Supplementary material

Targeted multiplex proteomics for molecular prescreening and biomarker discovery in metastatic colorectal cancer

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Supplementary tables and figures legend

Supplementary table 1. Colorectal cancer dataset included in the study: individual patient clinicopathological features and SRM-MS protein levels. Non-detectable proteins are indicated as “0”.

Supplementary table 2. List of proteins quantified by SRM-MS. Targets detectable in at least one samples are indicated.

Supplementary table 3. Descriptive statistics of detectable proteins in colorectal cancer samples. Percentage of samples classified as “High” is indicated in the bottom row.

Supplementary table 4. Comparison of protein expression levels between unmatched primary and metastatic samples. P-values calculated using non-parametric Kruskal-Wallis test with adjustment for multiple testing according to Benjamini and Hochberg (BH) method are indicated.

Supplementary table 5. Comparison of protein expression levels between RAS-mutated versus RAS-wild type samples. P-values calculated using non-parametric Kruskal-Wallis test with adjustment for multiple testing according to Benjamini and Hochberg (BH) method are indicated.

Supplementary table 6. Association between selected proteins and overall survival. Proteins were classified as low versus high as described in the main text. Log-rank test was used for statistical comparison. P-values are indicated.

Supplementary table 7. Multivariable model of overall survival including mesothelin together with known prognostic factor in the metastatic setting. Cox proportional-hazard model was used to obtain hazard ratios (HRs) with 95% CIs.

Supplementary table 8. Primary antibody sources, dilutions and incubation time and temperatures used in this study.

Supplementary Figure 1. Distribution of protein expressions across samples. In the bottom panel dark shadow represents high expression. (A) Receptors and downstream markers. Differentiation and histotypes markers. Apoptosis, immunotherapy and ADC proteins. (B) Chemotherapy markers. The definition of high protein expression varied by protein: ≥ 4,000 amol/µg for EGFR13, ≥750 amol/µg for HER2 and ≥1500 amol/µg for cMET. For the remaining proteins, SRM-MS levels above the upper 95% confidence interval of the mean were defined as high expression.

Supplementary Figure 2. Representative immunohistochemistry images of CHGA (A), CK7 (B) and HER2 (C). Sample ID (white) and target protein levels by SRM-MS (colored) are shown on the top right side of each image. ND, not detectable.

Supplementary Figure 3. Association between MSLN levels and clinicopathological features. (A) Data are displayed as box plots. Protein levels

by immunohistochemistry (Hscore, left plot) and mass spectrometry (MS, right plot) are shown on the y-axis for each subgroup. P-values (calculated using Wilcoxon test for two variables and Kruskal test for three variable) are indicated. (B) Kaplan-Meier overall survival curves according to MSLN expression levels determined by immunohistochemistry (left side) and mass spectrometry (right side). Hazard ratios together with 95% CI and p-values are shown.

Supplementary Figure 4. ROUT outlier analysis. The twenty-four patients with outlier expression in at least one protein are shown together with the affected target. Presence of mutations in APC, TP53, KRAS, and PIK3CA are indicated by the orange boxes. Outlier protein values (expressed in amol/µg) are shown in the greed boxes for each individual sample.

Supplementary table 2

Family	Protein	SRM-MS
Differenciation/Histotype	**KRT5**	detactable in at least one sample
	KRT7	detactable in at least one sample
	Vimentin	detactable in at least one sample
	CHGA	detactable in at least one sample
	SYP	not detectable
	P63	detactable in at least one sample
	TTF1	detactable in at least one sample
HER	**EGFR**	detactable in at least one sample
	Her2	detactable in at least one sample
	Her3	detactable in at least one sample
	Her4	not detectable
Protein targets	**cMet**	detactable in at least one sample
	HGF	not detectable
	IGF1R	not detectable
	PTEN	detactable in at least one sample
	KRAS	detactable in at least one sample
	ALK	not detectable
	ROS1	not detectable
	FRalpha	not detectable
	AR	not detectable
	ER	not detectable
	FGFR1	not detectable
	FGFR2	not detectable
	FGFR3	not detectable
	AURKA	not detectable
	Axl	not detectable
	E-CAD	detactable in at least one sample
Immuno/ADC	**PDL1**	not detectable
	MSLN	detactable in at least one sample
	IDO1	detactable in at least one sample
	GPNMB	detactable in at least one sample
	TROP2	detactable in at least one sample
Apoptosis	**MCL1**	detactable in at least one sample
	MDM2	not detectable
	P16	detactable in at least one sample
Chemotherapy	**SPARC**	detactable in at least one sample
	RRM1	detactable in at least one sample
	hENT1	detactable in at least one sample
	TOPO1	detactable in at least one sample
	TOP2A	detactable in at least one sample
	ERCC1	detactable in at least one sample
	MGMT	detactable in at least one sample
	XRCC1	detactable in at least one sample
	MRP1	detactable in at least one sample
	MDR1	detactable in at least one sample
	TUBB3	detactable in at least one sample
	DHFR	detactable in at least one sample
	RFC	detactable in at least one sample
	GRAFT	detactable in at least one sample
	TLE3	detactable in at least one sample
	ALDHA1	detactable in at least one sample
	FPGS	not detectable
	TYMS	not detectable
	TS	detactable in at least one sample
Housekeeping	**Actin**	detactable in at least one sample
	Tubulin	detactable in at least one sample

Supplementary table 3

	HER			Protein markers				Differenciation/Histotype						Apoptosis		Immunotherapy/ADC			
	EGFR	Her2	Her3	cMet	PTEN	KRAS	E-CAD	KRT5	KRT7	Vimentin	CHGA	P63	TTF1	MCL1	P16	GPNMB	IDO1	MSLN	TROP2
Number of detectable	48	46	22	44	26	47	50	50	22	50	5	1	1	15	4	50	3	15	36
Number of non detectable	2	4	28	6	24	3	0	0	28	0	45	49	49	35	46	0	47	35	14
Total number of samples	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Minimum	106.2	207.9	168.8	217	107.9	487	1855	344.6	168.3	3480	1399	533.7	561	109.5	377.5	298.6	214.5	348.9	168.9
25% Percentile	184.9	332.3	204.3	317.7	171.1	745.5	3181.2	683.6	558.4	23104	1556.1	533.7	561	134.5	385.3	977.9	214.5	459.8	293.1
Median	207.9	383.9	249.9	370.3	216.6	1003.5	4482.9	1151.5	1715	32061	1719.5	533.7	561	181.8	506.9	1315	346.7	583.3	610.9
75% Percentile	268.1	485.2	327.1	455.6	228.4	1209.5	5586.7	2508.8	3569.6	38526	59275	533.7	561	216.6	632.8	2317.3	493.2	861.7	1597.1
Maximum	7635	14530	519.7	757	281.1	1669	8706.7	229167	17333	65050	116667	533.7	561	355.4	642	9386.7	493.2	1422	5365
Mean	465.6	767.3	269.6	396.6	203.9	995.9	4426.9	8314.9	2943.7	31910	24676	533.7	561	178.4	508.3	1829.3	351.5	683.6	1079.8
Std. Deviation	1201.2	2100.7	83.9	125.9	42.9	301.8	1584.2	35206.8	3909.3	13112	51425	0	0	62.7	134.5	1515.3	139.4	329.8	1132
Std. Error of Mean	173.4	309.7	17.9	18.9	8.4	44.0	224.0	4978.9	833.5	1854.3	22998	0	0	16.2	67.2	214.3	80.5	85.2	188.7
Lower 95% CI of mean	116.8	143.4	232.4	358.4	186.6	907.3	3976.7	-1690.6	1210.4	28184	-39176	0	0	143.7	294.3	1398.7	5.2	500.9	696.8
Upper 95% CI of mean	814.4	1391.1	306.8	434.9	221.2	1084.5	4877.1	18320.5	4677	35636	88529	0	0	213.1	722.3	2259.9	697.8	866.2	1462.8
% above 95% CI (HIGH)	4%	8%	12%	0%	24%	30%	36%	4%	8%	30%	2%	2%	2%	8%	0%	26%	0%	6%	20%

Supplementary table 3 (con't)

	Chemotherapy																	Housekeeping	
	ALDHA1	DHFR	ERCC1	GRAFT	hENT1	MDR1	MGMT	MRP1	RFC	RRM1	SPARC	TLE3	TOPO1	TOP2A	TS	TUBB3	XRCC1	Actin	Tubulin
Number of detectable	42	23	11	50	40	15	17	1	2	38	38	46	50	43	1	48	50	50	50
Number of non detectable	8	27	39	0	10	35	33	49	48	12	12	4	0	7	49	2	0	0	0
Total number of samples	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50
Minimum	468.7	194	74.4	931.7	129.1	175.4	207.3	445.2	58.7	309.2	341.2	96.4	297.1	255.2	1179.5	297	295.7	165.3	109.2
25% Percentile	888.6	271.8	86.2	1785.2	182.7	291.4	394.4	445.2	58.7	394.1	712.5	171.7	987.9	457.5	1179.5	777.2	503.5	452.9	154.3
Median	1399.9	298.3	96.2	2226.7	222.5	410	455.7	445.2	69.7	459.4	845.8	199.6	1171	681.8	1179.5	1162.6	646.9	509.4	173.5
75% Percentile	2879.8	361.1	118.1	2752.5	257.9	710.5	797.4	445.2	80.7	601.5	959.1	270.9	1555.2	809.5	1179.5	1912	788.1	582.0	204.4
Maximum	25817	555.5	130.4	4401.3	423.8	1349.5	1083.3	445.2	80.7	762	1231.2	399.8	3240.3	7465	1179.5	8100	1935.7	1870.3	274.3
Mean	2775.1	315.8	100.5	2327.5	232.8	486.1	567.9	445.2	69.7	497.9	838.3	219.2	1320.9	954.5	1179.5	1737.9	689.4	537.8	178.7
Std. Deviation	4630.6	76.3	18.7	768.6	69.9	302.0	276.7	0	15.6	135.2	193.8	68.8	579.6	1161.4	0	1592.1	272.7	224.4	33.6
Std. Error of Mean	714.5	15.9	5.7	108.7	11.1	77.9	67.1	0	11	21.9	31.4	10.1	81.9	177.1	0	229.8	38.6	31.7	4.7
Lower 95% CI of mean	1332.1	282.8	87.9	2109.1	210.4	318.8	425.6	0	-70.1	453.4	774.6	198.7	1156.2	597.1	0	1275.6	611.9	473.9	169.2
Upper 95% CI of mean	4218.1	348.8	113.1	2545.9	255.1	653.3	710.2	0	209.5	542.3	901.9	239.6	1485.6	1311.9	0	2200.2	766.9	601.5	188.3
% above 95% CI (HIGH)	10%	12%	6%	30%	20%	8%	8%	2%	0%	26%	30%	26%	30%	12%	2%	20%	28%		

Supplementary table 4

	pvals.prim_met.adj
KRT7	0.060
EGFR	0.634
Her3	0.634
PTEN	0.634
MSLN	0.634
CHGA	0.634
TTF1	0.634
P16	0.634
SPARC	0.634
RRM1	0.634
MGMT	0.634
MRP1	0.634
MDR1	0.634
DHFR	0.634
GRAFT	0.634
TLE3	0.634
GPNMB	0.634
TS	0.636
MCL1	0.737
ERCC1	0.737
TROP2	0.737
TOPO1	0.785
XRCC1	0.785
TUBB3	0.835
IDO1	0.835
hENT1	0.877
Vimentin	0.899
ALDHA1	0.899
KRAS	0.978
Her2	0.988
cMet	0.988
E.CAD	0.988
KRT5	0.988
TOP2A	0.988
RFC	1.000

Supplementary table 5

	pvals.ras.adj
EGFR	0.023
KRAS	0.023
TOPO1	0.023
TOP2A	0.023
PTEN	0.284
cMet	0.330
XRCC1	0.330
ERCC1	0.340
DHFR	0.340
RRM1	0.370
E.CAD	0.494
MSLN	0.494
Her3	0.535
MCL1	0.535
MGMT	0.535
MDR1	0.535
Her2	0.541
KRT5	0.541
CHGA	0.541
TTF1	0.588
MRP1	0.588
IDO1	0.588
TS	0.588
TUBB3	0.595
ALDHA1	0.595
hENT1	0.601
KRT7	0.732
Vimentin	0.732
TROP2	0.842
P16	0.878
GPNMB	0.878
RFC	0.890
TLE3	0.924
SPARC	0.946
GRAFT	0.984

Supplementary table 6

Protein marker	records	events	median	0.95LCL	0.95UCL	Log-Rank
MET HIGH	17	12	48.0	41.5	NA	p=0.144
MET LOW	33	24	43.1	35.7	65.7	
ECAD HIGH	25	18	44.2	38.9	NA	p=0.400
ECAD LOW	25	18	50.1	37.3	66.1	
MSLN LOW	35	24	51.2	44.3	66.1	p=0.004
MSLN HIGH	15	12	37.5	23.4	NA	
MCL1 LOW	35	27	44.3	37.5	65.7	p=0.683
MCL1 HIGH	15	9	44.2	41.5	NA	
MCL1 LOW*1	30	30	8.0	6.1	12.8	p=0.260
MCL1 HIGH*1	15	15	10.1	6.1	11.9	
MCL1 LOW*2	30	30	7.4	6.0	10.5	p=0.665
MCL1 HIGH*2	15	15	6.4	3.1	15.5	
MDR1 LOW	35	26	43.1	35.7	65.7	p=0.255
MDR1 HIGH	15	10	51.2	44.3	NA	
MDR1 LOW*1	34	34	7.6	6.1	10.7	p=0.673
MDR1 HIGH*1	11	11	11.7	7.6	NA	
MDR1 LOW*2	34	34	6.9	6.0	9.4	p=0.064
MDR1 HIGH*2	11	11	9.3	5.9	NA	
PTEN LOW	24	15	48.0	42.9	NA	p=0.805
PTEN HIGH	26	21	44.3	35.6	70.3	

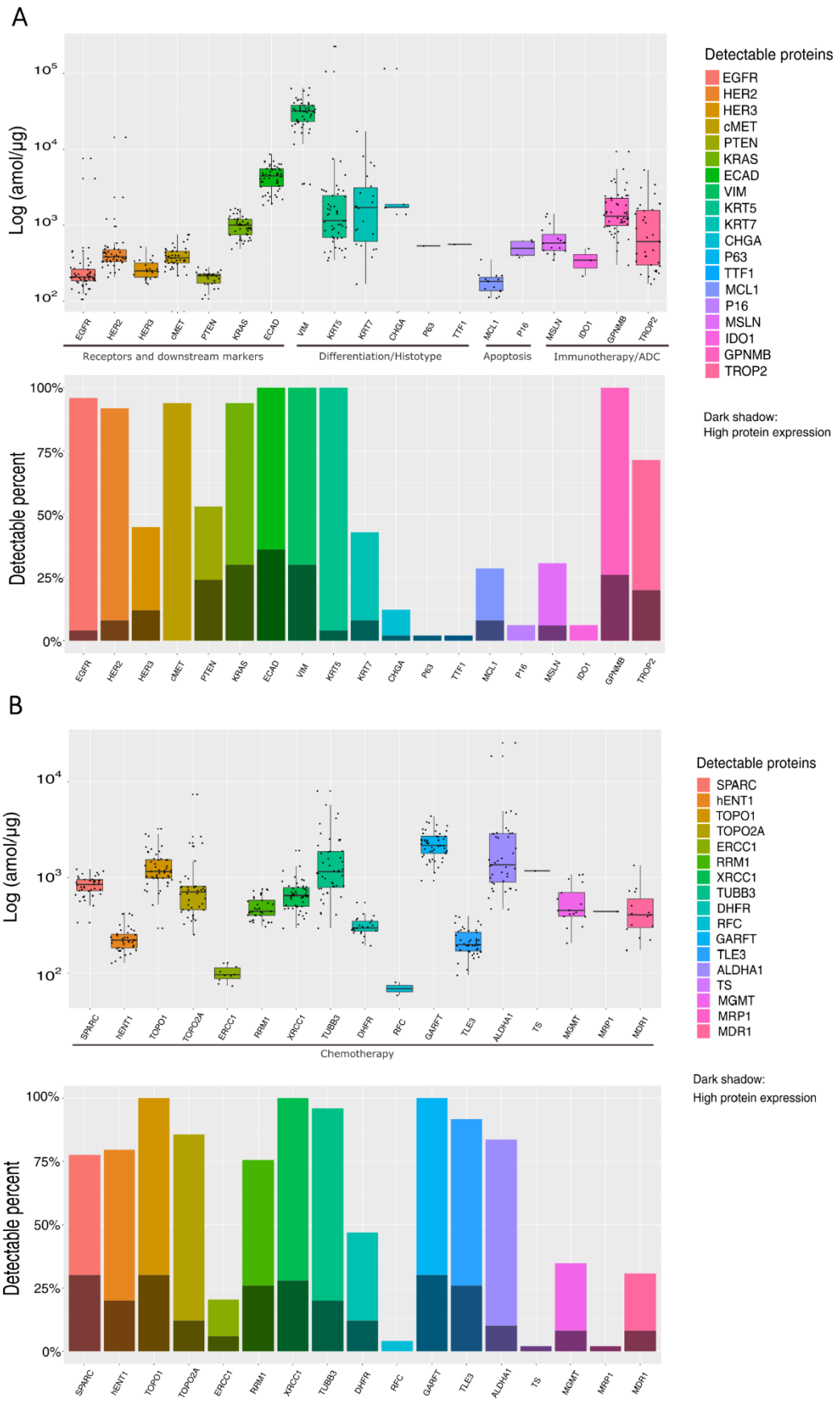
Supplementary table 7

Factor	HR	CI95% low	CI95% high	P value
Mesothelin high vs. low	2.36	1.04	5.24	0.04
Liver metastasis vs. no	1.45	0.55	3.83	0.45
RAS mut vs. wild-type	1.17	0.54	2.52	0.69
Right colon vs. left colon/ rectum	0.54	0.18	1.62	0.28
Curative resection of metastasis vs. no	0.53	0.19	1.45	0.21

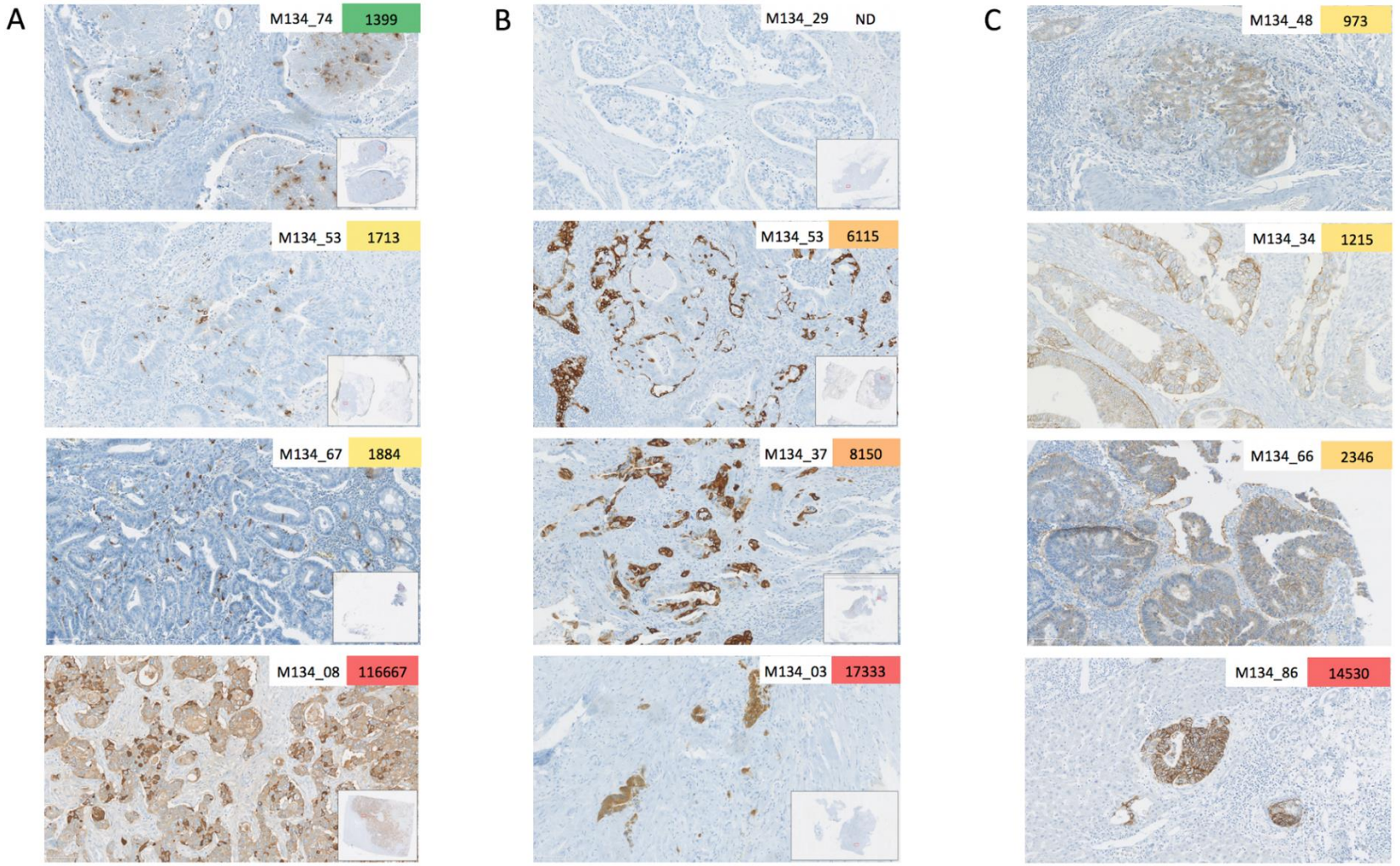
Supplementary table 8

Antigen	Clone	Dilution	Manufacturer and reference	Primary antibody incubation time and temperature
CK7	SP1	Ready-to-use	Ventana Medical Systems (#790-4462)	20 min Room Temperature
Chromogranin A	LK2H10	Ready-to-use	Ventana Medical Systems (#760-2519)	12 min 36ºC
HER2	4B5	Ready-to-use	Ventana Medical Systems (#790-2991)	20 min 37ºC

Supplementary figure 1

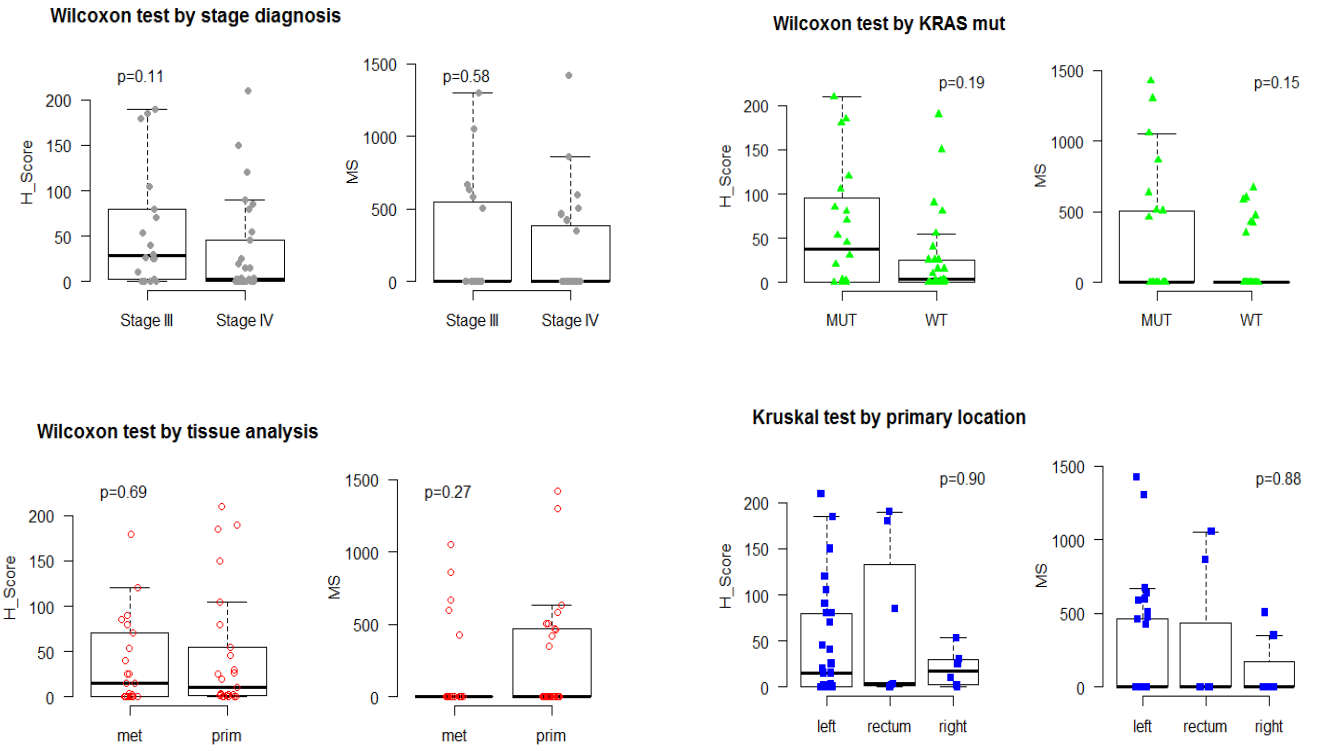


Supplementary figure 2



Supplementary Figure 3

A



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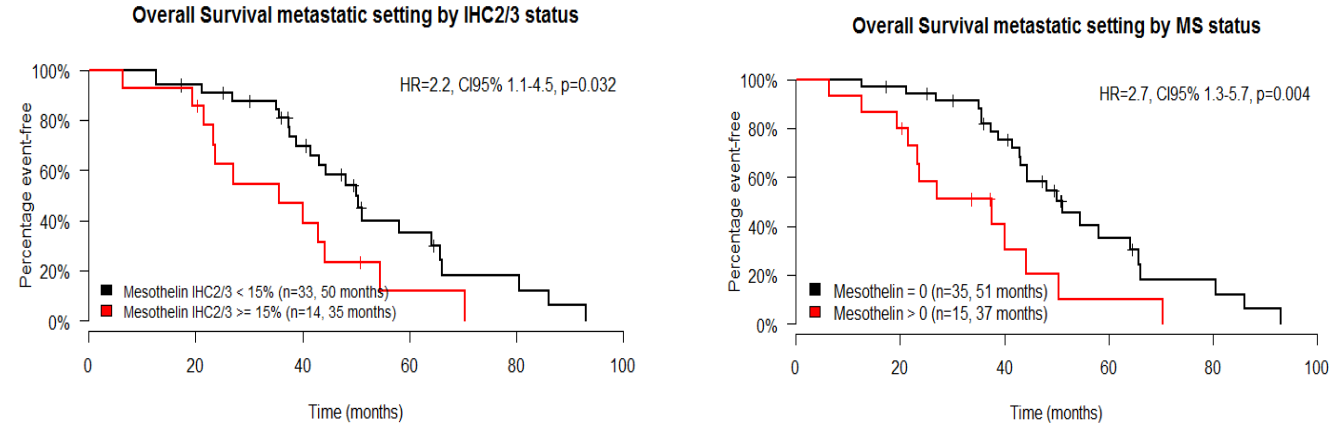

Supplementary figure 4