

Supplementary table S1. Baseline characteristics of the immunotherapy untreated NSCLC cohorts

	YTMA423	CIMA-CUN
Characteristic	N (%)	N (%)
Total	252	124
Gender	98 (39)	98 (79)
Male	154 (61)	26 (21)
Female		
Age		
<70 yo	141 (55.9)	88 (71)
≥70 yo	111 (44.1)	36 (29)
Smoking history		
Never smoker	31 (12)	15 (12)
Current smoker	62 (25)	28 (22.7)
Former smoker	159 (63)	81 (65.3)
Histology		
Adenocarcinoma	174 (69)	67 (54)
Squamous-cell carcinoma	66 (26)	46 (37.1)
Large-cell carcinoma	6 (2.4)	5 (4)
Other	5 (2)	6 (4.9)
Stage		
I	200 (79)	55 (44.4)
II	37 (14.7)	30 (24.2)
III	12 (4.8)	34 (27.4)
IV	3 (1.2)	5 (4)

Supplementary Table S2. Human IO DSP panels for protein detection used in this study

Core/Module	Target	YTMA471 cohort	H12O_ITX1 cohort	CIMA-CUN cohort
Immune Cell Profiling Panel				
Human Protein Core	Beta-2-microglobulin	X	X	X
	CD11c	X	X	X
	CD20	X	X	X
	CD3	X	X	X
	CD4	X	X	X
	CD45	X	X	X
	CD56	X	X	X
	CD68	X	X	X
	CD8	X	X	X
	CTLA4	X	X	X
	Granzyme B (GZMB)	X	X	X
	Ki-67	X	X	X
	PD-1	X	X	X
	PD-L1	X	X	X
	Pan-cytokeratin	X	X	X
	HLA-DR	X	X	X
	SMA	X	X	X
	Fibronectin	X	X	X
	6 Controls (Histone H3, S6, GAPDH, Mouse IgG1, Mouse IgG2a, Rabbit IgG)	X	X	X
IO Drug Target Panel				
Human Protein Module	4-1BB	X	X	X
	ARG1	X	X	X
	B7-H3	X	X	X
	GITR	X	X	X
	IDO1	X	X	X
	LAG3	X	X	X
	OX40L	X	X	X
	STING	X	X	X
	TIM-3	X	X	X
	VISTA	X	X	X
Immune Activation Status Panel				
Human Protein Module	CD127	X	X	X
	CD25	X	X	X
	CD27	X	X	X
	CD40	X	X	X
	CD44	X	X	X
	CD80	X	X	X
	ICOS	X	X	X
	PD-L2	X	X	X

Cell Death Panel				
Human Protein Module	BAD	X		
	BCL6	X		
	BCLXL	X		
	BIM	X		
	CD95/Fas	X		
	GZMA	X		
	P53	X		
	PARP	X		
	Cleaved Caspase 9	X		
Immune Cell typing Panel				
Human Protein Module	CD14	X		
	CD163	X		
	CD34	X		
	CD45RO	X		
	CD66b	X		
	FAPalpha	X		
	FOXP3	X		
Pan-Tumor Panel				
Human Protein Module	BCL-2	X		
	EpCAM	X		
	ER alpha	X		
	HER2/ERBB2	X		
	MART1	X		
	NY-ESO-1	X		
	PR	X		
	PTEN	X		
	S100B	X		
MAPK Signaling Panel				
Human Protein Module	EGFR	X		
	Pan-RAS	X		
	BRAF	X		
	Phospho-c-RAF (S338)	X		
	Phospho-JNK (T183/Y185)	X		
	Phospho-MEK1 (S217/S221)	X		
	Phospho-p38 MAPK (T180/Y182)	X		
	Phospho-p44/42 MAPK ERK1/2 (T202/Y204)	X		
	P44/42 MAPK ERK1/2	X		
	Phospho-p90 RSK (T359/S363)	X		

Supplementary Table S3. Anti-CD44 antibody clone information

Antibodies	Source	Catalog #	Clone	Isotype	Concentration
CD44	Cell Signaling Technology (CST)	3570	156-3C11	mIgG2a	0.215 ug/ml
CD44	Abcam	ab51037	EPR1013Y	rIgG	0.192 ug/ml
Cytokeratin	DAKO	M351501-2	AE1/AE3	mIgG1	0.157 ug/ml
Cytokeratin	DAKO	Z062201-2	Polyclonal	rIgG	123 ug/ml

Supplementary Table S4. Association of CD44 expression measured by DSP in tumor and immune compartment with Overall Survival (OS) and Progression free Survival (PFS) in YTMA471 cohort

Overall survival (OS)						
Compartment	N	Marker	Univariate HR (95% CI)	P-value	Multivariate HR (95% CI)	P-value
Tumor compartment (panCK+)	54	CD44	0.82 (0.64-1.05)	0,13	NA	NA
Immune compartment (panCK-/CD45+/CD68+)	54	CD44	0.93 (0.58-1.48)	0,8	NA	NA
Progression-free survival (PFS)						
Compartment	N	Marker	Univariate HR (95% CI)	P-value	Multivariate HR (95% CI)	P-value
Tumor compartment (panCK+)	54	CD44	0.76 (0.61-0.96)	0,024	0.68 (0.46, 0.99)	0,043
Immune compartment (panCK-/CD45+/CD68+)	54	CD44	0.84 (0.55-1.28)	0,4	NA	NA

Supplementary tables S5. Impact of tumor cell CD44 expression in progression-free survival (PFS) in the univariate and bi-variate Cox Regression analysis after correcting for PD-L1 tumor proportion score (TPS) in the discovery set (a) and the validation set (b)

a)

Univariate Cox Regression analysis for PFS (YTMA471 cohort)			
Marker	HR	CI 95%	p value
PD-L1 IHC (TPS \geq 1% vs. TPS<1%)	0.53	0.24-1.18	0.14
PD-L1 IHC (TPS \geq 50% vs. TPS<50%)	0.48	0.21-1.09	0.064
Tumor CD44 QIF scores (highest quartile vs. rest)	0.29	0.11-0.75	0.004
Multivariate Cox Regression analysis for PFS (YTMA471 cohort)			
Co-variates	HR	CI 95%	p value
- PD-L1 IHC (TPS \geq 1% vs. TPS<1%)	0.83	0.36-1.91	0.70
- Tumor CD44 QIF scores (highest quartile vs. rest)	0.25	0.09-0.69	0.003
- PD-L1 IHC (TPS \geq 50% vs. TPS<50%)	0.72	0.31-1.67	0.40
- Tumor CD44 QIF scores (highest quartile vs. rest)	0.26	0.10-0.72	0.004

b)

Univariate Cox Regression analysis for PFS (H12O_ITX1 cohort)			
Marker	HR	CI 95%	p value
PD-L1 IHC (TPS \geq 1% vs. TPS<1%)	0.91	0.62-1.34	0.66
PD-L1 IHC (TPS \geq 50% vs. TPS<50%)	0.62	0.38-1.01	0.057
Tumor CD44 DSP counts (highest tertile vs. rest)	0.59	0.39-0.90	0.014
Multivariate Cox Regression analysis for PFS (H12O_ITX1 cohort)			
Co-variates	HR	CI 95%	p value
- PD-L1 IHC (TPS \geq 1% vs. TPS<1%)	0.95	0.65-1.39	0.81
- Tumor CD44 DSP counts (highest tertile vs. rest)	0.59	0.39-0.90	0.015
- PD-L1 IHC (TPS \geq 50% vs. TPS<50%)	0.68	0.41-1.12	0.13
- Tumor CD44 DSP counts (highest tertile vs. rest)	0.62	0.41-0.96	0.032

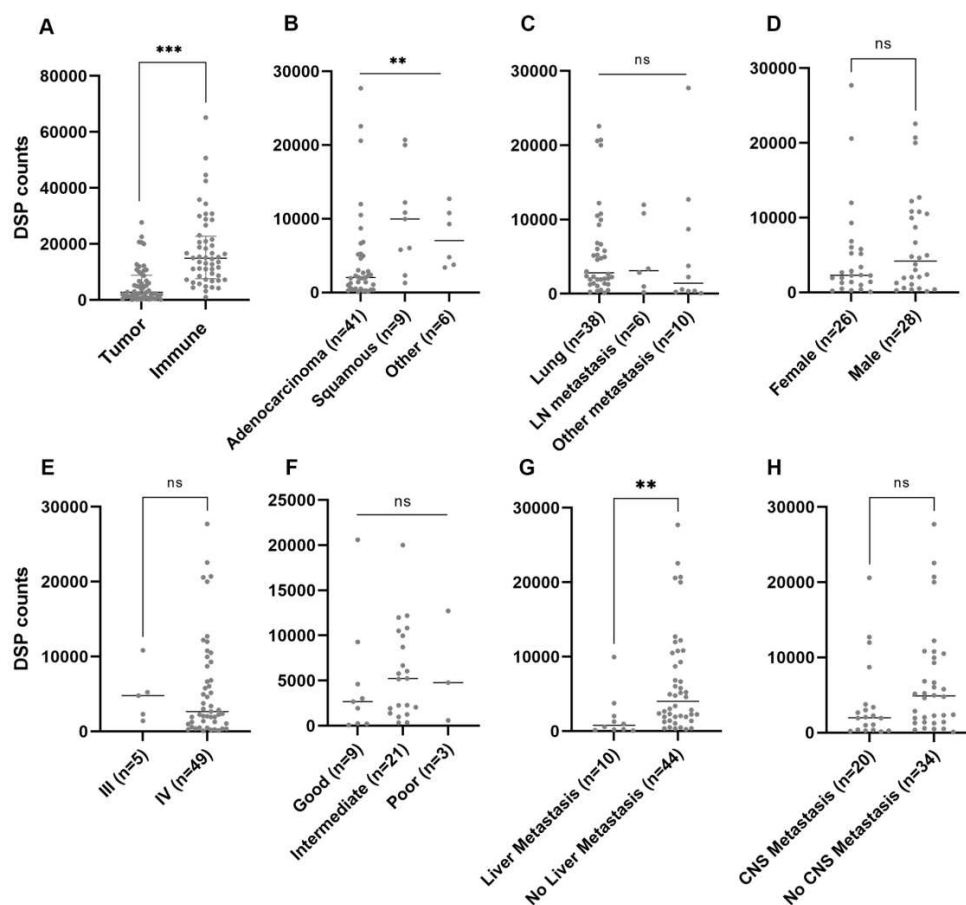
Supplementary Table S6. Correlation between CD44 expression and each individual protein marker all measured in the tumor compartment

H12O_ITX1			CIMA-CUN		
Protein	R ²	P value	Protein	R ²	P value
CD25	0,2432	<0,0001	CD80	0,3155	<0,0001
CD80	0,2038	<0,0001	CD40	0,3085	<0,0001
CD27	0,1429	<0,0001	PD-L1	0,2942	<0,0001
ICOS	0,1127	<0,0001	B7-H3	0,2934	<0,0001
GZMB	0,1071	<0,0001	ICOS	0,2316	<0,0001
CD40	0,09296	<0,0001	Tim-3	0,1968	<0,0001
PD-L2	0,0883	<0,0001	HLA-DR	0,1827	<0,0001
ARG1	0,06669	<0,0001	Beta-2-microglobulin	0,1406	<0,0001
CTLA4	0,058	<0,0001	CD27	0,1308	<0,0001
B7-H3	0,04519	<0,0001	CD3	0,08658	<0,0001
CD127	0,04186	<0,0001	CD8	0,08351	<0,0001
SMA	0,03677	<0,0001	GITR	0,08256	<0,0001
Tim-3	0,02972	<0,0001	VISTA	0,07979	<0,0001
Ki-67	0,02951	<0,0001	CD45	0,07487	<0,0001
LAG3	0,02208	0,0002	LAG3	0,06371	<0,0001
CD4	0,01726	0,0011	4-1BB	0,06155	<0,0001
VISTA	0,01655	0,0014	CD11c	0,05514	0,0002
PanCk	0,01638	0,0015	CD4	0,04975	0,0003
GITR	0,01404	0,0033	CD25	0,04683	0,0005
Fibronectin	0,01272	0,0052	STING	0,0458	0,0006
CD20	0,009499	0,0159	IDO1	0,04073	0,0012
4-1BB	0,007197	0,0359	GZMB	0,03753	0,0019
CD11c	0,006873	0,0403	PD-L2	0,0234	0,0145
CD68	0,005247	0,0733	CD68	0,02306	0,0152
CD45	0,004803	0,0867	PD-1	0,02232	0,017
CD8	0,003201	0,1621	CD127	0,01721	0,0363
PD-L1	0,002959	0,179	Fibronectin	0,01162	0,0858
CD3	0,001606	0,3223	PanCk	0,0107	0,0993
PD-1	0,001513	0,3367	Ki-67	0,008848	0,1341
STING	0,000521	0,5731	CD20	0,006468	0,2005
HLA-DR	0,000481	0,588	ARG1	0,003447	0,3504
CD56	0,000328	0,6549	SMA	0,003372	0,3558
Beta-2-microglobulin	0,000274	0,683	CTLA4	0,00151	0,5368
IDO1	0,000159	0,7553	OX40L	0,000829	0,6473
OX40L	2,08E-06	0,9716	CD56	0,000615	0,6934

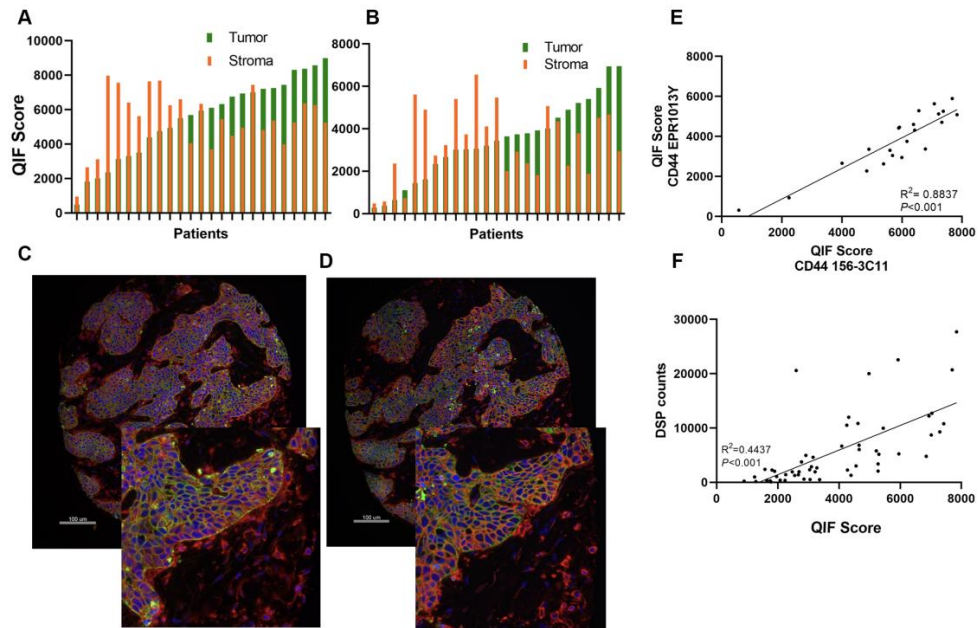
Supplementary Table S7. Differentially expressed protein markers between ROIs with elevated CD44 expression in the tumor compartment relative to ROIs with low CD44 expression in the tumor compartment

H12O_ITX1			CIMA-CUN		
Targets	Log2foldChange (high-low)	FDR-adjusted p-value	Targets	Log2foldChange (high-low)	FDR-adjusted p-value
PD-L1	1,2257	1,49E-08	Tim-3	0,80017	0,001035
IDO1	0,90745	0,0054572	CD40	0,79803	1,18E-05
VISTA	0,90323	8,54E-13	B7-H3	0,74146	1,18E-05
ICOS	0,88904	2,64E-13	ICOS	0,72723	1,24E-06
Tim-3	0,80696	1,09E-09	PD-L1	0,71736	0,011757
CD27	0,73234	1,49E-08	CD45	0,59472	0,002038
CD40	0,66583	2,11E-11	CD11c	0,57923	0,001035
ARG1	0,64844	2,11E-11	CD3	0,52081	0,003133
CD25	0,64208	7,42E-08	CD27	0,42725	0,002441
4-1BB	0,58834	9,08E-06	CD8	0,42541	0,006465
CD8	0,55807	1,61E-08	VISTA	0,38779	0,012837
CD45	0,53941	3,18E-06	Ki-67	0,21365	0,013243
B7-H3	0,52212	1,09E-09	IDO1	-0,42921	0,019737
GITR	0,4729	2,93E-09			
Ki-67	0,47068	9,32E-08			
Fibronectin	0,44567	0,00014619			
CD11c	0,44359	7,42E-06			
GZMB	0,37976	1,03E-10			
CD3	0,30722	0,0055482			
CD4	0,3037	1,06E-06			
CD68	0,22418	0,0043512			
PanCk	0,1781	0,00091907			
CD56	-0,32645	0,034899			

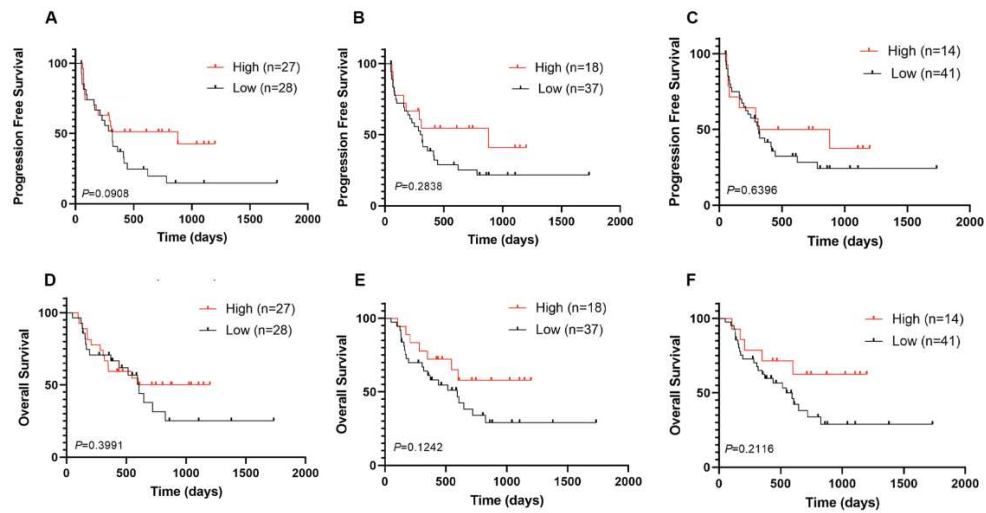
Only markers with a fold change (FC) ≥ 1 and significant FDR-adjusted p-value (< 0.05) are shown



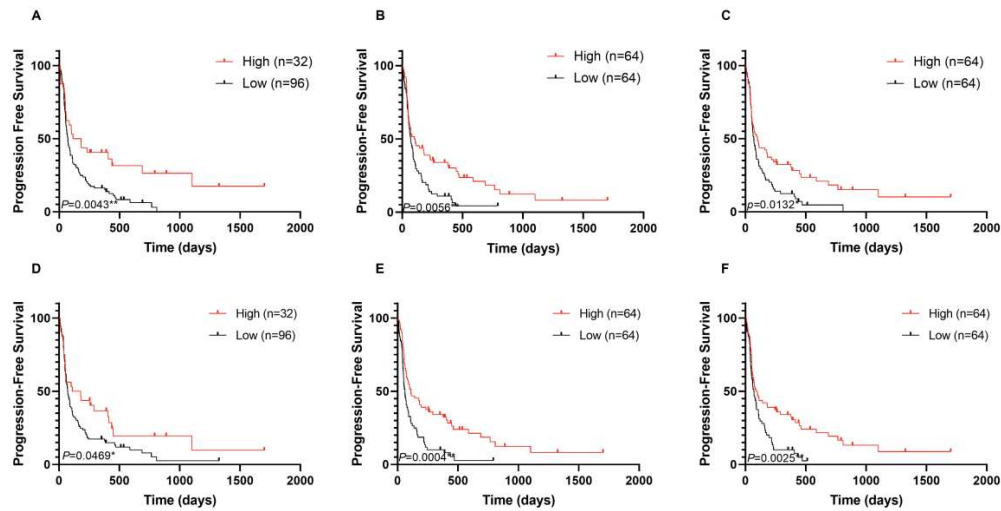
Supplementary Figure S1. (A) Comparative analysis of CD44 levels measured by DSP in the tumor compartment (panCK+) and in the immune compartment (panCK-/CD45+/CD68+) in YTMA471 cohort. (B-H) CD44 levels measured by DSP according to patients' clinical characteristics in YTMA471 cohort. p-values legends *: P<0.05; **: P<0.01, ***:P<0.001, ns: not significant; LN: lymph node; CNS: central nervous system



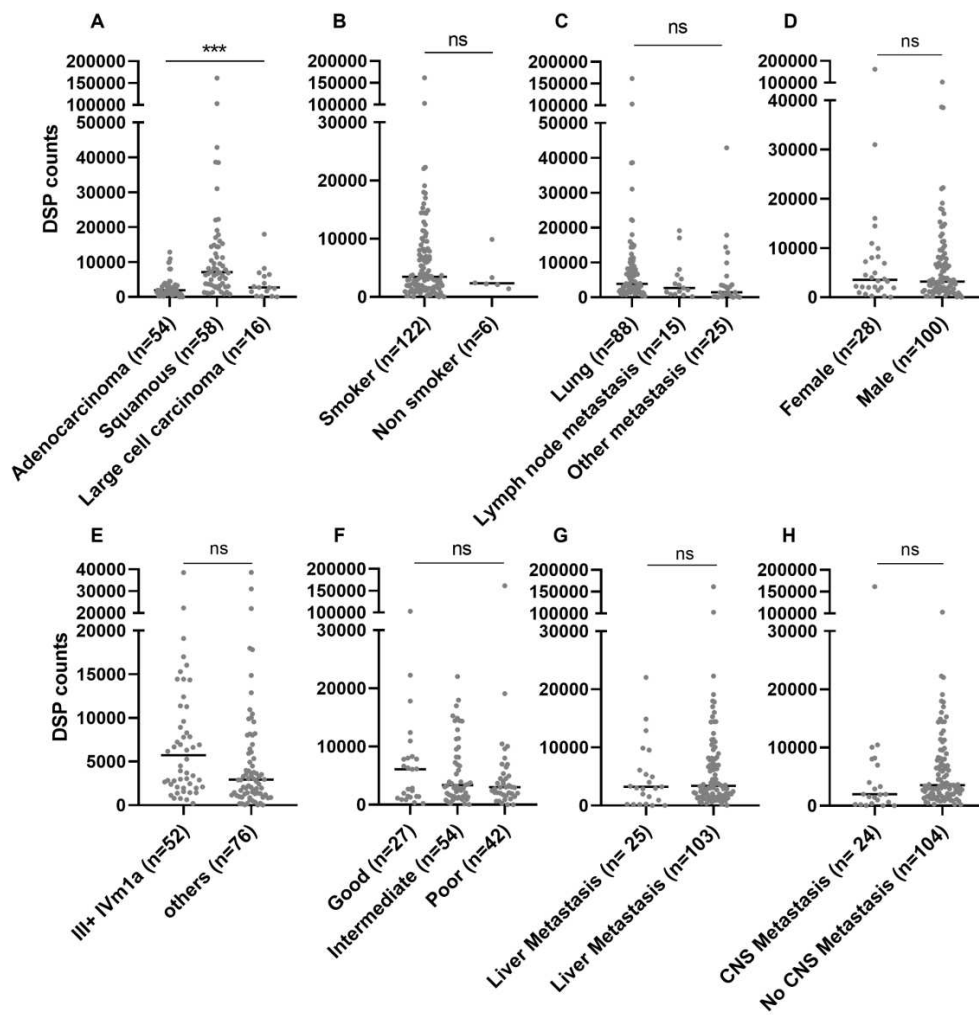
Supplementary Figure S2. (A-B). Dynamic range of CD44 QIF levels using two different anti-CD44 clones in a control lung cancer array (YTMA295); CD44 156-3C11 (A) and CD44 EPR1013Y (B). (C-D). Representative images of CD44 expression in the same TMA spot using CD44 156-3C11 (C) and CD44 EPR1013Y (D). (E) Correlation analysis of QIF scores obtained with the two different CD44 clones in YTMA295. (F) Correlation analysis of CD44 quantitative measurements obtained by DSP counts and QIF scores in YTMA471.



Supplementary Figure S3. (A-C) Kaplan-Meier PFS curves according to CD44 expression (QIF scores) in the immune compartment using the median (A), tertile (B), and quartile (C) cutpoints in YTMA471 cohort. (D-F) Kaplan-Meier OS curves according to CD44 expression (QIF scores) in the immune compartment using the median (D), tertile (E), and quartile (F) cutpoints in YTMA471 cohort.

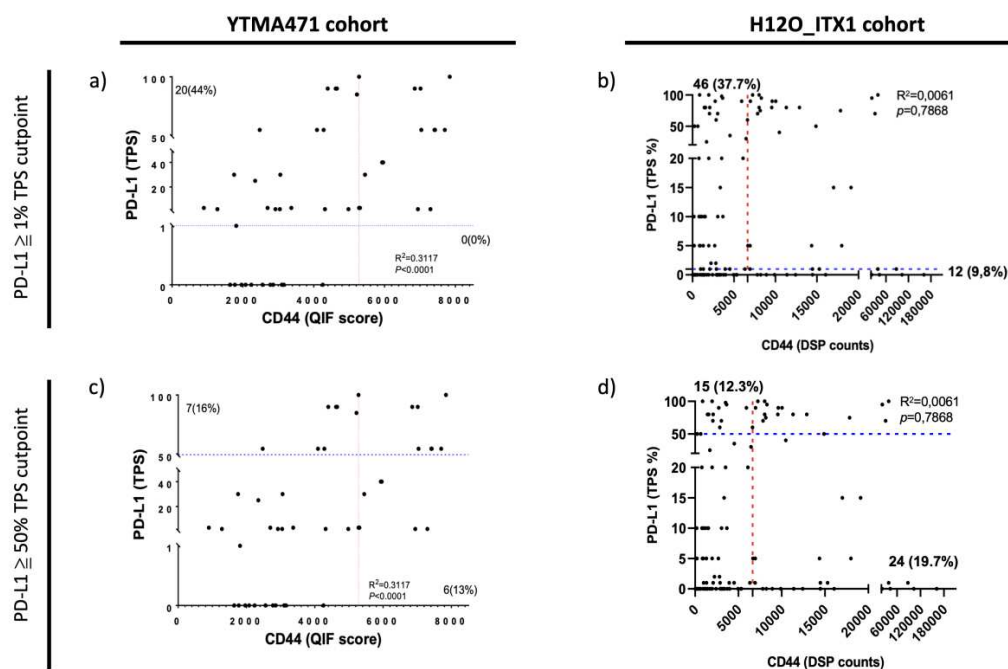


Supplementary Figure S4. (A-C) Kaplan-Meier PFS curves according to PD-L1 (A), CD3 (B), and CD8 (C) expression in the tumor compartment in H120_ITX1 cohort. (D-F) Kaplan-Meier PFS curves according to PD-L1 (D), CD3 (E), and CD8 (F) expression in the immune compartment in H120_ITX1 cohort.

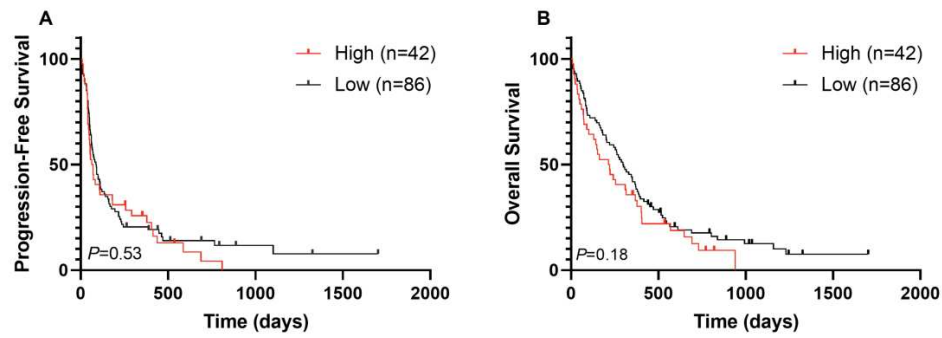


Supplementary Figure S5. CD44 levels measured by DSP according to patients' clinical characteristics in H12O_ITX1 cohort.

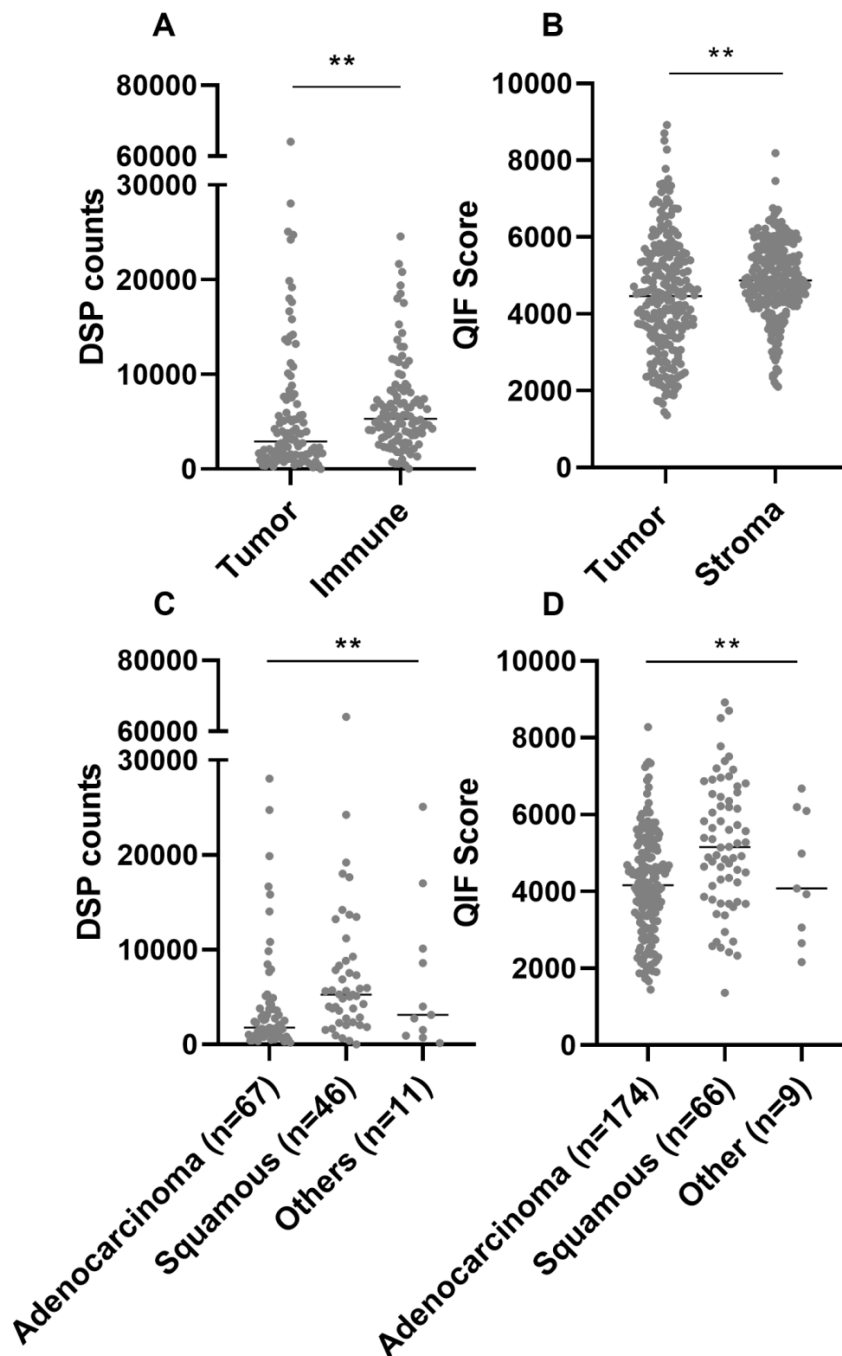
p-values legends *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$, ns: not significant



Supplementary figure S6. Correlation charts illustrating the association of tumor cell CD44 and PD-L1 TPS in the YTMA471 and H12O_ITX1 cohorts. Red line highlights tumor cell CD44 cutpoints, blue line highlights PD-L1 TPS cutpoints. (A-B) Graphs illustrating the number (%) of discordant cases using the PD-L1 $\geq 1\%$ TPS cutpoint in both cohorts. (C-D) Graphs illustrating the number (%) of discordant cases in both cohorts using the PD-L1 $\geq 50\%$ TPS cutpoint in both cohorts.

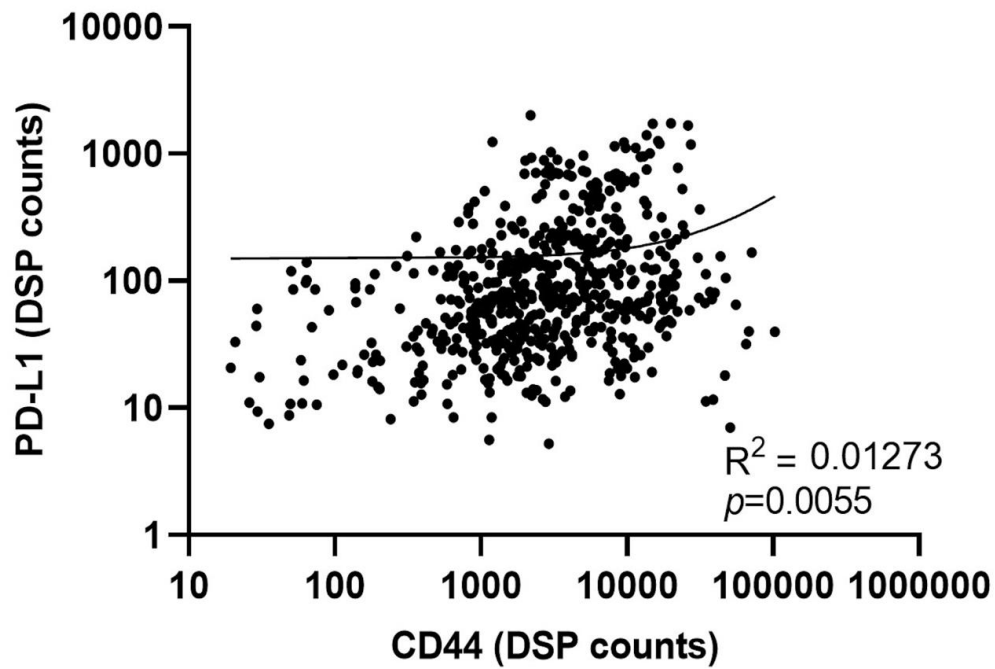


Supplementary Figure S7. (A-B) Kaplan-Meier PFS (A) and OS (B) curves according to CD44 expression in the immune compartment using the top tertile cutpoint in H12O_ITX1 cohort.



Supplementary Figure S8. (A-B) Comparative analysis of CD44 levels in the tumor and immune compartments using DSP in CIMA-CUN cohort (A) and tumor and stroma compartments using QIF in YTMA423 cohort (B). (C-D) CD44 levels according to histology measured by DSP in CIMA-CUN cohort (C) and by QIF in YTMA423 cohort (D).

p-values legends *: $P < 0.05$; **: $P < 0.01$, ***: $P < 0.001$, ns: not significant



Supplementary figure S9. Correlation chart showing the association between tumor cell CD44 and PD-L1 in the H12O_ITX1 cohort after removing outlier values exceeding the upper limits of expression of tumor cell CD44 and PD-L1 in the CIMA-CUN cohort