

BACKGROUND & OBJECTIVE

- Early-stage NSCLC patients are at much higher risk of developing second primary tumors (2P-T), which can be difficult to distinguish from recurrent tumors (REC-T), and require different clinical management.
- We previously identified spatial RNA and protein biomarkers (MS1 2P predictors) in primary tumors (2P Primary) associated with subsequent primary tumor development (2P-T) compared to primary tumors without subsequent tumor development (NONE)¹.
- Here, we evaluated the concordance of spatial RNA and protein biomarkers between 2P Primary and matched 2P-T by (a) validating all 2P predictors in 2P-T and (b) cross-validating newly identified 2P-T markers in 2P Primary tumors.

METHODS 1. SPATIAL PROFILING

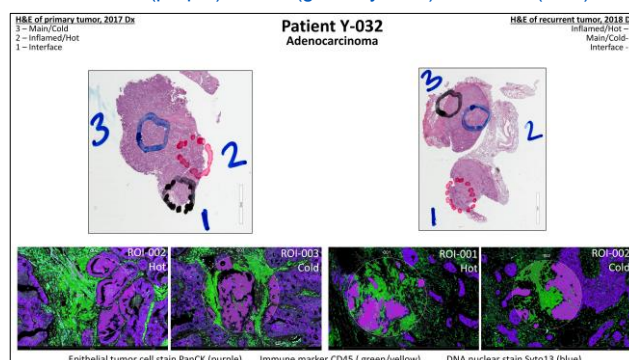
- TABLE 1 & FIGURE 1

- Profiled RNA & protein expression across 312 ROIs from 52 tumors, including 7 matched primary 2P/2P-T pairs (5-µm FFPE tumor sections) using GeoMx DSP.
- RNA profiling of Immune Pathways Panel (73 genes); protein profiling 7 GeoMx modules of 71 proteins.
- ROIs were morphologically classified as Hot/inflamed or Cold/uninflamed and segmented into PanCK+ tumor vs. CD45+ tumor immune microenvironment (TIME) compartments by immunofluorescence.

TABLE 1: Study Cohort & Samples for RNA & Protein Analyses

Total (n = 52)	Group	Number of samples	
		RNA (n=52)	Protein (n=48)
2P (n = 38)	REC	8	7
	2P	8	8
	EQU	5	4
2P-T (n = 14)	None	17	16
	Rec-T	2	2
	2P-T	7	7
	Equ-T	5	4

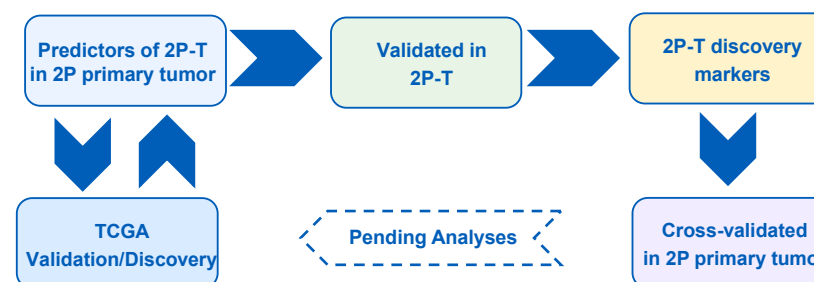
Figure 1: Representative paired 2P Primary & 2P-T samples: H&E-guided ROIs & GeoMx morphology staining; PanCK (purple), CD45 (green/ yellow), SYTO13 (blue).



METHODS 2: STATISTICAL ANALYSIS AND VALIDATION WORKFLOW

- Compared to NONE, GEE and GLMER statistical models were used to identify markers associated with 2P-T, accounting for repeated spatial measurements within patients.
- 2P-T discovery markers were cross-validated against 2P Primary Cox model results
- TCGA LUAD/LUSC datasets^{2,3,4} provided external support for MS1 2P predictors and enabled discovery of additional survival associated markers

Figure 2: Bidirectional Biomarker Validation Between 2P Primary and 2P-T Tumors with External TCGA Support



RESULTS 1

Reciprocal Validation of Spatial RNA and Protein Biomarkers

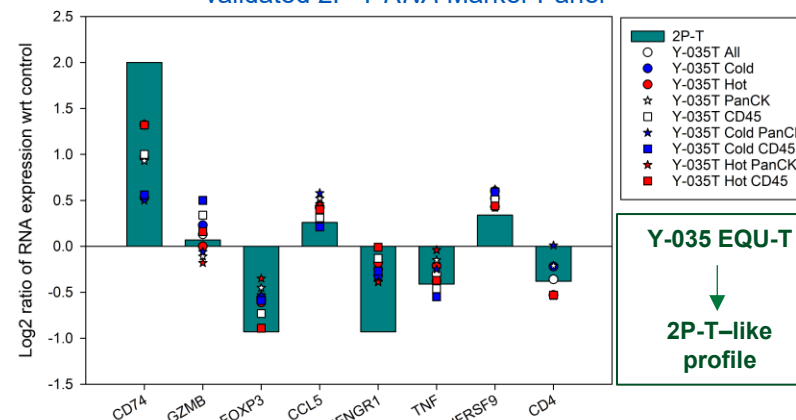
TABLE 2: Summary of Validated and Cross-Validated RNA and Protein Markers

Marker Category	Markers (RNA, protein)	Main Finding
2P predictors in primary tumor validated by matched secondary tumors 2P-T	CD274/PD-L1, FAS, HAVCR2, MS4A1/CD20, PDCD1, PTEN	Risk direction retained; several markers expanded spatially
2P-T RNA discovery markers cross-validated in matched primary tumor	Risk: CCL5, CD4, FOXP3, GZMB, IFNGR1, TNF, TNFRSF9; Protective: CD74	Concordant associations in matched primary tumor Cox models
2P-T protein discovery markers cross-validated in matched primary tumor	ARG1, EPCAM, LAG3, 4-1BB	Concordant associations in matched primary tumor
Cross-omic highlights	CD274/PD-L1, MS4A1/CD20	RNA-protein concordance supports shared 2P/2P-T biology

Application of the Validated 2P-T RNA Marker Panel to an Equivocal Tumor – Figure 3

- Distinguishing 2P-T from recurrent tumors (REC-T) is often complicated by morphologically and pathologically indeterminate “equivocal” secondary tumors (EQU-T)
- Using biomarkers identified in REC or 2P primary tumors that predict the development of REC-T and 2P-T, we previously aligned four primary equivocal tumors with either REC (n=3) or 2P (n=1, Y-035).
- Here, we assessed whether Y-035T, the matched secondary tumor of Y-035 EQU-T, aligned with 2P-T using the eight 2P-T discovery markers shown in table 2.

Figure 3: Alignment of an equivocal secondary tumor with validated 2P-T RNA Marker Panel



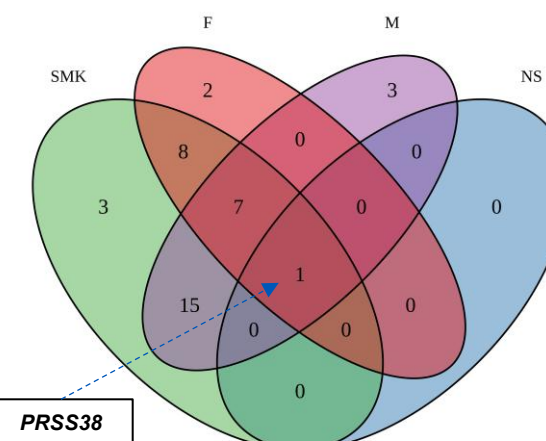
- Figure 3 shows that the expression of these markers in Y-035T strongly aligns with 2P-T, further supporting our previously published predictive model that identified Y-035 as a primary tumor that would give rise to a 2P-T.

RESULTS 2 – FIGURE 4

External Validation & Exploratory Analysis: TCGA LUAD/LUSC

- TCGA LUAD/LUSC overall-survival analyses supported the MS1 2P predictors **MS4A1** and **PDCD1**.
- Additional TCGA discovery markers were validated in 2P Primary tumors (Figure 4).

Figure 4: Subgroup-Specific Overlap of Significant TCGA mRNA Markers



DISCUSSION & CONCLUSION

- Spatial RNA and protein biomarkers showed strong concordance between 2P Primary and subsequent 2P-T tumors, with several markers retaining their prediction while expanding into additional spatial compartments.
- Reciprocal validation of 2P-T discovery markers in 2P Primary, together with TCGA support for **MS4A1** and **PDCD1**, strengthens the evidence for a reproducible 2P-T biomarker signature.
- Application of the validated RNA-marker panel to an equivocal tumor demonstrated a 2P-T-like expression profile, supporting its potential utility for risk stratification and classification of clinically uncertain secondary tumors.

REFERENCES

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- TCGA-LUAD: TCGA Research Network. *Nature*. 2014;511:543–550.
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- TCGAbiolinks: Colaprico A, et al. *Nucleic Acids Res*. 2016;44:e71.