Development and validation of a combined hypoxia and immune prognostic classifier for head and neck cancer

Brooks, Jill; Antao Mobre De Menezes, Albert Rahul Eugene; Ibrahim, Maha; Archer, Lucinda; Lal, Neeraj; Bagnall, Chris; Zeidler, Sandra Ventorin von; Valentine, Helen; Spruce, Rachel; Batis, Nikolaos; Bryant, Jennifer; Hartley, Margaret; Kaul, Baksho; Ryan, Gordon; Bao, Riyue; Khattri, Arun; Lee, Steve; Ogbureke, Kalu U.E.; Middleton, Gary; Tennant, Daniel

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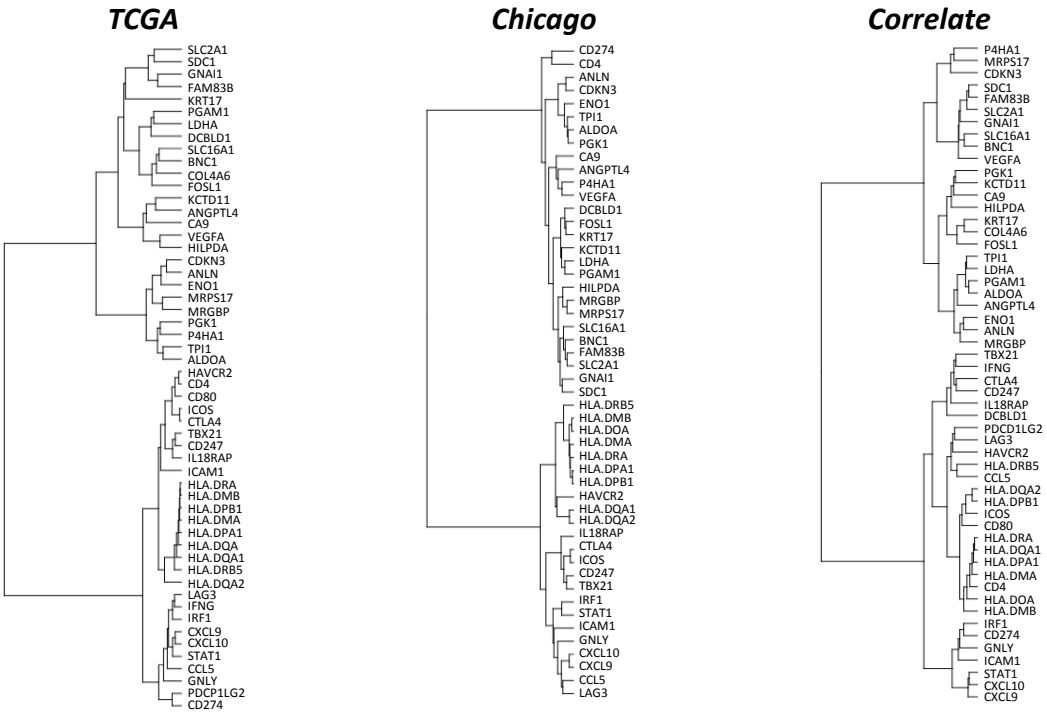
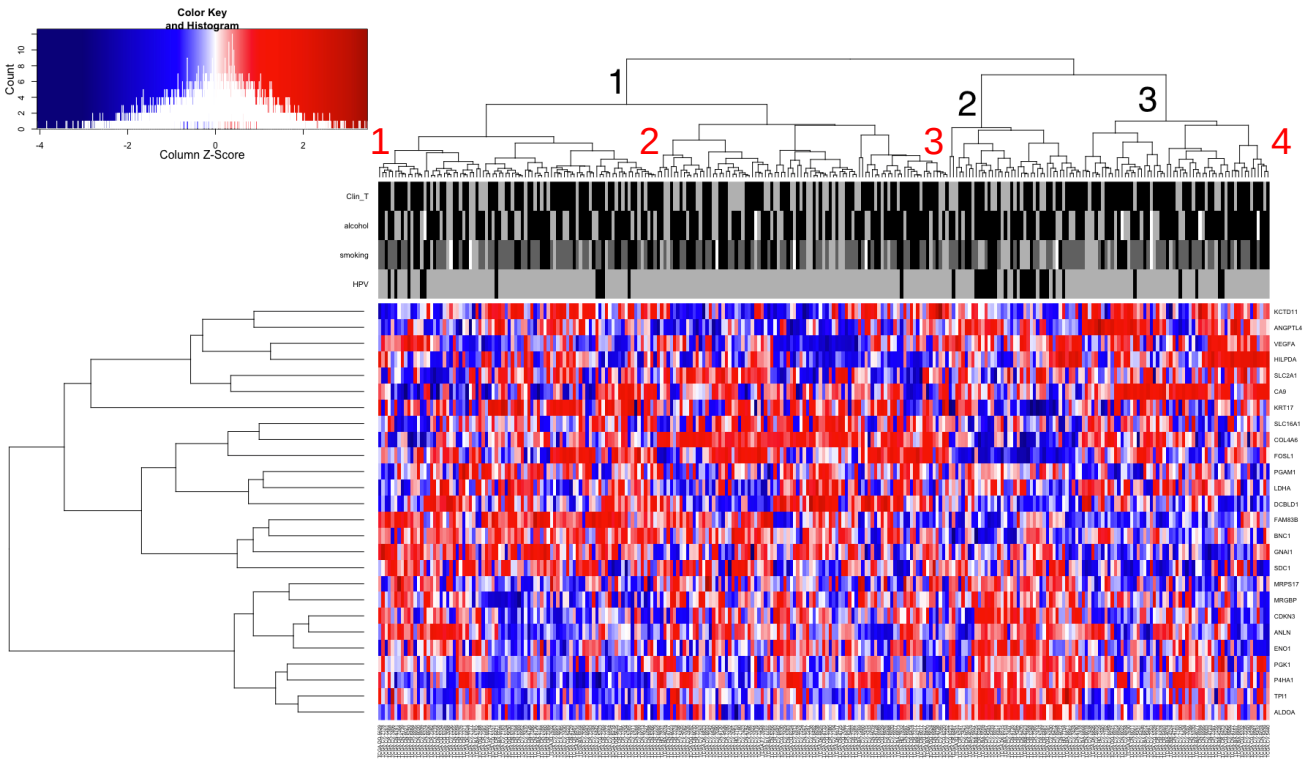
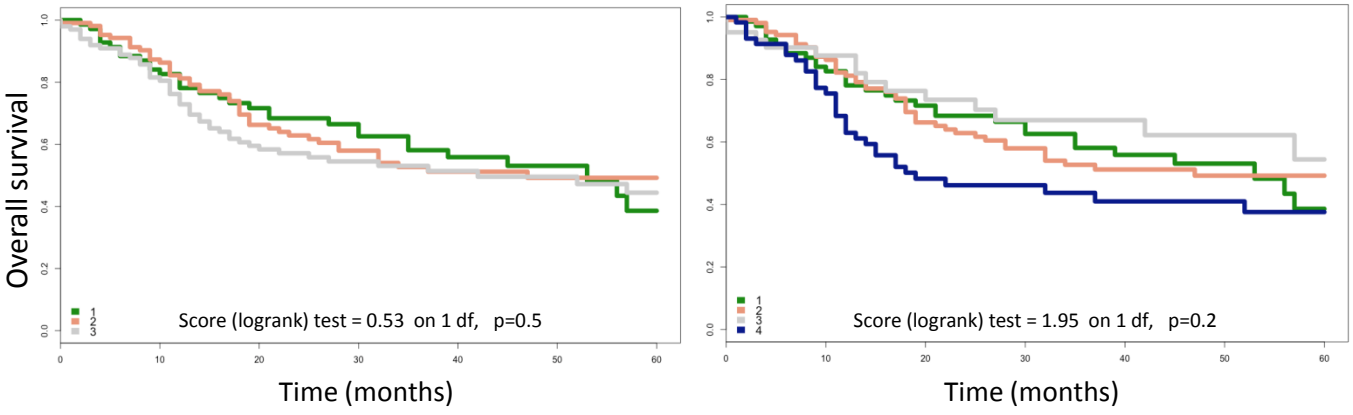


Figure S1. Gene order within the cluster defined groups.

A


Eustace hypoxia signature
Color Key and Histogram
Count
Column Z-Score
1
2
3
4
Clin_T
alcohol
smoking
HPV
KCTD11
ANGPTL4
VEGFA
HILPDA
SLC2A1
CA9
KRT17
SLC16A1
COL4A6
FOSL1
PGAM1
LDHA
DCBLD1
FAM83B
BNC1
GNAI1
SDC1
MRPS17
MRGBP
CDKN3
ANLN
ENO1
PGK1
P4HA1
TPI1
ALDOA

B


Overall survival
Score (logrank) test = 0.53 on 1 df, p=0.5
Time (months)
Score (logrank) test = 1.95 on 1 df, p=0.2
Time (months)

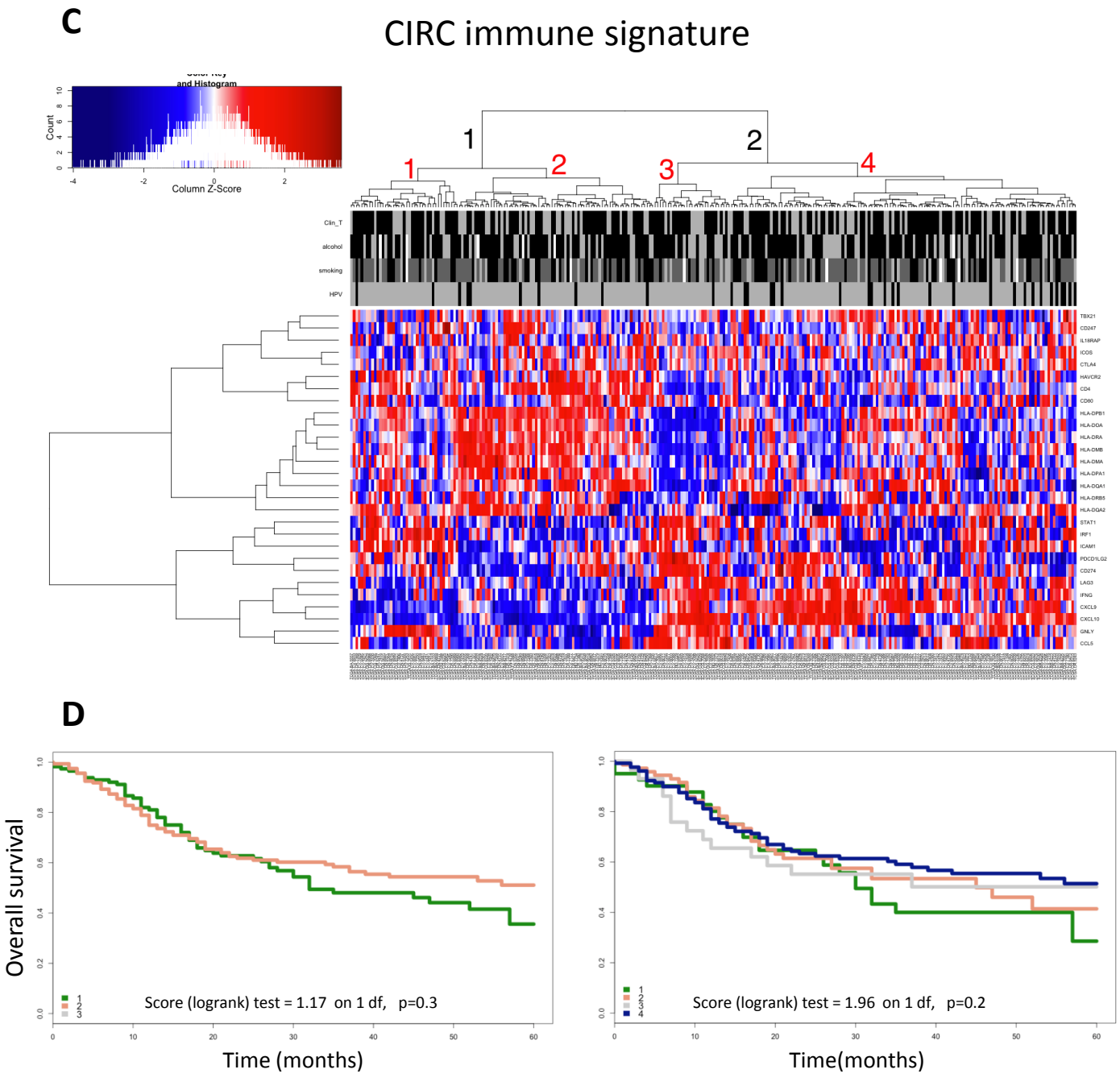
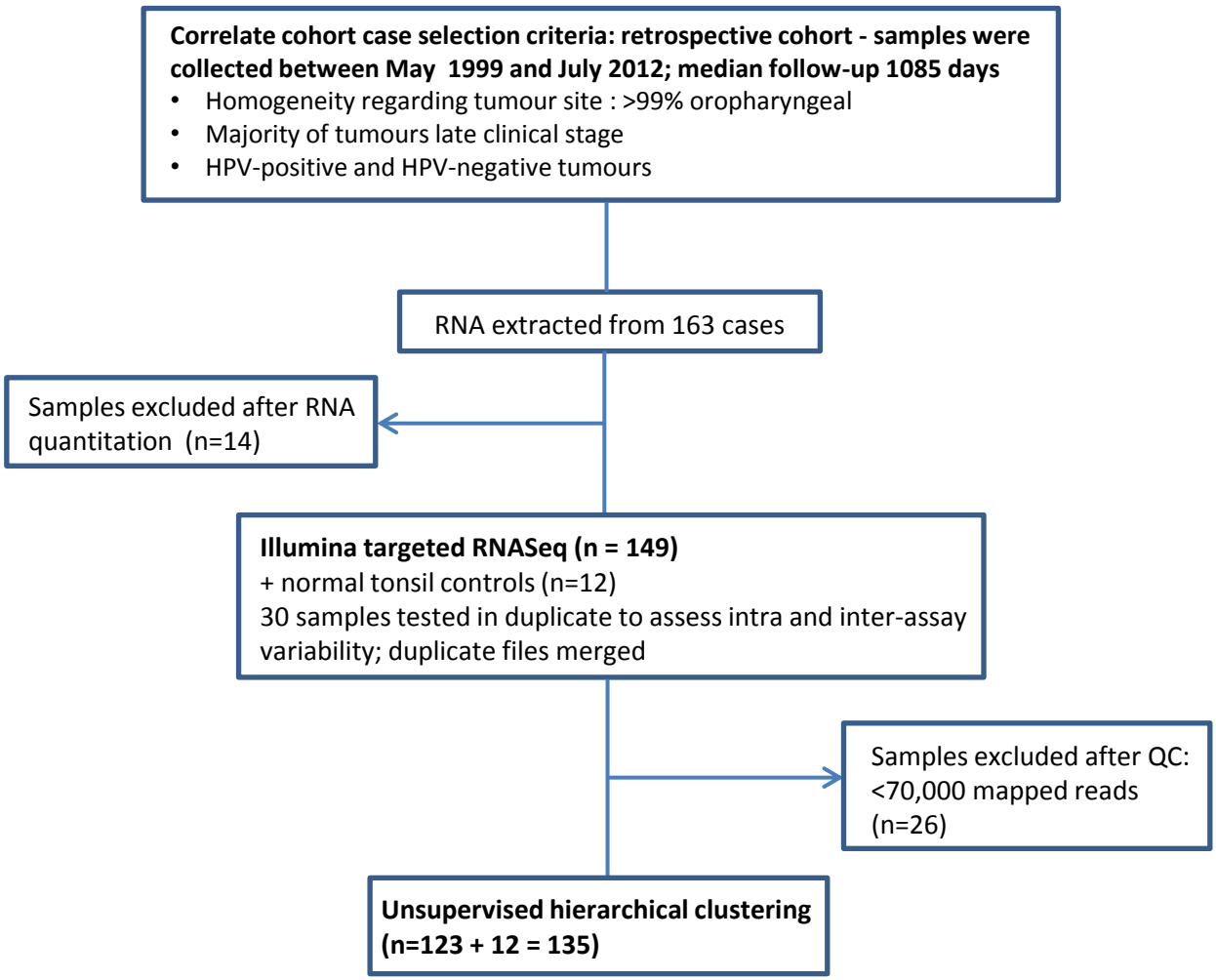


Figure S2. Analysis of individual hypoxia and immune gene signatures. Two dimensional hierarchical clustering of (A) hypoxia- and (C) immune response-related genes for the TCGA dataset does not identify comparable HNC patient subgroups with co-ordinate gene expression. (B and D) Kaplan-Meier survival plots for OS stratified according to the heatmap cluster-defined subgroups, for (B) Eustace hypoxia signature analysed using three or four highest hierarchical sample groups, and (D) CIRC immune signature analysed using two or four groups. Data are censored at 5 years.

Figure S3. Flow diagram showing progress of patient samples through study for Correlate cohort



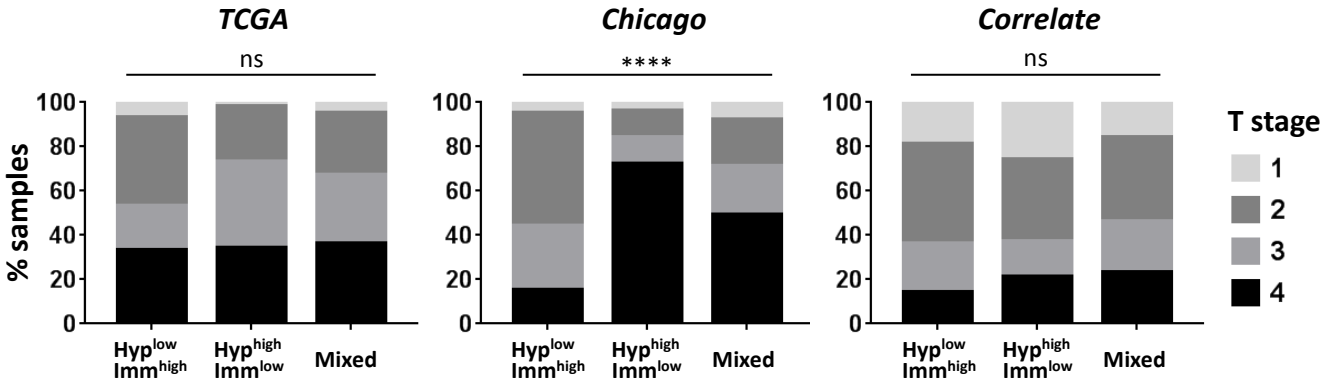


Figure S4. Relationship between heatmap defined hypoxia-immune subgroups and T stage. Graphs display percentage of samples classified as T stage 1, 2, 3 or 4 belonging to the hypoxia^{low}immune^{high}, hypoxia^{high}immune^{low} and mixed subgroups, for the TCGA (left), Chicago (middle) and Correlate (right) cohorts. Data were analysed using Fisher's exact test. p values are reported as: ns non-significant *<0.05, **<0.005, ***<0.0005, ****<0.0001.

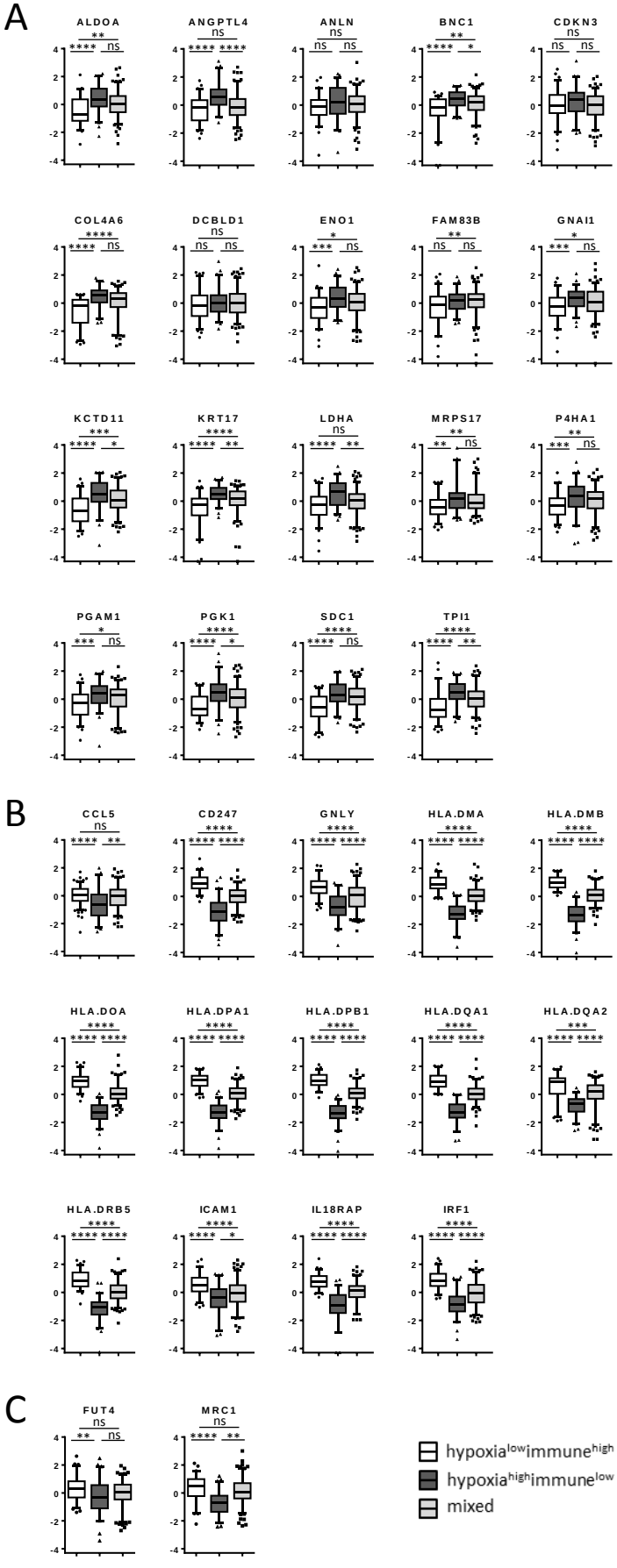


Figure S5 Expression of individual hypoxia- or immune-response related genes within the heat-map defined subgroups. Box-and-whisker plots showing normalised z scores for (A) Eustace hypoxia, (B) CIRC immune and (C) myeloid-related genes acquired from TCGA dataset. The hypoxia^{low}immune^{high}, hypoxia^{high}immune^{low} and mixed subgroups (as defined by two dimensional unsupervised hierarchical clustering of gene expression) are represented as white, dark grey and light grey respectively. Data were analysed using Kruskal-Wallis test with Dunn's correction; p values are reported as: ns non-significant *<0.05, **<0.005, ***<0.0005, ****<0.0001.

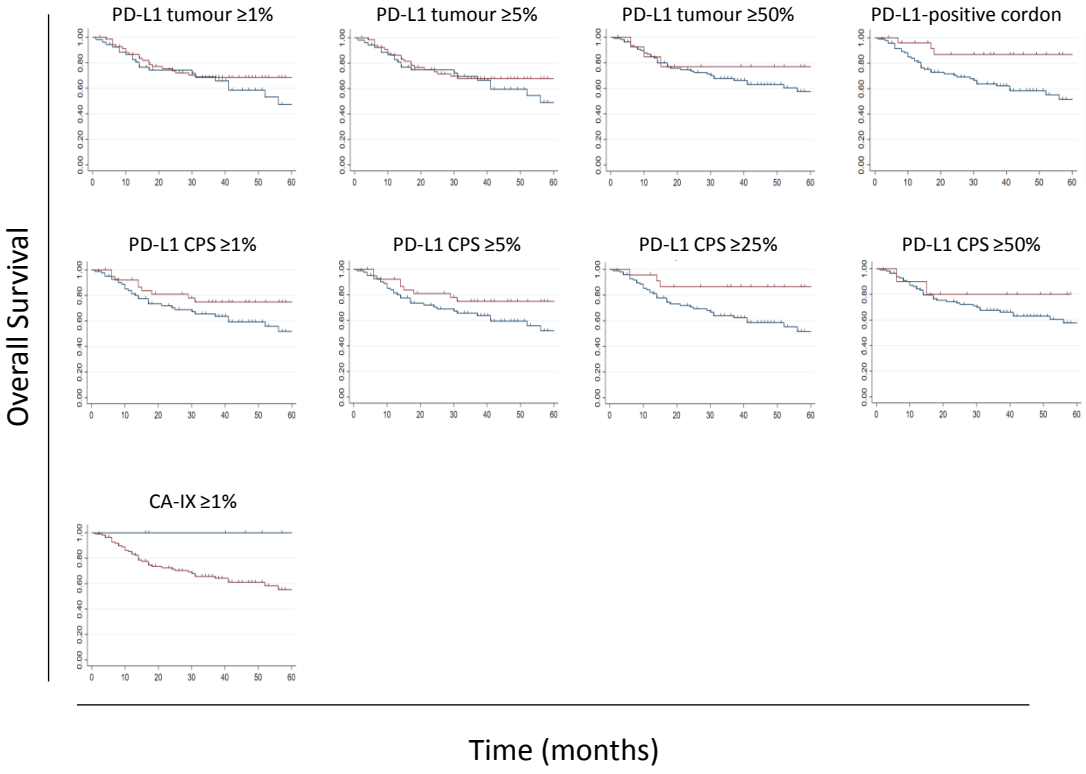
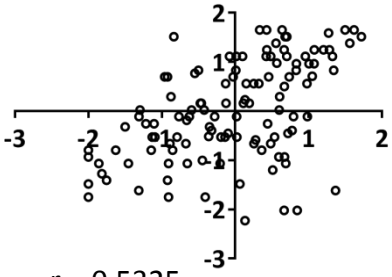


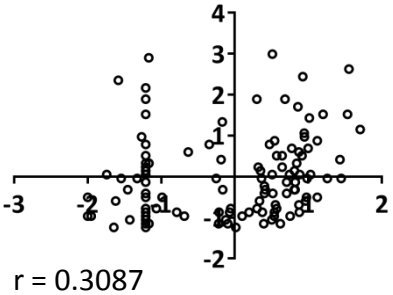
Figure S6. Correlation of PD-L1 or CA-IX protein expression with survival. Kaplan-Meier curves for OS survival relative to immune (PD-L1) or hypoxia (CA-IX) marker expression. Data are censored at 5 years.

CD3ε



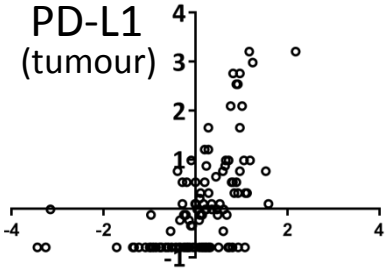
r = 0.5325
p = <0.0001

LAG3



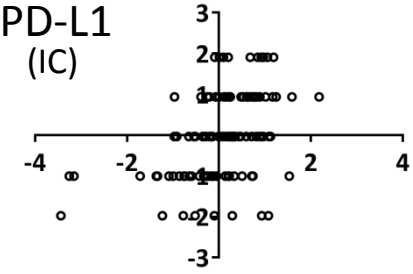
r = 0.3087
p = 0.0006

PD-L1
(tumour)



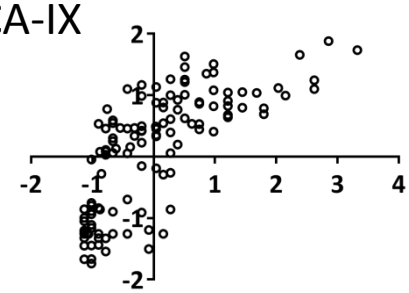
r = 0.5578
p = <0.0001

PD-L1
(IC)



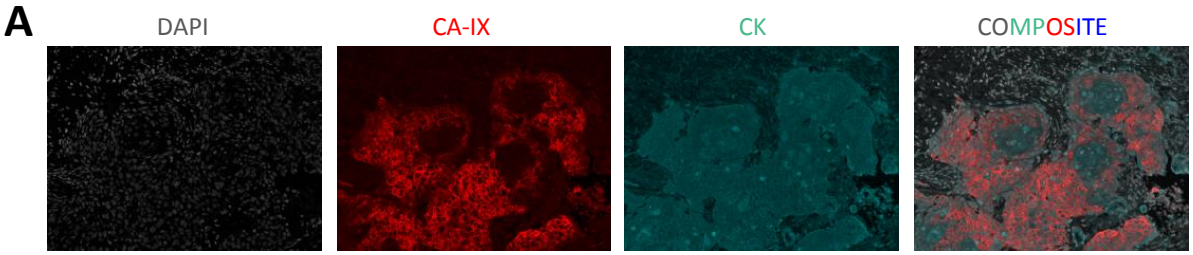
r = 0.4371
p = <0.0001

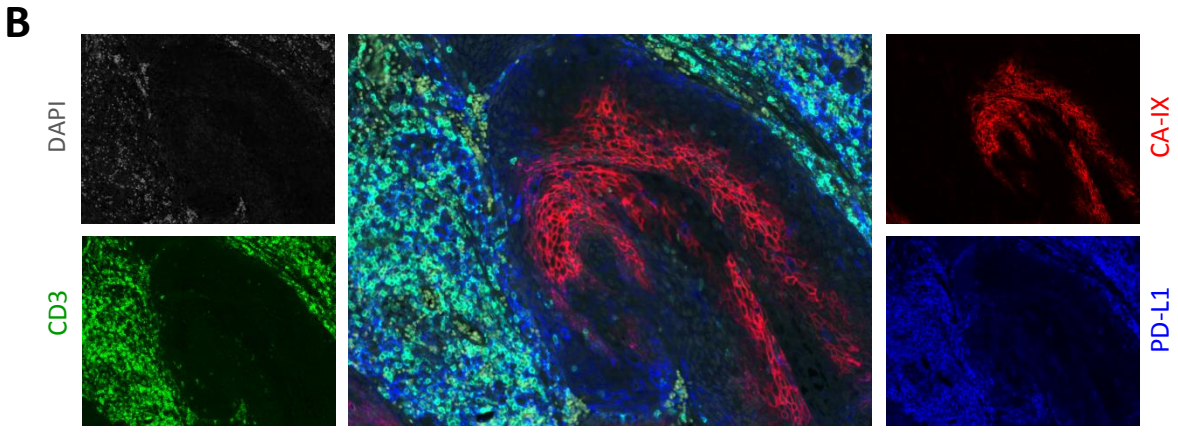
CA-IX

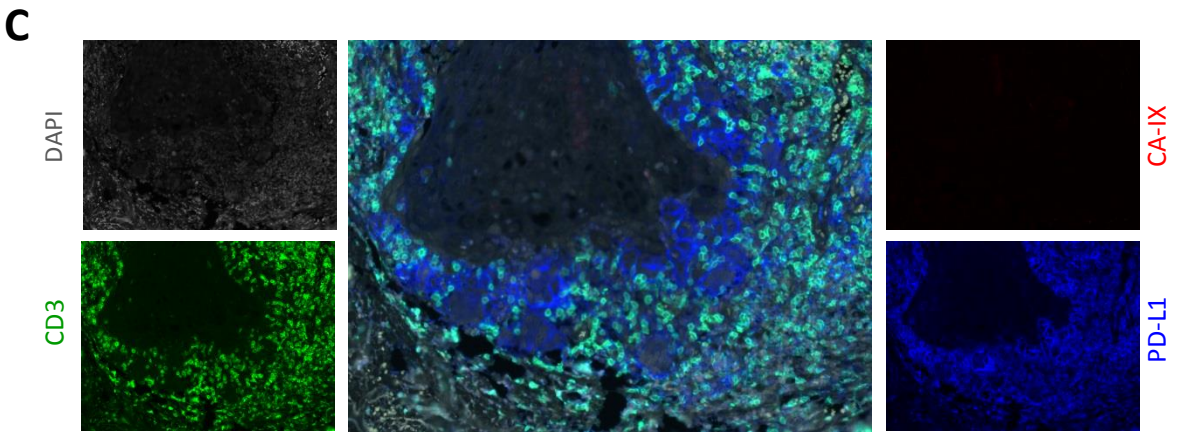


r = 0.7935
p = <0.0001

Figure S7 Correlation of gene and protein expression. Normalised gene expression versus IHC scores for CD3e, LAG3, PD-L1 and CA-IX show strong positive correlation. Data were analysed using Spearman's correlation.







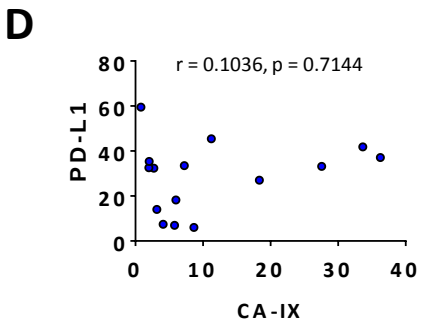


Figure S8. Spatial interactions of hypoxia and immune cells: Vectra images of multiplex stained HNC sections. (A) Co-localisation of CA-IX staining (red) with pan-cytokeratin staining (aqua) as a marker of tumour cells. (B and C) High power (20x) images from the same tumour section as in Figure 5B illustrating immune exclusion in the presence (B) and absence (C) of CA-IX. Note the presence of PD-L1-positive cells with morphology consistent with macrophage identity surrounding tumour nest. (D) InForm image analysis showed no significant correlation between CA-IX and PD-L1 expression within the tumour compartment; graph shows percentage of tumour cells positive for these markers, number of cases = 15. Data were analysed using Spearman's correlation.

Table S1 Treatment schedules

TCGA cohort

Primary surgery + adjuvant C/RT	Primary CRT	Primary RT	Palliative C/RT	Other C/RT*	NA
73	17	3	5	13	164

* received C/RT, but schedule not specified.

NA: not available

Chicago cohort*

CRT only	Induction CT + CRT	NA
37	93	4

* all patients received organ preserving chemoradiotherapy with curative intent

Correlate cohort

Study of origin	Primary surgery + adjuvant C/RT	Primary CRT	Primary RT	Other*	NA
PET-NECK	12	21	39	1	0
PredicTR	23	41	18	7	1

* includes CT only, surgery only and none.

Patients within the PET-NECK trial were randomised to receive primary surgery + adjuvant C/RT versus primary C/RT; patients within the PredicTR study received treatment based on clinician's choice.

Table S2. Genes in the RNASeq panel/TCGA dataset

Genes in hypoxia-immune signature				Additional genes in RNASeq panel						Analysed using TCGA dataset
Eustace		CIRC		Other immune function-related		Frequently mutated in HNC		Reference control		
ALDOA	NM_000034.3	CCL5	NM_001278736.1	CD163	XM_005253529.4	CASP8	NM_001080124.1	ACTB	NM_001101.3	
ANGPTL4	NM_001039667.2	CD247	NM_000734.3	CD3D	NM_000732.4	EGFR	NM_005228.3			ARG1
ANLN	NM_001284301.2	CD274	NM_001267706.1	CD3E	NM_000733.3	NOTCH1	NM_017617.4			CD14
BNC1	NM_001301206.1	CD4	NM_000616.4	CD3G	NM_000073.2	PIK3CA	NM_006218.3			CD27
CA9	NM_001216.2	CD80	NM_005191.3	CD68	NM_001040059.1	PTEN	NM_000314.6			CD33
CDKN3	NM_001130851.1	CTLA4	NM_001037631.2	CD8A	NM_001145873.1	TP53	NM_000546.5			CD40
COL4A6	NM_001287758.1	CXCL10	NM_001565.3	CD8B	NM_001178100.1					CD163
DCBLD1	NM_173674.2	CXCL9	NM_002416.2	CDKN2A	NM_000077.4					ENTPD1
ENO1	NM_001201483.1	GNLY	NM_001302758.1	CIITA	NM_000246.3					FUT4
FAM83B	NM_001010872.2	HAVCR2	NM_032782.4	FOXP3	NM_001114377.1					IDO1
FOSL1	NM_001300844.1	HLA-DMA	NM_006120.3	GZMB	NM_004131.4					MRC1
GNAI1	NM_001256414.1	HLA-DMB	NM_002118.4	HLA-A	NM_001242758.1					NOS1
HILPDA	NM_001098786.1	HLA-DOA	NM_002119.3	HLA-B	NM_005514.7					NT5E
KCTD11	NM_001002914.2	HLA-DPA1	NM_001242524.1	HLA-C	NM_001243042.1					ORL1
KRT17	NM_000422.2	HLA-DPB1	NM_002121.5	IL12A	NM_000882.3					PRF1
LDHA	NM_001135239.1	HLA-DQA1	NM_002122.3	IL12B	NM_002187.2					TNFRSF4
MRGBP (C20orf20)	NM_018270.5	HLA-DQA2	NM_020056.4	IL18	NM_001243211.1					
MRPS17	NM_015969.2	HLA-DRA	NM_019111.4	IL6	NM_000600.4					
P4HA1	NM_000917.3	HLA-DRB5	NM_002125.3	PDCD1	NM_005018.2					
PGAM1	NM_001317079.1	ICAM1	NM_000201.2	PIAS1	NM_001320687.1					
PGK1	NM_000291.3	ICOS	NM_012092.3	STAT3	NM_003150.3					
SDC1	NM_001006946.1	IFNG	NM_000619.2	TNFRSF9	XM_011541386.2					
SLC16A1	NM_001166496.1	IL18RAP	NM_003853.3							
SLC2A1	NM_006516.2	IRF1	NM_002198.2							
TPI1	NM_000365.5	LAG3	NM_002286.5							
VEGFA	NM_001025366.2	PDCD1LG2	NM_025239.3							
		STAT1	NM_007315.3							
		TBX21	NM_013351.1							

Table S3. Relationship of hypoxia-immune classifier to other prognostic variables

	TCGA	Chicago	Correlate
Hypoxia-immune gene classifier	**10.08 on 1 df, p=0.0015**	**6.32 on 1 df, p=0.012**	2.76 on 1 df, p=0.097
Age	2.18 on 1 df, p=0.1402	1.09 on 1 df, p=0.2964	3.26 on 1 df, p=0.07
Gender	3.5 on 1 df, p=0.0614	0.22 on 1 df, p=0.6395	0.85 on 1 df, p=0.3553
Tobacco use	1.73 on 1 df, p=0.1885	1.37 on 1 df, p=0.2419	0.68 on 1 df, p=0.4083
Alcohol consumption	0.15 on 1 df, p=0.6993	2.93 on 1 df, p=0.09	2.55 on 1 df, p=0.1101
T-stage	0.2 on 1 df, p=0.6583	0.83 on 1 df, p=0.3628	2.39 on 1 df, p=0.122
N-stage	2.17 on 1 df, p=0.1408	0.07 on 1 df, p=0.7976	2.19 on 1 df, p=0.1389
Clinical stage	0.11 on 1 df, p=0.7391	0.94 on 1 df, p=0.331	0.35 on 1 df, p=0.6
HPV	**6.66 on 1 df, p=0.00983**	**8.83 on 1 df, p=0.00296**	
p16			**16.14 on 1 df, p=5.868e-05**
Hypoxia-immune gene classifier + age	**12.02 on 2 df, p=0.002**	**7.72 on 2 df, p=0.02**	4.24 on 2 df, p=0.1
Hypoxia-immune gene classifier + gender	**13.95 on 2 df, p=9e-04**	**6.32 on 2 df, p=0.04**	3.58 on 2 df, p=0.2
Hypoxia-immune classifier + tobacco use	**10.61 on 2 df, p=0.005**	**8.71 on 2 df, p=0.01**	3.9 on 2 df, p=0.1
Hypoxia-immune classifier + alcohol consumption	**9.37 on 2 df, p=0.009**	**6.82 on 2 df, p=0.03**	**6.5 on 2 df, p=0.04**
Hypoxia-immune gene classifier + T-stage	**10.78 on 2 df, p=0.005**	5.54 on 2 df, p=0.06	3.6 on 2 df, p=0.2
Hypoxia-immune gene classifier + N-stage	**12.33 on 2 df, p=0.002**	5.5 on 2 df, p=0.06	4.33 on 2 df, p=0.1
Hypoxia immune classifier + clinical stage	**10.08 on 2 df, p=0.006**	**6.33 on 2 df, p=0.04**	2.48 on 2 df, p=0.3

Table shows log-rank p-values for single prognostic variables and for the hypoxia-immune classifier adjusted for each of these clinical prognosticators. Values in bold indicate statistical significance (<0.05).

Table S4. Summary of immunohistochemistry staining

	All OPSCC n = 163 (%)		P16-positive n = 93 (%)		P16-negative n = 70 (%)		P-value*
TILs							
High	52	(31.9)	46	(49.5)	6	(8.6)	**<0.0001**
Low	110	(67.5)	47	(50.5)	63	(90.0)	
Not available	1	(0.6)	0	(0.0)	1	(1.4)	
CD3							
Intratumoural							
High	86	(52.8)	61	(65.6)	25	(35.7)	**0.0002**
Low	75	(46.0)	31	(33.3)	44	(62.9)	
Not available	2	(1.2)	1	(1.1)	1	(1.4)	
Peritumoural							
Stroma	66	(40.5)	52	(55.9)	14	(20.0)	**<0.0001**
High	94	(57.7)	39	(41.9)	55	(78.6)	
Low	3	(1.8)	2	(2.2)	1	(1.4)	
Not available							
Tumour margin	72	(44.2)	56	(60.2)	16	(22.8)	**<0.0001**
High	80	(49.1)	32	(34.4)	48	(68.6)	
Low	11	(6.7)	5	(5.4)	6	(8.6)	
Not available							
Combined	74	(45.4)	59	(63.4)	15	(21.4)	**<0.0001**
High	87	(53.4)	33	(35.5)	54	(77.2)	
Low	2	(1.2)	1	(1.1)	1	(1.4)	
Not available							
PD-L1							
Tumour cells							
Positive (≥1%)	83	(50.9)	55	(59.1)	28	(40.0)	**0.0126**
Negative (<1%)	79	(48.5)	37	(39.8)	42	(60.0)	
Positive (≥5%)	81	(49.7)	5339	(57.0)	28	(40.0)	**0.0264**
Negative (<5%)	81	(49.7)		(41.9)	42	(60.0)	
			31				
Positive (≥25%)	48	(29.5)	61	(33.3)	17	(24.3)	0.1938
Negative (<25%)	114	(69.9)		(65.6)	53	(75.7)	
			10				
Positive (≥50%)	17	(10.4)	82	(10.7)	7	(10.0)	0.8580
Negative (<50%)	145	(90.0)		(88.2)	63	(90.0)	
Immune cells			36				
High	50	(30.7)	56	(38.7)	14	(20.0)	**0.009**
Low	112	(68.7)		(60.2)	56	(80.0)	
PD-L1$^+$ cordon			24				
Present	30	(18.4)	68	(25.8)	6	(8.6)	**0.0045**
Absent	132	(81.0)	1	(73.1)	64	(91.4)	
Not available	1	(0.6)		(1.1)	0	(0.0)	
LAG3							
Intratumoural							
High	83	(50.9)	62	(66.7)	21	(30.0)	**<0.0001**
Low	73	(44.8)	29	(31.2)	44	(62.9)	
Peritumoural							
High	72	(44.2)	53	(57.0)	19	(27.2)	**0.0003**
Low	84	(51.5)	38	(40.9)	46	(65.7)	
Combined							
High	81	(49.7)	60	(64.5)	21	(30.0)	**<0.0001**
Low	75	(46.0)	31	(33.3)	44	(62.9)	
Not available	7	(4.3)	2	(2.2)	5	(7.1)	
CA-IX							
Positive (≥10%)	109	(66.9)	65	(69.9)	44	(62.9)	0.2949
Negative	53	(32.5)	27	(29.0)	26	(37.1)	
Positive (>1%)	144	(88.4)	85	(91.4)	60	(85.7)	0.1696
Negative	18	(11.0)	7	(7.5)	10	(14.3)	
Not available	1	(0.6)	1	(1.1	0	(0.0)	

*comparison of immune/hypoxia marker expression p16-positive versus negative cases (Fisher's exact test)

Table S5. Summary of statistical analyses for IHC staining

Variable	Cut-off	HR (CI)	p value	HR (CI)*	p value*
TILs	1 + 2 vs. 3	0.27 (0.12 – 0.66)	**0.004**	0.53 (0.2 – 1.43)	0.208
CD3					
Intratumoural	≤2.3 vs. >2.3	0.33 (0.16 – 0.67)	**0.002**	0.43 (0.19 – 0.97)	**0.043**
Peritumoural stroma	≤2.7 vs. >2.7	0.40 (0.21 – 0.77)	**0.006**	0.51 (0.24 – 1.08)	0.078
Tumour margin	≤2.7 vs. >2.7	0.63 (0.34 – 1.17)	0.146	1.23 (0.58 – 2.65)	0.589
Combined	≤2.6 vs.> 2.6	0.39 (0.20 – 0.77)	**0.007**	0.70 (0.32 – 1.53)	0.366
PD-L1					
Tumour cells	≥1%	0.71 (0.38 – 1.31)	0.273	0.78 (0.38 – 1.60)	0.493
	≥5%	0.75 (0.30 – 1.39)	0.354	0.80 (0.39 – 1.64)	0.537
	≥25%	0.39 (0.17 – 0.89)	**0.025**	0.33 (0.13 – 0.87)	**0.025**
	≥50%	0.63 (0.19 – 2.04)	0.441	0.63 (0.19 – 2.12)	0.458
Immune cells combined	≤1 vs. >1	0.55 (0.27 – 1.12)	0.101	0.45 (0.19 – 1.05)	0.066
Combined positive score	≥1%	0.54 (0.26 – 1.14)	0.105	0.41 (0.17 – 0.99)	**0.048**
	≥5%	0.54 (0.26 – 1.14	0.105	0.41 (0.17 – 0.99)	**0.048**
	≥25%	0.27 (0.08 – 0.86)	**0.028**	0.09 (0.02 – 0.45)	**0.004**
	≥50%	0.53 (0.13 – 2.16)	0.368	0.43 (0.10 – 1.85)	0.255
Cordon	No vs. yes	0.25 (0.08 – 0.82)	**0.022**	0.19 (0.05 – 0.68)	**0.011**
LAG3					
Intratumoural	≥10%	0.60 (0.32 – 1.12)	0.106	0.94 (0.46 – 1.93)	0.866
	≥25%	0.34 (0.12 – 0.96)	**0.042**	0.46 (0.15 – 1.43)	0.179
Peritumoural	≥10%	0.67 (0.36 – 1.27)	0.219	0.77 (0.37 – 1.63)	0.499
	≥25%	0.19 (0.03 – 1.39)	0.103	0.22 (0.03 – 1.74)	0.151
Combined	≥10%	0.53 (0.28 – 1.00)	0.051	0.66 (0.32 – 1.36)	0.261
	≥25%	0.30 (0.07 – 1.25)	0.099	0.30 (0.06 – 1.52)	0.147
CA-IX	≥10%	1.23 (0.62 – 2.41)	0.555	1.29 (0.59 – 2.84)	0.521
	≥25%	1.10 (0.59 – 2.04)	0.774	0.79 (0.40 – 1.57)	0.501
	≥50%	0.99 (0.41 – 2.35)	0.977	0.66 (0.26 – 1.70)	0.387

* adjusted for age, gender, T stage, N stage, p16 status, smoking status and alcohol consumption