Tumor DNA-Methylome derived Epigenetic Fingerprint Identifies HPV-negative Head and Neck Patients at Risk for Locoregional Recurrence after Postoperative Radiochemotherapy

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Table of Contents

Supplementary Table 1. Univariate Cox Regression for Progression, for clinical parameters

	Training Cohort (n=128)		**Validation Cohort (n=125)**	
Progression				
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Clinical parameters				
HICR Class	***9.47 (5.13-17.5)***	***<10^{-13}***	***2.09 (1.13-3.89)***	***0.020***
Female Gender	1.03 (0.51-2.08)	0.94	0.93 (0.43-2.00)	0.85
Age	0.97 (0.94-1.01)	0.13	0.98 (0.96-1.01)	0.29
Current Smoker	1.46 (0.35-6.17)	0.60	1.27 (0.44-3.62)	0.66
Never Smoker	0.68 (0.16-2.88)	0.60	0.79 (0.27-2.26)	0.66
Never Drinker	1.07 (0.39-2.65)	0.97	NA	NA
p16 IHC positive	0.49 (0.17-1.38)	0.18	NA	NA
Localization				
Oral Cavity	Reference		Reference	
Oropharynx	***0.50 (0.26-0.97)***	***0.04***	0.53 (0.26-1.08)	0.08
Hypopharynx	0.76 (0.33-1.73)	0.51	0.71 (0.29-1.72)	0.45
pT-stage				
T1-T2	***0.43 (0.24-0.80)***	***0.007***	***0.44 (0.24-0.80)***	***0.0075***
T3-T4	***2.3 (1.26-4.2)***	***0.007***	***2.27 (1.24-4.13)***	***0.0075***
pN-stage				
N0-N1	0.56 (0.27-1.24)	0.16	0.79 (0.42-1.48)	0.46
N2-N3	1.74 (0.80-3.76)	0.16	1.26 (0.67-2.37)	0.46
UICC stage				
II	Reference		*No events**	
III	0.97 (0.11-8.13)	0.98	NA	
IV	1.39 (0.19-10.25)	0.75	NA	
Resection Margins				
R0	0.82 (0.45-1.50)	0.53	1.08 (0.53-2.20)	0.83
R1	1.21 (0.66-2.21)	0.53	0.92 (0.45-1.88)	0.83
ECE				
Positive	***1.90 (1.02-3.55)***	***0.043***	1.50 (0.91-2.75)	0.20
Negative	***0.53 (0.28-0.98)***	***0.043***	0.67 (0.36-1.24)	0.20
Chemotherapy				
None	Not applicable		Reference	
Cisplatin	Not applicable		0.79 (0.37-1.61)	0.50
Other	Not applicable		0.53 (0.21-1.36)	0.50
Radiation Dose	1.01 (0.89-1.14)	0.89	1.10 (0.94-1.3)	0.24

Supplementary Table 2. Univariate Cox Regression for LR for clinical parameters

	Training Cohort (n=128)		Validation Cohort (n=125)	
Local Recurrence				
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Clinical parameters				
HICR Class	***13.1 (5.91-29.0)***	***<2x10^{-10}***	***2.90 (1.33-6.34)***	***0.008***
Female Gender	1.49 (0.67-3.40)	0.35	1.04 (0.42-2.53)	0.94
Age	0.97 (0.94-1.01)	0.13	0.98 (0.95-1.02)	0.25
Current Smoker	0.86 (0.20-3.78)	0.84	1.74 (0.40-7.49)	0.50
Never Smoker	1.24 (0.29-5.30)	0.77	0.58 (0.13-2.49)	0.46
Never Drinker	1.33 (0.44-3.99)	0.61	NA	NA
p16 IHC positive	0.64 (0.19-2.12)	0.46	NA	NA
Localization				
Oral Cavity	Reference		Reference	
Oropharynx	***0.42 (0.19-0.95)***	***0.037***	0.42 (0.17-1.04)	0.06
Hypopharynx	0.34 (0.10-1.18)	0.089	0.63 (0.21-1.82)	0.39
pT-stage				
T1-T2	***0.38 (0.17-0.82)***	***0.013***	***0.42 (0.21-0.87)***	***0.02***
T3-T4	***2.65 (1.22-5.74)***	***0.013***	***2.35 (1.15-4.83)***	***0.02***
pN-stage				
N0-N1	0.73 (0.29-1.82)	0.50	1.01 (0.49-2.11)	0.97
N2-N3	1.37 (0.55-3.40)	0.50	0.99 (0.48-2.05)	0.97
UICC stage				
II	Reference		*No events**	
III	0.69 (0.08-6.26)	0.74	NA	
IV	0.89 (0.11-6.73)	0.91	NA	
Resection Margins				
R0	0.79 (0.37-1.68)	0.54	0.81 (0.37-1.80)	0.50
R1	1.27 (0.59-2.71)	0.54	1.31 (0.60-2.87)	0.50
ECE				
Positive	***1.60 (0.74-3.48)***	***0.23***	1.29 (0.61-2.71)	0.51
Negative	***0.62 (0.29-1.35)***	***0.23***	0.78 (0.37-1.64)	0.51
Chemotherapy				
None	Not applicable		Reference	
Cisplatin	Not applicable		0.45 (0.17-1.18)	0.10
Other	Not applicable		0.26 (0.06-1.09)	0.066
Radiation Dose	1.11 (0.94-1.32)	0.23	1.05 (0.89-1.23)	0.59

Supplementary Table 3. Univariate Cox Regression, for OS, for clinical parameters

	Training Cohort (n=128)		Validation Cohort (n=125)	
Overall Survival				
Clinical parameters	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
HICR Class	***5.5 (3.09-9.77)***	***<10^{-9}***	***1.81 (1.13-2.89)***	***0.014***
Female Gender	1.17 (0.62-2.20)	0.63	0.86 (0.47-1.57)	0.49
Age	0.98 (0.95-1.02)	0.35	1.01 (0.99-1.03)	0.38
Current Smoker	1.91 (0.45-8.15)	0.38	1.13 (0.53-2.41)	0.74
Never Smoker	0.52 (0.12-2.23)	0.38	0.88 (0.41-1.87)	0.74
Never Drinker	1.23 (0.51-3.0)	0.64	NA	NA
p16 IHC positive	0.69 (0.29-1.61)	0.39	NA	NA
Localization				
Oral Cavity	Reference		Reference	
Oropharynx	0.59 (0.33-1.07)	0.08	0.66 (0.38-1.13)	0.13
Hypopharynx	***0.34 (0.13-0.91)***	***0.03***	0.77 (0.39-1.54)	0.46
pT-stage				
T1-T2	***0.54 (0.31-0.95)***	***0.031***	0.67 (0.42-1.08)	0.1
T3-T4	***1.85 (1.06-3.2)***	***0.031***	1.48 (0.93-2.38)	0.1
pN-stage				
N0-N1	0.87 (0.46-1.65)	0.68	0.88 (0.55-1.42)	0.6
N2-N3	1.14 (0-61-2.16)	0.68	1.14 (0.70-1.83)	0.6
UICC stage				
II	Reference		Reference	
III	1.01 (0.22-4.66)	0.99	1.49 (0.43-5.12)	0.53
IV	0.88 (0.21-3.7)	0.86	2.07 (0.64-6.63)	0.22
Resection Margins				
R0	0.89 (0.51-1.55)	0.67	0.96 (0.56-1.65)	0.89
R1	1.13 (0.65-1.97)	0.67	1.04 (0.61-1.78)	0.89
ECE				
Positive	***1.83 (1.03-3.24)***	***0.040***	1.45 (0.91-2.32)	0.12
Negative	***0.55 (0.31-0.97)***	***0.040***	0.69 (0.43-1.10)	0.12
Chemotherapy				
None	Not applicable		Reference	
Cisplatin-based	Not applicable		0.90 (0.52-1.58)	0.72
Other	Not applicable		0.66 (0.33-1.31)	0.24
Radiation Dose	1.09 (0.96-1.23)	0.18	1.03 (0.92-1.15)	0.24

Supplementary Table 4. Methylation Probes Comprising the Signature

cg	Illumina 450K Manifest (UCSC)/ Additional TSS Annotation[1]	Gene Name	Progression as function of Methylation
cg03234186	ZNF154:TSS200;	Zinc finger protein 154	Lower Risk
cg16638540	ZNF135:TSS200;	Zinc finger protein 135	Higher Risk
cg04294888	TTC28:Body;	Tetratricopeptide repeat domain 28	Lower Risk
cg26755141	SIM1:Body;	SIM bHLH transcription factor 1	Lower Risk
cg05361262	SHB:Body;	SH2 containing adaptor protein B	Higher Risk
cg12413421	RSPO1:3'UTR;	G protein nucleolar 2	Lower Risk
cg17073323	RPL32:3'UTR	ribosomal protein L32	Lower Risk
cg00126261	PRDM13:TSS1500;	PR/SET domain 13	Lower Risk
cg17288142	PCDHB4:TSS200;	Protocadherin-beta-4 precursor	Lower Risk
cg24818200	PCDHB4:TSS1500;	Protocadherin-beta-4 precursor	Lower Risk
cg20768342	MCOLN3:3'UTR;	mucolipin 3	Lower Risk
cg06139099	INPP5A:Body;	inositol polyphosphate-5-phosphatase A	Higher Risk
cg14546128	INPP5A:Body;	inositol polyphosphate-5-phosphatase A	Higher Risk
cg03215137	HOXC9:TSS200;	Homeoboc C9	Higher Risk
cg19802649	GPR153:Body;	G protein-coupled Recepted 154	Lower Risk
cg14431587	FUBP3:Body;	Far Upstream Element Binding Protein 3	Lower Risk
cg00158227	ESPN:Body;	ESPIN	Lower Risk
cg06014401	EOMES:TSS1500;	Eomesodermin	Lower Risk
cg01511557	ELAVL4:Body;	ELAV like RNA binding protein 4	Lower Risk
cg10312566	EEF1AL7:Body;	Eukaryotic Translation Elongation Factor 1 Alpha 1 Pseudogene 9	Lower Risk
cg09893431	DIP2C:Body;	Disco interacting protein homolog C	Lower Risk
cg21623566	DCC:Body;	Netrin 1 receptor [Deleted in Colorectal Carcinoma]	Lower Risk
cg06866237	CUX1:Body;	Cut like homeobox 1	Lower Risk
cg02310658	C12orf62:Body;	Cytochrome c oxidase assembly factor COX14	Lower Risk
cg09045262	AKR7L:TSS1500;	Aldo keto reductase family 7 like	Higher Risk

cg12255897	AKR1E2:TSS200;	Ado-keto reductase family 1 member E2)	Lower Risk
cg14614844	ESM1	endothelial cell specific molecule 1	Lower Risk
cg13869401	:; ESM1	endothelial cell specific molecule 1	Lower Risk
cg00459623	:; EVX2	even skipped homeobox 2	Lower Risk
cg00760950	:; FLJ34747		Lower Risk
cg24399430	:; IRGC	immunity related GTPase cinema	Lower Risk
cg11130317	:; JA429130		Higher Risk

Supplementary Table 5. Effect of biological parameters (hypoxia, CSCs and immune cell markers) on disease progression and OS in the methylation high risk group

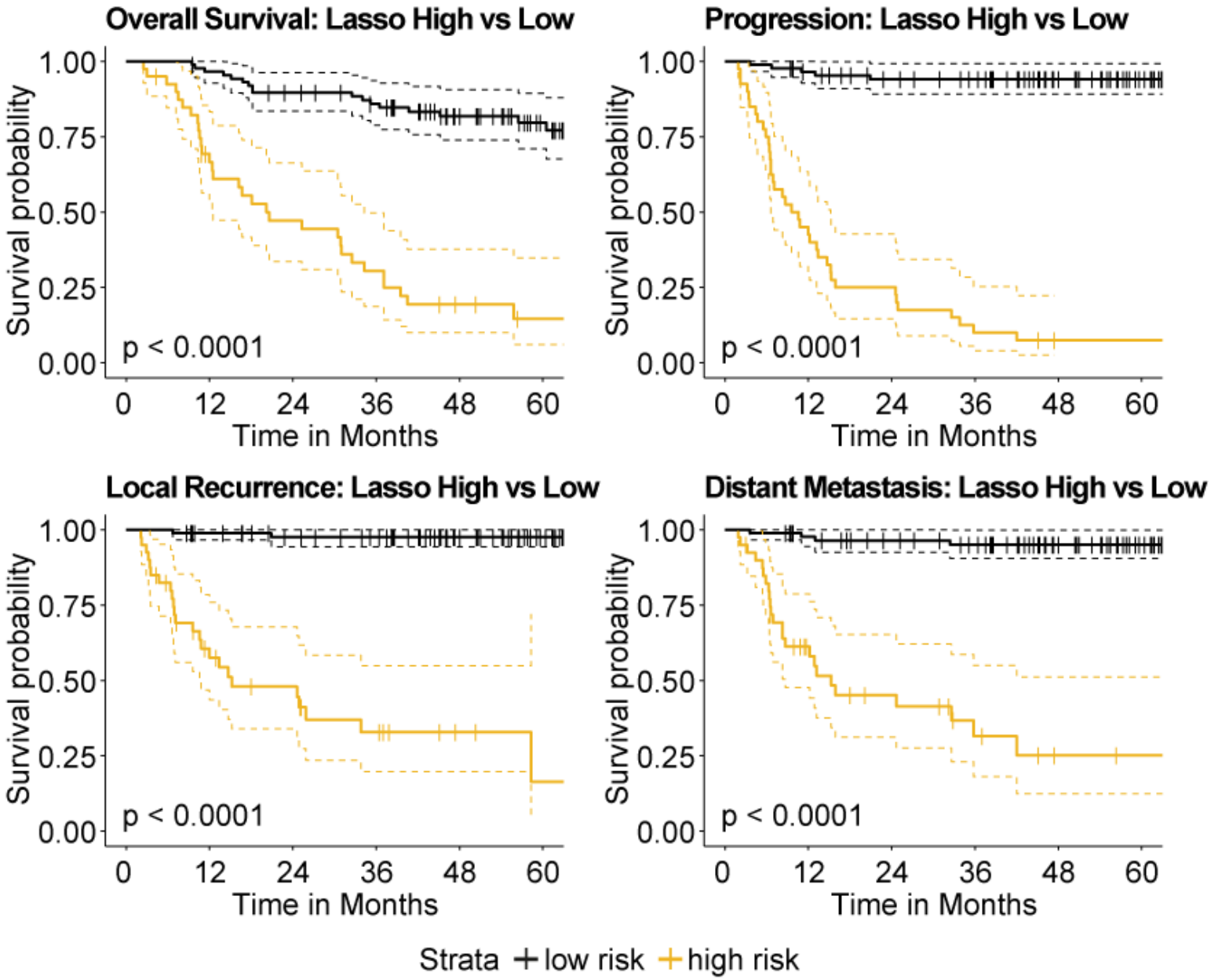
	High Risk (n=25)					
	LR		Progression		Overall Survival	
Biological parameters	HR, 95%CI	p-value	HR, 95%CI	p-value	HR, 95%CI	p-value
p16 IHC		NA		NA		NA
Positive	NA		NA		NA	
Negative	NA		NA		NA	
Hypoxia (15-gene signature)		0.17		0.07		0.27
High	2.45 (95%CI 0.68-8.83)		2.65 (0.92-7.61)		1.79 (0.63-5.03)	
Low	0.41 (95%CI 0.11-1.48)		0.38 (0.13-1.08)		0.56 (0.20-1.59)	
Hypoxia (26-gene signature)		**0.043**		**0.009**		0.068
High	**8.32 (95%CI 1.07-65)**		**5.57 (1.53-20.35)**		3.21 (0.92-11.22)	
Low	**0.12 (95%CI 0.016-0.93)**		**0.18 (0.05-0.65)**		0.31 (0.89-1.09)	
Missing						
CD44 protein		0.99		0.45		0.65
High	Inf (0-Inf)		2.21 (0.29-16.99)		1.602 (0.21-12.25)	
Low	0 (0-Inf)		Reference		0.62 (0.08-4.77)	
Missing						
SCL3A2 mRNA/CD98H		0.99		0.103		0.25
High	Inf (0-Inf)		3.44 (0.78-15.2)		2.42 (0.54-10.72)	
Low	0 (0-Inf)		0.29 (0.66-1.28)		0.41 (0.09-1.83)	
Missing						
CD8 IHC (≥6)		0.94		0.71		0.17
Positive	0.94 (95%CI 0.19-4.57)		1.25 (0.39-4.01)		2.40 (0.70-8.26)	
Negative	1.06 (95%CI 0.22-5.15)		0.80 (0.25-2.58)		0.42 (0.12-1.44)	
Missing						
CD3 IHC (≥6)		0.32		0.92		0.57

Positive	1.9 (95% 0.54-6.72)	1.06 (0.33-3.37)	1.41 (0.43-4.61)
Negative	0.53 (95%CI 0.15-1.86)	0.94 (0.30-2.99)	0.71 (0.22-2.31)
Missing			
PD1 IHC (≥4)	0.91	0.66	0.28
Positive	0.92 (95%CI 0.24-3.61)	0.78 (0.26-2.34)	0.50 (0.14-1.74)
Negative	1.08 (95%CI 0.28-4.23)	1.28 (0.43-3.85)	2.00 (0.58-6.95)
Missing			
PDL1 IHC	0.15	0.38	0.14
Positive	2.52 (95%CI 0.71-8.97)	1.70 (0.52-5.55)	2.57 (0.72-9.18)
Negative	0.40 (95%CI 0.11-1.41)	0.59 (0.18-19.21)	0.39 (0.11-1.39)
Missing			
5-miRNA signature	0.93	0.48	0.66
High risk	0.94 (95%CI 0.24-3.64)	1.58 (0.44-5.65)	1.34 (0.37-4.89)
Low risk	1.06 (95%CI 0.27-4.1)	0.63 (0.17-2.25)	0.74 (0.20-2.71)
Missing			

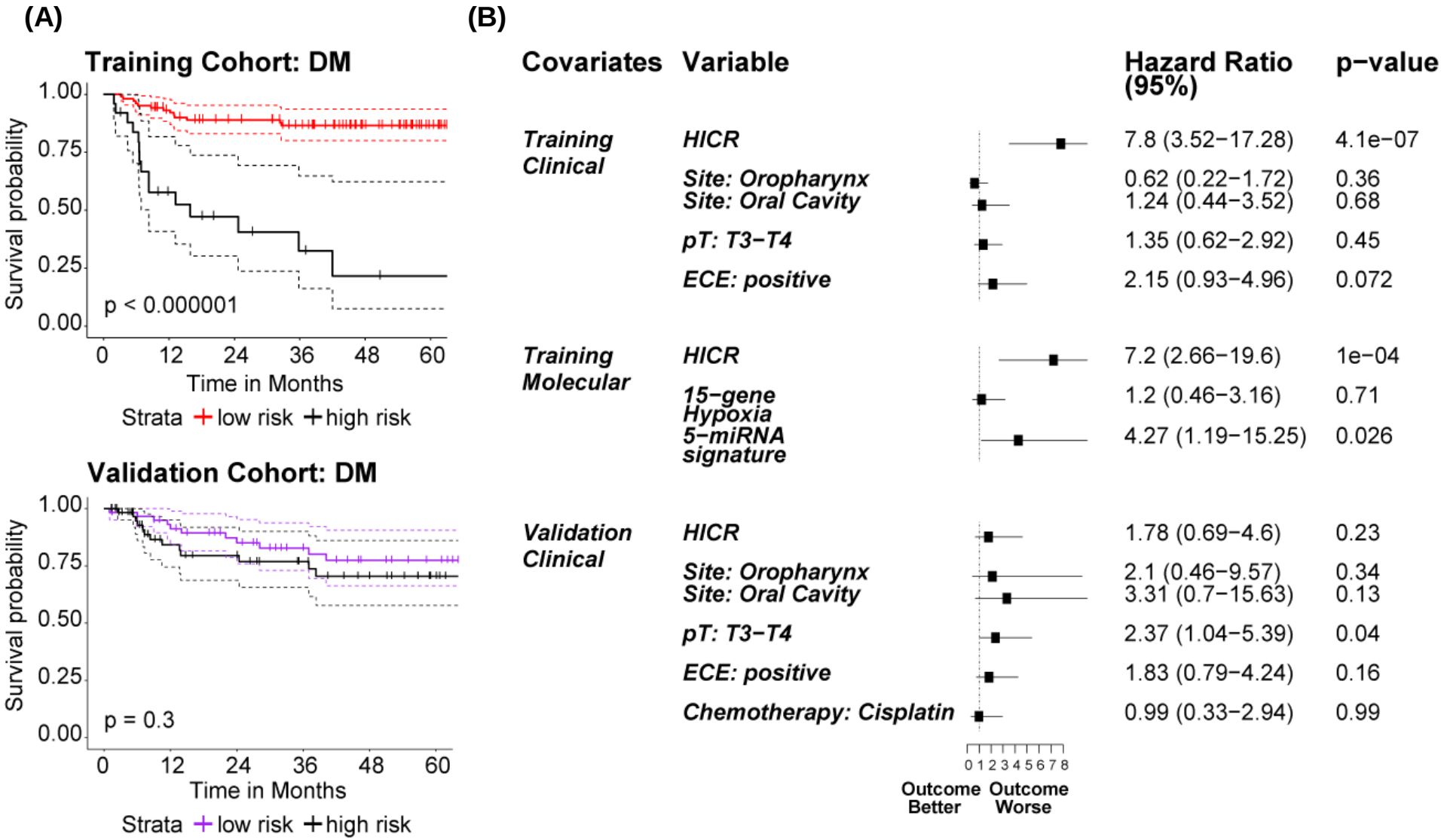
Supplementary Table 6. Effect of biological parameters (hypoxia, CSCs and immune cell markers) on disease progression and overall survival in the methylation- low risk group:

	Low Risk (n=103)					
	LR		Progression		Overall Survival	
Biological parameters	HR, 95%CI	p-value	HR, 95%CI	p-value	HR, 95%CI	p-value
p16 IHC		0.41		0.99		0.86
Positive	1.71 (0.56-6.85)		1.01 (0.34-3.02)		1.09 (0.44-2.66)	
Negative	0.57 (0.15-2.19)		0.99 (0.33-2.96)		0.92 (0.38-2.269	
Hypoxia (15-gene signature)		0.084		0.22		0.20
High	3.91 (0.83-18.44)		1.79 (0.71-4.59)		1.62 (0.77-3.41)	0
Low	0.26 (0.054-1.20)		0.56 (0.23-1-41)		0.62 (0.29-1.30)	
Hypoxia (26-gene signature)		0.99		0.16		0.11
High	Inf (0-Inf)		2.41 (0.70-8.21)		2.19 (0.84-5.72)	
Low	0 (0-Inf)		0.42 (0.12-1.42)		0.46 (0.17-1.20)	
Missing						
CD44 protein		0.99		0.988		0.61
High	Inf (0-Inf)		Infinite		1.46 (0.35-6.13)	
Low	0 (0-Inf)		0		0.69 (0.16-2.88)	
Missing						
SCL3A2 mRNA/CD98H		0.99		0.998		0.22
High	Inf (0-Inf)		Infinite		1.83 (0.70-4.80)	
Low	0 (0-Inf)		0		0.55 (0.21-1.43)	
Missing						
CD8 IHC (≥6)		0.37		0.65		0.081
Positive	0.45 (0.10-2.37)		0.79 (0.29-2.15)		0.44 (0.17-1.11)	
Negative	2.1 (0.42-10.4)		1.26 (0.47-3.41)		2.28 (0.90-5.77)	
Missing						
		0.42		0.183		***0.02***

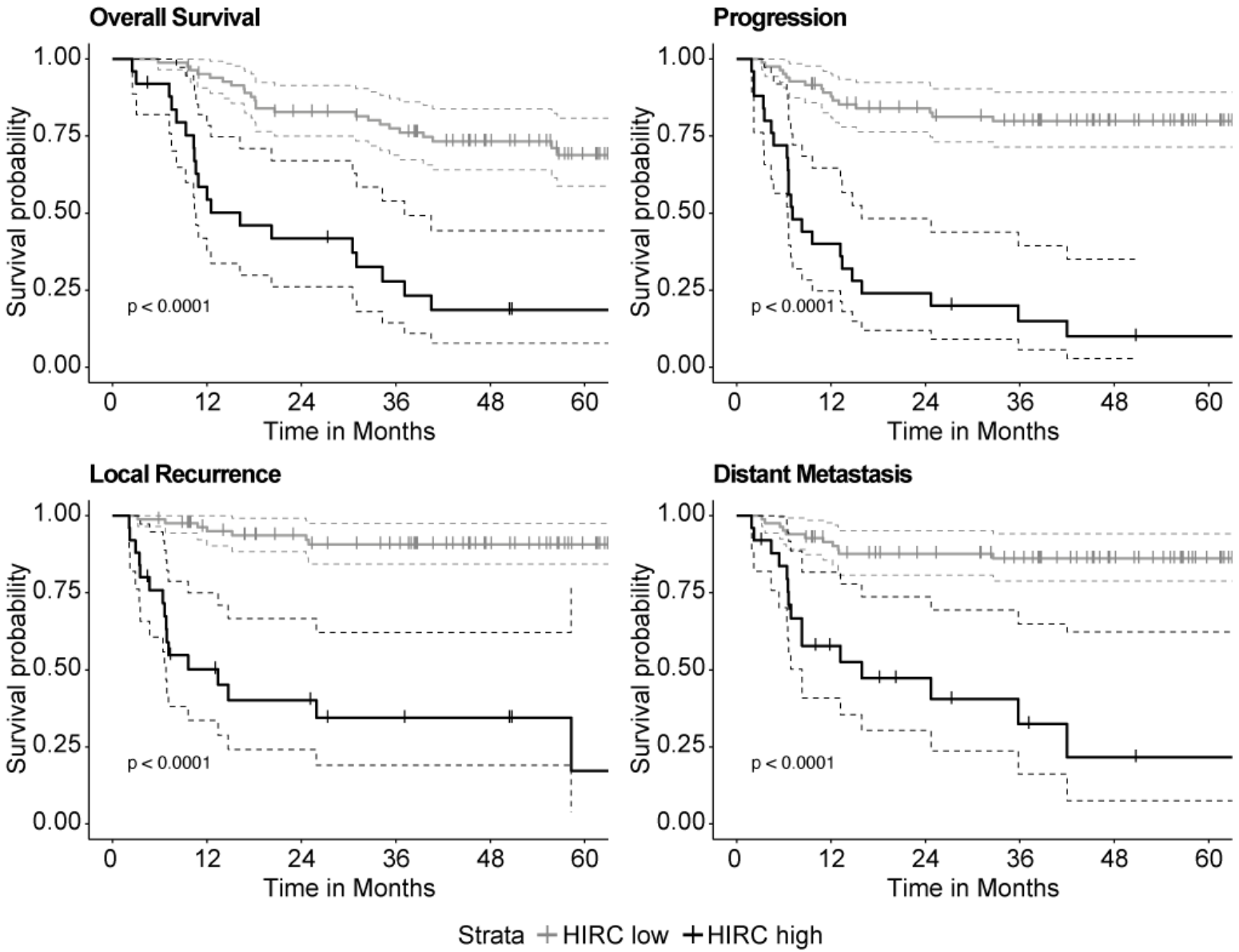
CD3 IHC (≥6) Positive Negative Missing	 0.52 (0.10-2.57) 1.93 (0.39-9.6)	 0.47 (0.15-1-43) 2.14 (0.70-6.58)	 ***0.28 (0.09-0.82)*** ***3.56 (1.22-10.5)***
PD1 IHC (≥3) Positive Negative Missing	0.19 0.39 (0.09-1.62) 2.58 (0.62-10.8)	0.51 0.73 (0.28-1.89) 1.37 (0.53-3.56)	0.13 0.53 (0.24-1.20) 1.21 (0.50-2.92)
PDL1 IHC Positive Negative Missing	0.19 0.24 (0.03-2.02) 4.03 (95%CI 0.50-32.8)	0.13 0.38 (0.11-1.33) 2.62 (0.75-9.12)	***0.02*** ***0.24 (0.07-0.81)*** ***4.15 (1.24-13.95)***
5-miRNA signature High risk Low risk Missing	***0.041*** ***9.44 (1.10-81.2)*** ***0.11 (0.012-0.91)***	***0.0064*** ***6.07 (1.66-22.17)*** ***0.17 (0.045-0.60)***	***0.02*** ***3.07 (1.19-7.96)*** ***0.32 (0.13-0.84)***



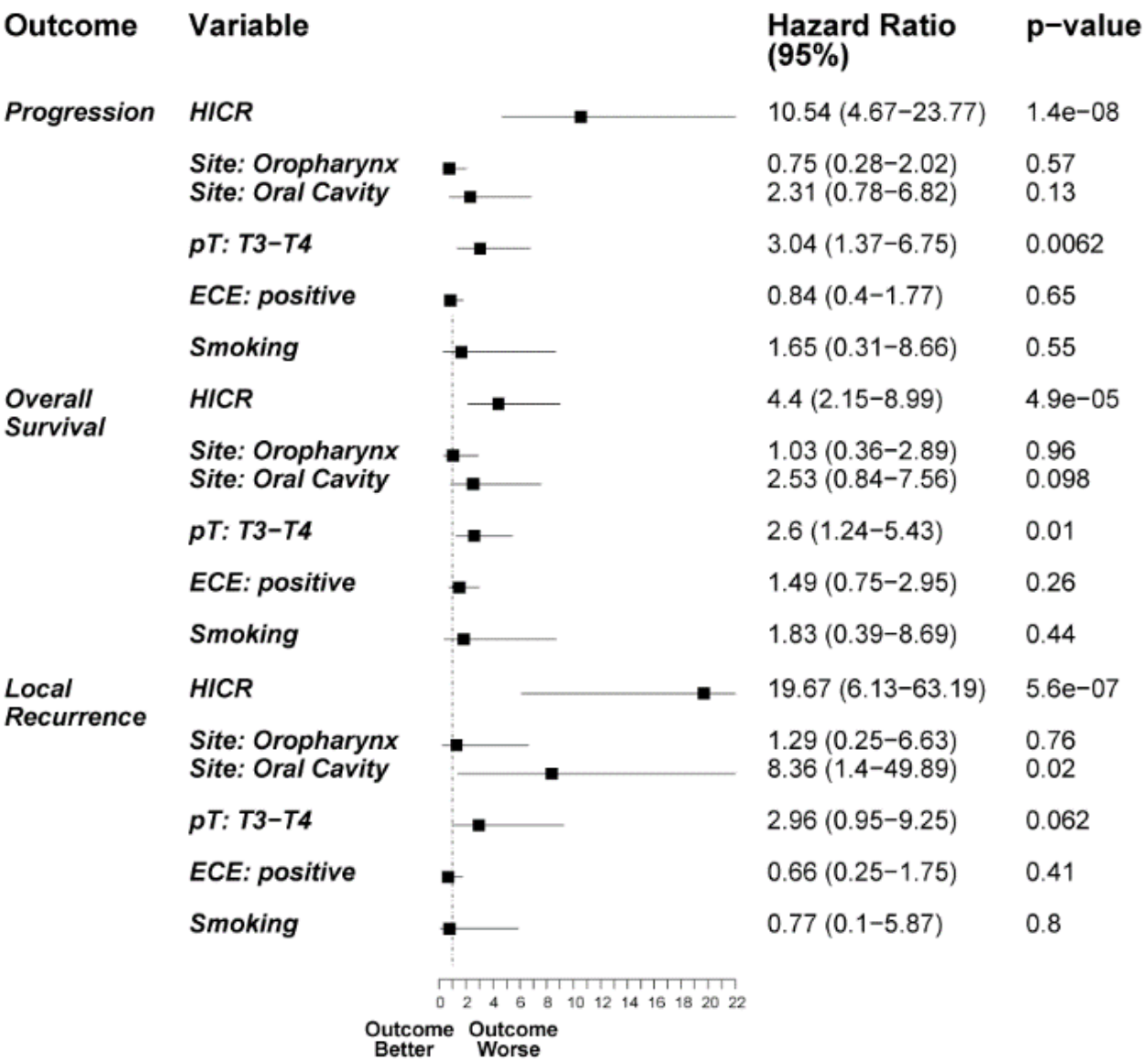
Supplementary Figure 1) Kaplan-Meier curves of OS, progression, LR and DM among patients with HPVDNA negative HNSCC using the Lasso assignment in the training cohort. Patients are stratified by Lasso high risk (n=40) vs low risk (n=88).



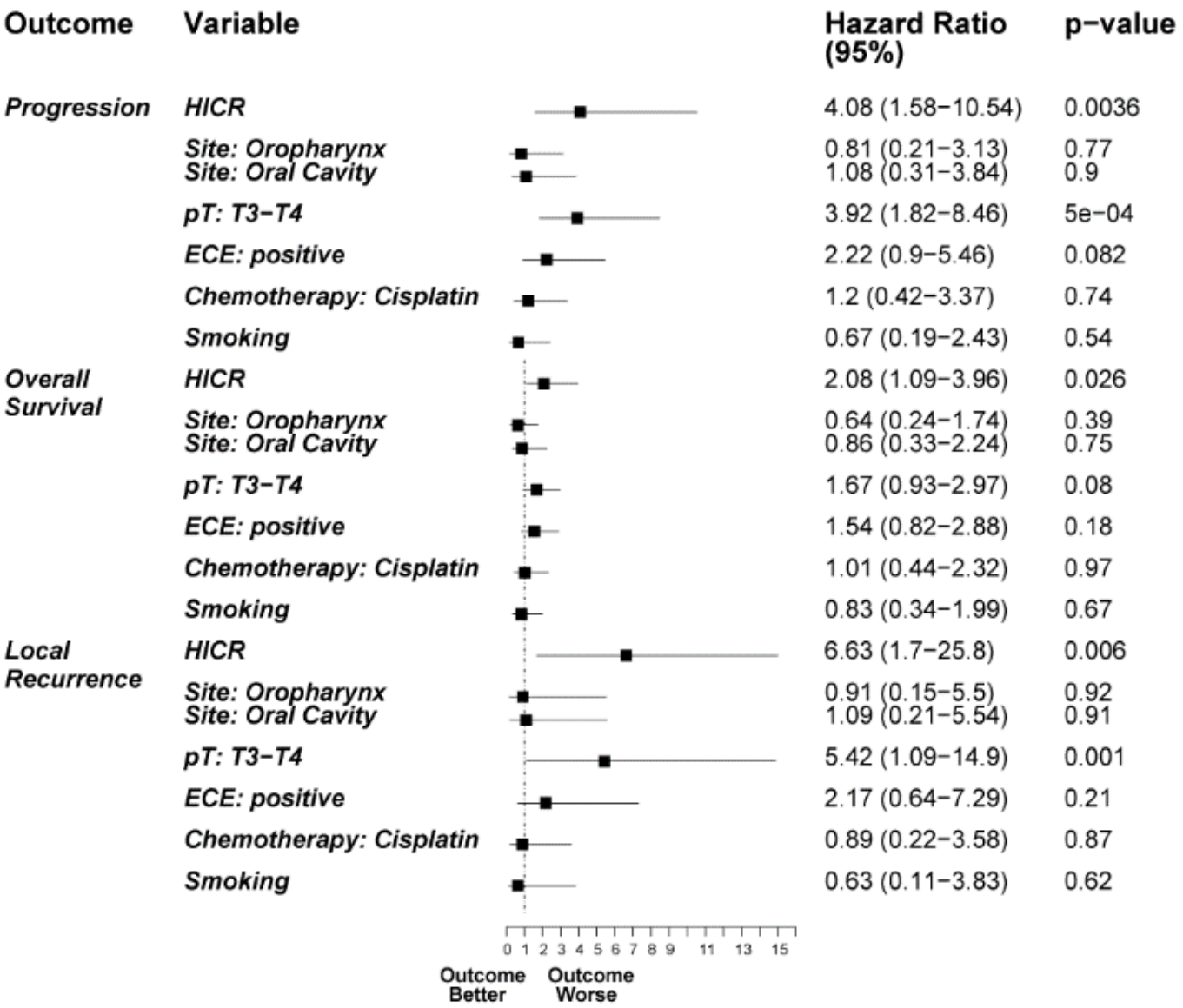
Supplementary Figure 2) a) Kaplan-Meier curves of DM among patients with HPVDNA negative HNSCC in the training cohort (top, red) and validation cohort (bottom, purple). b) Forest plot adjusting for clinicopathological variables, HICR remains significantly associated with DM in the training cohort but not in the validation cohort.



Supplementary Figure 3) Kaplan-Meier Curves for OS, PD, LR and DM in the retrospective PORT-C cohort of the DKTK-ROG (n=108). All Patients are HPVDNA(-) and p16-IHC(-). Patients classified as HICR high (black line) have a significantly worse clinical outcome than patients classified as HICR low ($p<0.05$)



Supplementary Figure 4) Forest plot adjusting for clinicopathological variable including smoking, in the training cohorts. HICR remains significantly associated with worsened all-event progression,OS and LR ($p<0.05$). Advanced T-stage is significantly associated with worsened all-event progression and OS, with a trend towards increased rates of LR. Oral cavity tumors had increased hazard for LR and a trend towards worsened OS.



Supplementary Figure 5) Forest plot adjusting for clinicopathological variable including smoking, in the validation cohort. HICR remains significantly associated with worsened all-event progression,OS and LR ($p<0.05$). Advanced T-stage was significantly associated with worsened all-event progression, LR and with a trend towards worsened OS ($p=0.08$). ECE showed a trend towards higher rates of all-event progression.

References

1. Price ME, Cotton AM, Lam LL, Farré P, Emberly E, Brown CJ, Robinson WP, Kobor MS. Additional annotation enhances potential for biologically-relevant analysis of the Illumina Infinium HumanMethylation450 BeadChip array. *Epigenetics Chromatin [Internet]* 2013 [cited 2019 Apr 1];6:4. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23452981