

Neo-fs index: a novel immunohistochemical biomarker panel predicts survival and response to anti-angiogenetic agents in clear cell renal cell carcinoma

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Table S1. Univariate analysis to identify a correlation between survival and clinicopathological factors and immunohistochemical results in patients receiving anti-angiogenetic therapies

	Overall survival (OS)		Disease-specific survival (DSS)		Recurrence-free survival (RFS)	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
Clinicopathological variables						
Female (vs Male)	0.874 (0.402-1.899)	0.734	0.874 (0.402-1.899)	0.734	1.049 (0.444-2.479)	0.914
Age ≥55 years	1.171 (0.624-2.199)	0.623	1.171 (0.624-2.199)	0.623	1.334 (0.596-2.985)	0.483
Radical nephrectomy (vs partial nephrectomy)	1.893 (0.675-5.304)	0.225	1.893 (0.675-5.304)	0.225	1.169 (0.502-2.722)	0.717
ISUP grade 3–4	3.452 (0.834-14.290)	0.087	3.452 (0.834-14.290)	0.087	2.294 (0.869-6.058)	0.094
Tumor size ≥4 cm	2.618 (1.026-6.679)	0.044	2.618 (1.026-6.679)	0.044	1.739 (0.807-3.749)	0.158
pT3–4	1.761 (0.807-3.844)	0.156	1.761 (0.807-3.844)	0.156	1.365 (0.649-2.871)	0.411
pN1 (vs pN0/pNx)	3.094 (1.566-6.113)	0.001	3.094 (1.566-6.113)	0.001	8.198 (2.323-28.932)	0.001
Lymphovascular invasion	2.541 (1.304-4.953)	0.006	2.541 (1.304-4.953)	0.006	2.307 (1.062-5.012)	0.035
Margin involvement	1.514 (0.634-3.615)	0.350	1.514 (0.634-3.615)	0.350	3.017 (1.146-7.944)	0.025
Necrosis	1.662 (0.796-3.472)	0.176	1.662 (0.796-3.472)	0.176	1.771 (0.865-3.626)	0.118
Sarcomatoid change	2.309 (1.247-4.278)	0.008	2.309 (1.247-4.278)	0.008	1.050 (0.449-2.455)	0.910
mTOR inhibitor recipient	2.781 (1.421-5.444)	0.003	2.781 (1.421-5.444)	0.003	1.869 (0.902-3.872)	0.092
Immunohistochemistry						
High APC expression	1.549 (0.685-3.505)	0.293	1.549 (0.685-3.505)	0.293	1.589 (0.600-4.210)	0.351
High NOTCH1 expression	1.814 (0.981-3.355)	0.058	1.814 (0.981-3.355)	0.058	1.951 (0.922-4.127)	0.080
High ARID1A expression	10.061 (1.211-83.584)	0.033	10.061 (1.211-83.584)	0.033	NA	NA
High FAT1 expression	0.814 (0.289-2.292)	0.697	0.814 (0.289-2.292)	0.697	0.918 (0.274-3.075)	0.890
High VHL expression	0.698 (0.382-1.273)	0.241	0.698 (0.382-1.273)	0.241	1.086 (0.519-2.273)	0.826
High EYS expression	2.718 (1.451-5.092)	0.002	2.718 (1.451-5.092)	0.002	2.848 (1.214-6.679)	0.016
High KMT2D expression	1.577 (0.846-2.940)	0.152	1.577 (0.846-2.940)	0.152	2.102 (0.939-4.707)	0.071
High Filamin A expression	1.928 (0.982-3.786)	0.057	1.928 (0.982-3.786)	0.057	2.213 (0.954-5.132)	0.064
High PTEN expression	0.717 (0.386-1.334)	0.294	0.717 (0.386-1.334)	0.294	1.412 (0.631-3.160)	0.402
High p53 expression	1.363 (0.487-3.818)	0.556	1.363 (0.487-3.818)	0.556	1.543 (0.531-4.480)	0.425

Neo-fs index	Log-rank p	0.001	Log-rank p	0.001	Log-rank p	0.096
0–1	1.953 (0.550-6.934)	0.300	1.953 (0.550-6.934)	0.300	3.188 (0.837-12.144)	0.089
2	6.678 (2.443-18.252)	<0.001	6.678 (2.443-18.252)	<0.001	3.101 (0.660-14.567)	0.152
3	3.370 (1.312-8.655)	0.012	3.370 (1.312-8.655)	0.012	2.739 (0.982-7.641)	0.054
4	2.373 (1.077-5.230)	0.032	2.373 (1.077-5.230)	0.032	2.862 (1.076-7.612)	0.035
5 (reference)	1	-	1	-	1	-
<i>p-for trend</i>	0.738 (0.599-0.909)	0.004	0.738 (0.599-0.909)	0.004	0.733 (0.562-0.957)	0.022
Neo-fs index						
Low (≤ 4)	1	-	1	-	1	-
High (>4)	0.349 (0.177-0.688)	0.002	0.349 (0.177-0.688)	0.002	0.347 (0.157-0.765)	0.009

Neo-fs index: The number of markers with low expression among the five independent prognosticators (APC, NOTCH1, ARID1A, EYS, and Filamin A)

The number of patients with the Neo-fs index 0–1, 2, 3, 4, and 5 was three, six, nine, 17, and 28, respectively

* mTOR inhibitor, mammalian target of rapamycin (mTOR) inhibitor; CI, confidence interval

Table S2. Multivariate analysis to identify a correlation between survival and clinicopathological factors and immunohistochemical results in patients receiving anti-angiogenic therapies

	Overall survival (OS)		Disease-specific survival (DSS)		Recurrence-free survival (RFS)	
	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>	HR (95% CI)	<i>p</i>
Clinicopathological variables						
Tumor size \geq 4 cm	1.429 (0.516-3.962)	0.492	1.429 (0.516-3.962)	0.492	NA	NA
pN1 (vs pN0/pNx)	1.702 (0.756-3.836)	0.199	1.702 (0.756-3.836)	0.199	4.681 (1.160-18.894)	0.030
Lymphovascular invasion	1.500 (0.698-3.225)	0.299	1.500 (0.698-3.225)	0.299	1.798 (0.750-4.312)	0.189
Margin involvement	NA	NA	NA	NA	2.450 (0.877-6.845)	0.087
Sarcomatoid change	1.496 (0.746-3.000)	0.257	1.496 (0.746-3.000)	0.257	NA	NA
mTOR inhibitor recipient	1.977 (0.947-4.124)	0.069	1.977 (0.947-4.124)	0.069	NA	NA
Immunohistochemistry						
High ARID1A expression	9.835 (1.100-87.939)	0.041	9.835 (1.100-87.939)	0.041	2.431 (0.964-6.130)	0.060
High EYS expression	2.433 (1.243-4.762)	0.009	2.433 (1.243-4.762)	0.009	NA	NA
Neo-fs index						
0–1	2.987 (0.766-11.646)	0.115	2.987 (0.766-11.646)	0.115	0.917 (0.131-6.431)	0.930
2	10.806 (2.897-40.308)	<0.001	10.806 (2.897-40.308)	<0.001	0.616 (0.140-2.697)	0.520
3	3.619 (1.356-9.659)	0.010	3.619 (1.356-9.659)	0.010	0.751 (0.170-3.305)	0.704
4	2.632 (0.995-6.962)	0.051	2.632 (0.995-6.962)	0.051	0.354 (0.086-1.460)	0.151
5 (reference)	1	-	1	-	1	-
<i>p-for trend</i>	0.662 (0.516-0.850)	0.001	0.662 (0.516-0.850)	0.001	0.778 (0.573-1.057)	0.108
Neo-fs index						
Low (\leq 4)	1	-	1	-	1	-
High ($>$ 4)	0.314 (0.146-0.679)	0.003	0.314 (0.146-0.679)	0.003	0.473 (0.198-1.132)	0.093

Neo-fs index: The number of markers with low expression among the five independent prognosticators (APC, NOTCH1, ARID1A, EYS, and Filamin A) # The number of patients with the Neo-fs index 0–1, 2, 3, 4, and 5 was three, six, nine, 17, and 28, respectively

* mTOR inhibitor, mammalian target of rapamycin (mTOR) inhibitor; CI, confidence interval.

Table S3. Clinicopathological characteristics of the study population based on the Neo-fs index

Clinicopathological variables	Neo-fs index					<i>p</i>
	0–1 (n=12)	2 (n=39)	3 (n=71)	4 (n=140)	5 (n=369)	
Sex						0.416
Male	9 (75.0%)	32 (82.1%)	57 (80.3%)	99 (70.7%)	277 (75.1%)	
Female	3 (25.0%)	7 (17.9%)	14 (19.7%)	41 (29.3%)	92 (24.9%)	
Age (years)						0.460
< 55 years	5 (41.7%)	21 (53.8%)	34 (47.9%)	62 (44.3%)	192 (52.0%)	
≥55 years	7 (58.3%)	18 (46.2%)	37 (52.1%)	78 (55.7%)	177 (48.0%)	
Procedure						0.425
Partial nephrectomy	6 (50.0%)	16 (41.0%)	41 (57.7%)	76 (54.3%)	200 (54.2%)	
Radical nephrectomy	6 (50.0%)	23 (59.0%)	30 (42.3%)	64 (45.7%)	169 (45.8%)	
ISUP grade						<0.001
1–2	3 (25.0%)	10 (25.6%)	34 (47.9%)	61 (43.6%)	221 (59.9%)	
3–4	9 (75.0%)	29 (74.4%)	37 (52.1%)	79 (56.4%)	148 (40.1%)	
Tumor size (cm)						0.873
<4 cm	6 (50.0%)	23 (59.0%)	45 (63.4%)	87 (62.1%)	223 (60.4%)	
≥4 cm	6 (50.0%)	16 (41.0%)	26 (36.6%)	53 (37.9%)	146 (39.6%)	
T stage						0.394
pT1–2	7 (58.3%)	29 (74.4%)	58 (81.7%)	109 (77.9%)	290 (78.6%)	
pT3–4	5 (41.7%)	10 (25.6%)	13 (18.3%)	31 (22.1%)	79 (21.4%)	
N stage						<0.001
pN0/pNx	11 (91.7%)	35 (89.7%)	70 (98.6%)	133 (95.0%)	368 (99.7%)	
pN1	1 (8.3%)	4 (10.3%)	1 (1.4%)	7 (5.0%)	1 (0.3%)	
Lymphovascular invasion						0.074
Absent	8 (66.7%)	29 (74.4%)	64 (90.1%)	114 (81.4%)	318 (86.2%)	
Present	4 (33.3%)	10 (25.6%)	7 (9.9%)	26 (18.6%)	51 (13.8%)	
Resection margin						0.033
Clear	10 (83.3%)	38 (97.4%)	69 (97.2%)	137 (97.9%)	363 (98.4%)	
Involved	2 (16.7%)	1 (2.6%)	2 (2.8%)	3 (2.1%)	6 (1.6%)	
Necrosis						0.093
Absent	8 (66.7%)	32 (82.1%)	59 (83.1%)	116 (82.9%)	318 (86.2%)	
Present	4 (33.3%)	7 (17.9%)	12 (16.9%)	24 (17.1%)	51 (13.8%)	
Sarcomatoid change						0.133
Absent	10 (83.3%)	37 (94.9%)	67 (94.4%)	130 (92.9%)	354 (95.9%)	
Present	2 (16.7%)	2 (5.1%)	4 (5.6%)	10 (7.1%)	15 (4.1%)	
Anti-angiogenetic agent						0.009
Not received	9 (75.0%)	33 (84.6%)	62 (87.3%)	123 (87.9%)	341 (92.4%)	
Received	3 (25.0%)	6 (15.4%)	9 (12.7%)	17 (12.1%)	28 (7.6%)	
mTOR inhibitor						0.016
Not received	10 (83.3%)	35 (89.7%)	65 (91.5%)	131 (93.6%)	353 (95.7%)	
Received	2 (16.7%)	4 (10.3%)	6 (8.5%)	9 (6.4%)	16 (4.3%)	

Neo-fs index: The number of markers with low expression among the five independent prognosticators (APC, NOTCH1, ARID1A, EYS, and Filamin A)

Of the 638 samples, seven lacked sufficient tumor cells for testing the expression of at least one marker among APC, NOTCH1, ARID1A, EYS, and Filamin A; these samples were excluded, and 631 samples were analyzed.

* mTOR inhibitor, mammalian target of rapamycin (mTOR) inhibitor

Table S4. Immune signature (mean z-scores) in clear cell renal cell carcinoma based on the Neo-fs index

Immune gene signature	Neo-fs index		p
	Low (0–1) (mean±SD)	High (4–5) (mean±SD)	
MHC Class I	-0.39±0.59	0.15±0.93	< 0.001
CD8+ T cells	-0.20±0.65	0.05±0.99	0.008
Cytolytic activity	-0.18±0.63	0.12±1.06	0.002
pDCs	-0.03±0.42	0.09±0.59	0.041
Co-stimulation, APC	-0.02±0.37	0.03±0.46	0.305
Co-inhibition, T cell	0.02±1.13	0.06±0.69	0.705
Co-inhibition, APC	0.20±1.97	-0.17±0.52	0.011
Type II IFN Response	0.39±0.75	-0.16±0.55	< 0.001

Neo-fs index: The number of markers with low expression among the five independent prognosticators (APC, NOTCH1, ARID1A, EYS, and Filamin A); **SD:** standard deviation; **pDCs:** plasmacytoid dendritic cells

Table S5. Comparison of mutational landscape in clear cell renal cell carcinoma based on Neo-fs index

	Neo-fs index			P
	Total	Low (0–2)	High (>4)	
Total mutation count	6.67±3.53	2.75±2.63	8.63±1.85	0.001
Single nucleotide variant count	5.17±2.89	2.25±2.06	6.63±2.00	0.005
Total indel count	1.33±1.56	0.25±0.50	1.88±1.64	0.030
Frameshift indel count	1.08±1.08	0.50±0.58	1.38±1.19	0.201

Neo-fs index: The number of markers with low expression among the five independent prognosticators (APC, NOTCH1, ARID1A, EYS, and Filamin A); **SD:** standard deviation

Table S6. The list of genes targeting in the OncoPanel AMC version 4.3

Genes	<p>1) Entire exonic sequence were included for detection of Base Substitution, Insertions/Deletions, and Copy Number Alterations</p> <p><i>ABL1, ABL2, AKT1, AKT2, AKT3, ALK, APC, AR, ARAF, ARID1A, ARID1B, ARID2, ASXL1, ATM, ATR, ATRX, AURKA, AURKB, AURKC, AXIN1, AXL, BAP1, BARD1, BRAF, BRCA1, BRCA2, BRD2, BRD3, BRD4, BRIP1, CBF, CCND1, CCND2, CCND3, CCNE1, CD274, CDH1, CDK12, CDK4, CDK6, CDKN1A, CDKN1B, CDKN2A, DKN2B, CDKN2C, CEBPA, CHEK2, CREBBP, CSF1R, CTNNA1, DDR1, DDR2, DDX3X, DNMT3A, DOT1L, DPYD, EGFR, EPHA3, EPHB4, ERBB2, ERBB3, ERBB4, ERCC2, ERCC4, ERG, ERFF1, ESR1, ETV1, ETV4, ETV5, ETV6, EWSR1, EZH2, FAM175A, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM, FBXW7, FGF19, FGF4, FGFR1, FGFR2, FGFR3, FGFR4, FLCN, FLT1, FLT3, FLT4, FOXL2, FUBP1, GATA2, GEN1, GNA11, GNAQ, GNAS, H3F3A, HDAC9, HGF, HLA-A, HLA-B, HLA-C, HLA-DRB1, HNF1A, HRAS, IDH1, IDH2, IGF1R, IGF2, JAK1, JAK2, JAK3, KDR, KIT, KMT2A, KRAS, LRP1B, LTK, MAP2K1, MAP2K2, MAP2K4, MAP3K1, MAP3K4, MAPK1, MAPK3, MAPK8, MCL1, MDM2, MDM4, MED12, MEN1, MET, MITF, MLH1, MPL, MRE11A, MSH2, MSH6, MTAP, MTOR, MYC, MYCN, NBN, NF1, NF2, NFKB1A, NKX2-1, NOTCH1, NOTCH2, NOTCH3, NOTCH4, NRAS, NTRK1, NTRK2, NTRK3, NUTM1, PALB2, PARP1, PBRM1, PDGFB, PDGFRA, DGFRB, PIK3CA, PIK3CB, PIK3CD, PIK3R1, PIK3R2, PMS2, POLE, PPARG, PTCH1, PTCH2, PTEN, PTPN11, RAD50, RAD51, RAD51C, RAD51D, RAF1, RARA, RB1, RET, RICTOR, RNF43, ROS1, RSPO1, RSPO2, RUNX1, SDHA, SDHB, SDHC, SDHD, SETD2, SLX4, SMAD2, SMAD4, SMARCA4, SMARCB1, SMO, SOX2, SOX9, SPOP, SRC, STK11, SYK, TERT, TET2, TMPRSS2, TOP1, TOP2A, TP53, TSC1, TSC2, UBE2T, VHL, WT1, XPO1, XRCC2, ZNRF3</i></p>
	<p>2) Partial intronic sequence were included for detection of Rearrangements</p> <p><i>ALK, EGFR, NTRK1, RET, ROS1, BRAF</i></p>
	<p>3) Hot spot was included for detection of Base Substitution, Insertions/Deletions</p> <p><i>A1BG, ABCC5, ACVR2A, ADAMTS18, ADNP, AKAP7, AP1S1, ARV1, ASH1L, BAX, CASD1, CBX4, CCDC73, CD3G, CDH26, CEBPZ, CENPV, CKAP2, CLOCK, COBLL1, CPEB2, CRIPAK, DLC1, DNAH12, DOCK3, DPAGT1, DYNC1I2, EBPL, EPPK1, FBXL3, FGFBP1, FMN2, FRG2B, FXR1, GRIN3B, GTPBP2, HIAT1, IMPA1, INO80E, IRS1, KCTD16, KIAA1919, KRT32, LIPT1, MADCAM1, MVK, MYO1A, NIPA2, NNAT, NOS3, NOTCH2NL, NUDT7, OR4M2, PABPC1, PCBP1, PCDHB16, PCMTD1, PREX2, PRIM2, RASA4, RBBP8, RGS12, RHOA, RUFY2, SEC63, SF3B1, SLC23A2, SPRR3, SSTR4, STAMBPL1, STAU2, SULT6B1, SYNJ2, TAS2R19, TAS2R31, TCF7L2, TEAD2, TMEM60, TMPRSS13, TPSD1, WDR55, WDR87, ZFP37, ZNF141, ZNF563, BTK, CBL, IFITM1, IFITM3, KNSTRN, MAX, NFE2L2, PPP2R1A, PTPN11, RAC1, STAT3, U2AF1, KLF4, CIC, GATA3, KIF5B, ADCYAP1, IMPA2, MIR3648-1, DX3X</i></p>

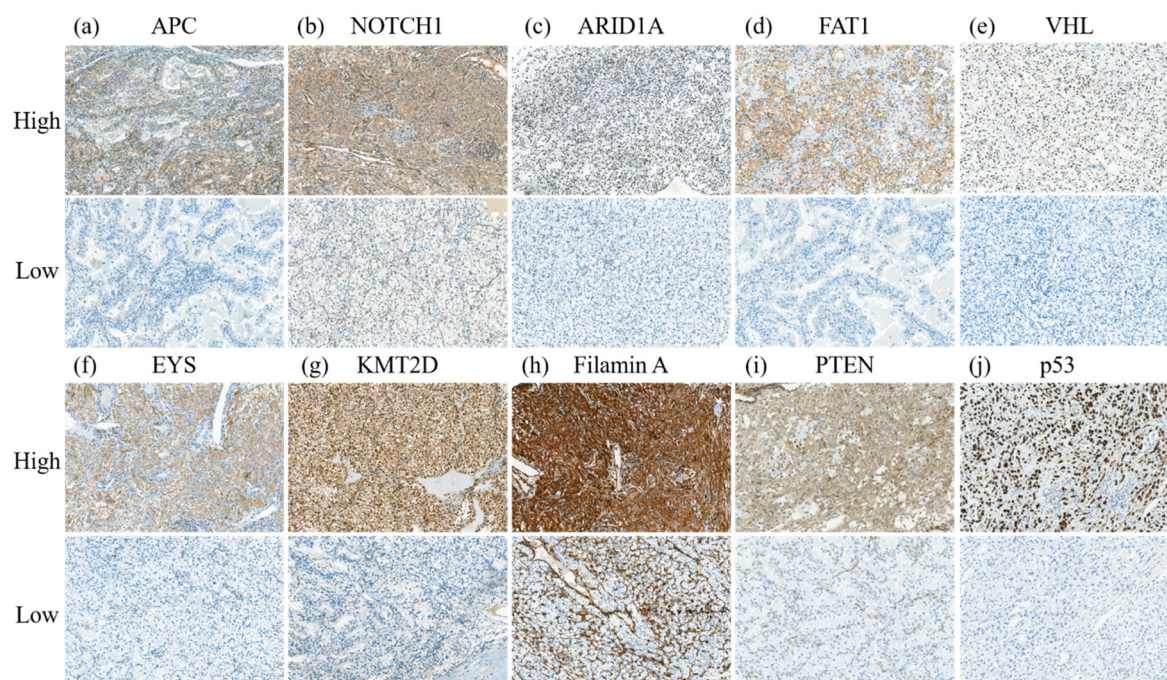


Figure S1. Representative images depicting high and low expression of APC (a), NOTCH1 (b), ARID1A (c), FAT1 (d), VHL (e), EYS (f), KMT2D (g), Filamin A (h), PTEN (i), and p53 (j) in clear cell renal cell carcinoma (x200). APC, NOTCH1, FAT1, EYS, Filamin A, and PTEN were expressed in the cytoplasm, and ARID1A, VHL, KMT2D, and p53 were expressed in the nucleus.

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