the morphological context.
- analysis software.
- different checkpoint pathways.

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Nov 29 – Dec 2, 2016

Figure 5. Co-expression profiles of immune checkpoint markers visualized in a tumor microenvironment (core 2E2)

In this study, the expression profiles of immune checkpoint and functional markers were evaluated in the tumor microenvironment of 60 NSCLC

• The immune infiltration in the tumor microenvironment, revealed by RNAscope ISH, is readily quantifiable in addition to visual

A large proportion of NSCLC cases (>80%) were identified as PD-L1 positive by RNAscope® ISH assay. In comparison with IHC, the RNAscope[®] assay revealed a dynamic range of PD-L1 expression with higher sensitivity, and the assay readily quantifiable by image

Detection of co-expression profiles of multiple checkpoint markers in the TME revealed a unique yet heterogeneous pattern of expression in different tumor tissues. This information may reveal potential insight into combination therapies targeted against