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Original Article

A tumor DNA-Methylome derived signature of Hypoxia Identifies HPV-negative head and neck cancer patients at risk for distant metastasis after postoperative radiochemotherapy (PORT-C)

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A B S T R A C T

Background and purpose: Tumor hypoxia is a predictive biomarker of treatment resistance in patients with head and neck squamous cell carcinoma (HNSCC). We previously reported the discovery of a tumor DNA methylation signature of hypoxia (Hypoxia-M), identifying HNSCC patients at risk for local recurrence (LR), all event progression, and death after primary radiochemotherapy (RCHT). We further validate Hypoxia-M in an independent cohort of HNSCC patients who underwent surgical resection followed by postoperative radiochemotherapy (PORT-C)

Methods: Hypoxia-M was validated in HPV-negative HNSCC patients (n = 134) homogeneously treated with PORT-C in the frame of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG) multicenter biomarker trial. DNA methylation was profiled using Illumina450K technology. The performance of Hypoxia-M was integrated with previously reported biomarkers, including gene expression signatures (GES) of hypoxia, a methylome-based HPV-Independent Classifier of disease Recurrence (HICR), and immune cell score using immunohistochemistry (CD3/CD8/PD-L1/PD1).

Results: Hypoxia-M was independently prognostic for overall survival (OS, HR = 2.34, p = 0.03) and distant metastasis (DM, HR = 4.3, p = 0.001), but not for LR after PORT-C. Hypoxia-M remained significant after adjusting for patientś age, gender, smoking status, tumor stage, and high-risk features (ECE&/R1 resection). Hypoxia-M status was inversely associated with CD8 T-cell infiltration. Patient stratification improved by integrating previously reported biomarkers, with Hypoxia-M demonstrating independent prognostic performance.

Conclusions: The prognostic utility of Hypoxia-M was validated in an independent cohort. Our results highlighted a difference in recurrence patterns of hypoxic tumors treated in the primary setting (local recurrence) versus postoperatively (distant metastasis) and the utility of Hypoxia-M for identifying the main pattern of recurrence.

Introduction

Tumoral hypoxia confers resistance to radiotherapy (RT) and contributes to aggressive remodeling of the tumor and immune microenvironment[1]. Although its prognostic and predictive utility is specially well-established in human papilloma virus negative (HPV negative) head and neck squamous cell carcinoma (HNSCC) [2,3], it has little to no consequence for patient treatment in current clinical practice. The development of robust and reliable molecular surrogates of tumor hypoxia is needed to identify those patients with hypoxic tumors and mitigate their high risk of disease recurrence. DNA methylation biomarkers can bridge this gap, given that DNA methylation is well-conserved and can be robustly profiled from tumor DNA [4].

We previously developed and validated a DNA-methylation based classifier of tumor hypoxia, Hypoxia-M, in a multicentric retrospective cohort of patients with HPV HNSCC treated with primary radiochemotherapy (RCHT)[5]. Hypoxia-M was prognostic for overall survival (OS), disease progression and local recurrence (LR) but not distant metastasis (DM). Hypoxia-M was developed in the cancer genome atlas cohort (TCGA)- HNSCC cohort after assigning patients into hypoxia high versus low groups, by using two gene-expression based signatures of hypoxia[6–8] and identifying associated differentially methylated probes (DMPs). Hypoxia-M was further validated in an independent retrospective multicentric cohort from the German Cancer Consortium (DKTK) of patients treated with primary radiochemotherapy (RCHT) [9]. Hypoxia-M was notably associated with lower T-cell infiltration, consistent with the reports of altered T-cell activation under hypoxia and immunosuppression in hypoxic niches [10,11].

Consequently, we sought to validate Hypoxia-M in an independent multicentric retrospective arm of the German Cancer Consortium – Radiation Oncology Group (DKTK-ROG) of patients with HNSCC treated with surgery followed by postoperative cisplatin-based radiochemotherapy (PORT-C). The PORT-C cohort is well-characterized, with selection of the radiation treatment information to identify in-field versus out of field recurrence[12]. As a biomarker discovery cohort, it has served for the basis of development/validation of gene-expression signatures (GES) of hypoxia[7,8,13], immune cell infiltration[14], DNA methylation classifier of disease recurrence (HICR)[15], 5-miRNA risk signatures[16] and a signature of cancer stem cells[13] to identify patients with HNSCC at high risk for treatment failure after radiotherapy (RT). Most recently, the molecular subtypes of HNSCC[17] and GES of epithelial-to-mesenchymal transition (EMT)[18], radiosensitivity and radioresistance[19–21] could be validated at the gene expression level for this cohort[19].

Additionally, we aimed to validate the association of Hypoxia-M with decreased immune cell infiltration and to use Hypoxia-M as a complementary classifier to HICR, a previously reported DNA-methylation-based classifier of disease recurrence with the aim to further improve molecular stratification of patients with HPV negative HNSCC prior to receiving radiotherapy treatment [15].

Materials and Methods

Patient cohorts

Inclusion criteria, data collection and handling have been reported in detail[12,15]. Briefly, the PORT-C cohort of the DKTK-ROG is a retrospective multicenter cohort of patients treated with postoperative RT and concurrent cisplatin-based chemotherapy, exhibiting at least one of the following high risk features: pT4 stage, more than three positive lymph nodes (LN), positive microscopic resection margins or extracapsular extension (ECE). The cohort consists of 221 patients, treated between 2004 and 2012, with histologically proven squamous cell carcinoma of the oropharynx, oral cavity and hypopharynx, recruited from eight DKTK partner sites in Germany[12]. Radiotherapy was prescribed to the surgically resected tumor bed and regional LNs in all patients. The primary tumor bed and regional LN-levels with ECE received 50 Gray (Gy) in 2 Gy fractions (fx) with a 16 Gy Boost. Electively irradiated LN-levels were treated with 50 Gy/2Gy fx. Genomic DNA from HPV negative tumors was profiled for this study.

Local ethics committees granted ethical approval for retrospective data collection and analysis of clinical and biological data (EA 312–12, EA 448–13, EA 17–116).

Clinical endpoints

Clinical outcomes (time to death, LR and DM) have been previously reported for this cohort and were defined as time (in months) from first day of RT treatment to time of first event[15]. Time to progression was defined as time to LR or DM, whichever occurred first. Censoring occurred at time of death or last follow-up. Clinical imaging and RT treatment plans were reviewed centrally for the cohort[12,22].

HPV DNA status of samples was assayed at the DKTK partner site Dresden, as previously described[12,23]. Extraction of genomic DNA was performed on 5-μm FFPE sections using QIAmp DNA FFPE tissue kit (Qiagen, Venlo, NL). The LCD-Array HPV 3.5 Kit (CHIPRON GmbH,

Berlin, DE) was used for analysis of HPVDNA status and genotyping as per manufacturer instructions. p16 immunohistochemistry status was assessed using the CINtec Histology kit (Roche mtm laboratories AG, Basel, CH) also, at the DKTK partner site Dresden. Tumors with strong and diffuse nuclear and cytoplasmic staining in $\geq$ 70% of tumor cells were considered as p16 positive[12,22,23]. HPV status was determined using a DNA-methylation based signature of HPV driven carcinogenesis, HPVM [24].

Methylation profiling

Illumina microarrays based on Infinium HumanMethylation 450 (450 K) array were used for methylation profiling, as previously described for the cohort[15], at the Genomics and Proteomics Core Facility of the German Cancer Research Center (DKFZ). Functional normalization including background correction, dye bias correction and probe type bias correction was performed in "minfi" package in R [25,26].

Hypoxia-M validation and statistical analysis

All statistical analyses were performed using R[27] in Rstudio environment[28].

Random Forest prediction

Hypoxia-M classifier was developed in HPV negative patients in the TCGA-HNSCC cohort, as previously described[5](Supplementary Material). First, using two previously validated GES of hypoxia ("Hypoxia 15-GES" and "Hypoxia-30 GES") [29,30], patients were assigned into into "consensus hypoxia high" versus "else" groups. Logistic regression was performed to identify differentially methylated probes (DMPs) between both groups at FDR < 0.05. A random forest was trained on the significant DMPs using quaternary assignment of patients' tumors into "Consensus High", "Hypoxia 30-GESHigh", "Hypoxia-15 GES- High" and "Consensus Low" using the randomforest package in R. The top 5th percentile of probes (n = 299) was selected, by decreasing importance of Gini Index to form Hypoxia-M, the random Forest classifier. The forest, Hypoxia-M, was used to predict the risk groups for patient samples in the PORT-C validation cohort (Supplementary Fig. 3).

Survival Analysis

Kaplan-Meier (KM) survival curves were compared to estimate the difference in clinical outcomes between Hypoxia-M assigned groups using $p < 0.05$ for significance in "survival"[31] and "ggsurv"[32] R packages. Median time to clinical event and 95% confidence intervals were calculated in "survival" package.

Analysis of clinicopathologic parameters

Multivariate Cox regression analysis was performed to adjust for demographic, treatment and molecular parameters. To test for differences in clinical parameters between Hypoxia-M high vs Hypoxia-M low groups, a permuted Fisher's test (two-sided) and a Student's *t*-test (two-sided) were applied for categorical and continuous data respectively.

Integration of previously reported biomarkers

The prognostic impact of gene expression biomarkers (two hypoxia GES and Cancer stem cell markers (*SLC3A2* gene/CD98H), methylation classifiers (HICR), miRNA signatures (5-miR) and immune markers staining by IHC (CD3, CD8, PD1 and PD-L1) has been reported for the PORT-C cohort[8,14–16,33]. Most recently, the molecular subtypes of HNSCC[17] and GES of epithelial-to-mesenchymal transition (EMT) [18], radiosensitivity[20] and radioresistance[19–21], GES of prognosis (12-gene SIG[34] and 7-gene GES[35]) were reported for this cohort [19]. Fisher's test tested for enrichments in these biomarkers between Hypoxia-M high-versus low- groups. Additionally, pearson's correlation between the Hypoxia-M assignment, all gene expression signatures, HICR, 5-mir and immune cell stainings was calculated using the stats package in R and a p-value was calculated for all correlations. Finally, univariate regressions tested impact of biomarkers on progression of disease (PD) and OS in Hypoxia-M high- vs. low-risk groups separately.

Results

Hypoxia-M Validation in the patient cohort

Clinicopathologic characteristics of the PORT-C cohort of the DKTK-ROG (n = 134) are displayed in Table 1. The Hypoxia-M forest was used to predict risk groups for the PORT-C cohort (n = 134) based on the methylation status of 299 probes. 38 patients were predicted to have Hypoxia-high tumors (28%) versus 96 patients with Hypoxia-M low tumors (72%). Compared to Hypoxia-M low patients, patients classified as Hypoxia-M high had higher rates of smoking (82% vs 55%. $p < 0.005$). There was no imbalance in age, gender, anatomical localization, stage (T or N) and distribution of high risk features (ECE or R1 margins), RT dose prescribed or in the time to radiotherapy. Patients predicted to belong to the Hypoxia-M high group had a significantly higher probability of disease recurrence compared to predicted Hypoxia-M Low patients ($p = 0.057$), death ($p = 0.026$) and distant metastasis ($p = 0.029$) compared to predicted Hypoxia-M low patients. LR rates were similar for Hypoxia-M low- vs. high-risk groups ($p = 0.93$) (Fig. 1).

Evaluation of clinical parameters

Multivariate cox regression adjusted for the impact of clinicopathologic parameters on DM, PD and OS (Fig. 2) and for LR (Supplementary Fig. 1). Evaluated parameters were age, smoking status, stage (8th AJCC I-II, III, IV), anatomical site (oropharynx, oral cavity, hypopharynx), radiotherapy dose and presence of high risk features (ECE and resection status).

On multivariate analysis, Hypoxia-M was independently prognostic for OS (HR = 1.95, $p < 0.03$), disease progression (HR = 1.91, $p < 0.02$). There was a statistical trend for increased rates of distant metastasis (HR = 2.09, $p < 0.067$) (Fig. 2). Hypoxia-M was not significantly associated with local recurrence (supplementary Fig. 2). Oropharyngeal tumor location was also independently associated with decreased rates of distant metastasis (HR = 0.39, $p < 0.02$), disease progression (HR = 0.56, $p < 0.03$) and showed a trend towards improved LR (HR = 0.46, $p < 0.07$). High risk features were an independent predictor of DM (ECE: HR = 3.3, $p < 0.01$, R1: HR = 2.16, $p < 0.064$) and disease progression (ECE: 1.8, $p < 0.037$, R1: HR = 2.2, $p < 0.007$). Finally, increased radiotherapy dose was associated with lower DM rates (HR = 0.85, $p < 0.04$) and age was associated with lower LR rates (HR = 0.95, $p < 0.03$).

Moreover, Hypoxia-M remained an independent prognosticator of OS (HR = 2.23, $p < 0.013$), PD (HR = 2.26, $p < 0.009$) and DM (HR = 2.56, $p < 0.04$) after adjusting for p16 immunohistochemistry (positive in 14% of patients). p16-IHC was not prognostic for OS, PD, DM or LR (Supplementary Fig. S1 and S2).

Integration of hypoxia-15 GES and previously reported biomarkers for the clinical cohort

Next, we tested whether the Hypoxia-M high vs low groups showed statistically significant enrichment for any of the previously reported biomarkers for the retrospective arm of the DKTK-ROG cohort (Table 2). Looking at immune markers by immunohistochemistry, Hypoxia-M high tumors showed significantly lower median staining scores for CD8 T cells and CD3 T cells compared to Hypoxia-M low tumors ($p < 0.03$). There was no difference in PD1 and PD-L1 staining scores. Hypoxia-M tumors also overexpressed the 5-miRNA signature associated with poor prognosis ($p = 0.053$). There was no significant enrichment for HICR, the DNA-methylation signature of disease recurrence.

With respect to gene expression signatures (GES), tumors in the

Table 1
Characteristics of retrospective cohort of the DKTK-ROG cohort with postoperative radiochemotherapy and comparison of Hypoxia-M high versus Low Patients.

Variable	All Patients n = 134		Hypoxia-M V2 High n = 38		Hypoxia-M V2 Low n = 96		p-value
Hypoxia-M2							
High	38	28%	38	100%			
Low	96	72%			96	100%	
HPVDNA status							0.19
Positive	13	10%	6	16%	7	7%	
Negative	121	90%	32	84	89	93	
p16 IHC							0.9
Positive	19	14%	5	13%	14	15%	
Negative	115	86%	33	87%	82	85%	
Gender							0.24
Male	107	80%	33	87%	74	77%	
Female	27	20%	5	13%	22	23%	
Anatomical Site							0.39
Oral Cavity	50	37%	12	32%	38	40%	
Oropharynx	61	46%	17	45%	44	46%	
Hypopharynx	23	17%	9	24%	14	15%	
Age							0.41
Median	55				55		
Range	24–74				32–74		
Smoker							**0.005**
Ever smoker	84	63%	31	82%	53	55%	
Never smoker	50	37%	7	18%	43	45%	
p53							0.45
overexpression	71	53%	18	47	53	55%	
none	63	47%	20	53	43	45%	
T stage							0.27
T1-T2	78	58%	19	50%	59	61%	
T3-T4	56	42%	19	50%	37	39%	
N stage							0.59
N0-N1	37	28%	13	34%	24	25%	
N2-N3	97	72%	25	66%	72	75%	
8th AJCC							0.32
I-II	12	9%	2	5%	10	10%	
III	28	21%	11	29%	17	18%	
IV	94	70%	25	66%	69	72%	
High Risk features							0.51
ECE+	51	38%	18	47%	33	34%	
R1 Resection	38	28%	8	21%	30	31%	
Both	21	16%	5	13%	16	17%	
None	24	18%	7	18%	17	18%	
Radiation Therapy							
Median Dose	64 Gy		64		64		0.22
Range	56–68.4		60–66		56–68.4		
Time to RT Start							0.09
Median t	44		43.5		44		
Range	35–57		33–55		36–57		

Hypoxia-M high group had significantly higher expression of the hypoxia-15 GES (63% vs. 51%, $p = 0.056$), cancer stemness marker SLC3A2/CD98H ($p < 0.002$), GES signatures of radiosensitivity (radiosensitivity GES and GARD-GES, $p < 0.05$), DNA repair and apoptosis (7-gene GES, $p < 0.05$) and tumor inflammation/progression (12-gene SIG, $p < 0.05$). Stratification by molecular subtypes of HNSCC demonstrated that Hypoxia-M high tumors were enriched in the classical subtype (24%) and underrepresented in the atypical subtype (0%) or mesenchymal subtype (3%), $p < 0.0005$.

Additionally, Hypoxia-M was significantly correlated with 15-Hypoxia GES ($r = 0.30$, $p < 0.003$) which provided the basis for training Hypoxia-M in the TCGA-HNSCC cohort (Fig. 3) and also correlated with the following GES: 12-gene SIG ($r = 0.41$, $p < 0.001$), radiosensitivity GES ($r = 0.31$, $p < 0.001$) and.CD98H/SLC3A2 expression ($r = 0.22$, $p < 0.03$). There was a significant negative correlation with total CD8 staining ($r = -0.26$, $p < 0.01$) and CD8 staining in the stroma ($r = -0.3$, $p < 0.02$), total CD3 staining ($r = -0.46$, $p < 0.003$) and samples with CD8/PDL1 positive staining ($r = -0.4$, $p < 0.04$).

Given the strong correlations with the 12-gene SIG and radiosensitivity GES, the composition of GES was scrutinized to see whether there was overlap with hypoxia-related genes (Fig. 3, Supplementary Table S1). The 12-gene SIG, GARD-GES and Radiosensitivity GES were composed of 33%, 30% and 23% of genes respectively that were either (a) shared in common with the Hypoxia-GES, (b) were differentially methylated as a function of Hypoxia (i.e., represented in Hypoxia-M) or (c) were differentially methylated with subsequent differential gene expression as a function of Hypoxia-M. For example, 25% of genes composing the 12-gene SIG GES (*ADM, DDIT4, ENO2*) are also part of the hypoxia-GES that were used for training Hypoxia-M (Fig. 2). In another instance, methylation probes mapping to four genes (19%) from the radiosensitivity signature were differentially methylated with corresponding differential gene expression in the TCGA-HNSCC cohort as a function of hypoxia (ANXA2 upregulated, HCLS1 downregulated, LAPTM5 downregulated, LRMP downregulated). For two additional genes, methylation probes were differentially methylated as a function of Hypoxia-M (PTPRC, DAG1).

Next, we aimed to evaluate whether the prognostic capacity of the aforementioned biomarkers may further improve the stratification of patients belonging to either the Hypoxia-M high (n = 38) or Hypoxia-M low (n = 96) groups. To this end, univariate cox regressions were performed univariate Cox regressions for HICR, immune stainings, GES and 5-miRNA scores separately in Hypoxia-M high (n = 36) vs low (n = 96) groups. (Supplementary Table S2). In the Hypoxia-M high group, HICR was independently prognostic of PD and DM (HR = 3.18 and HR = 4–61

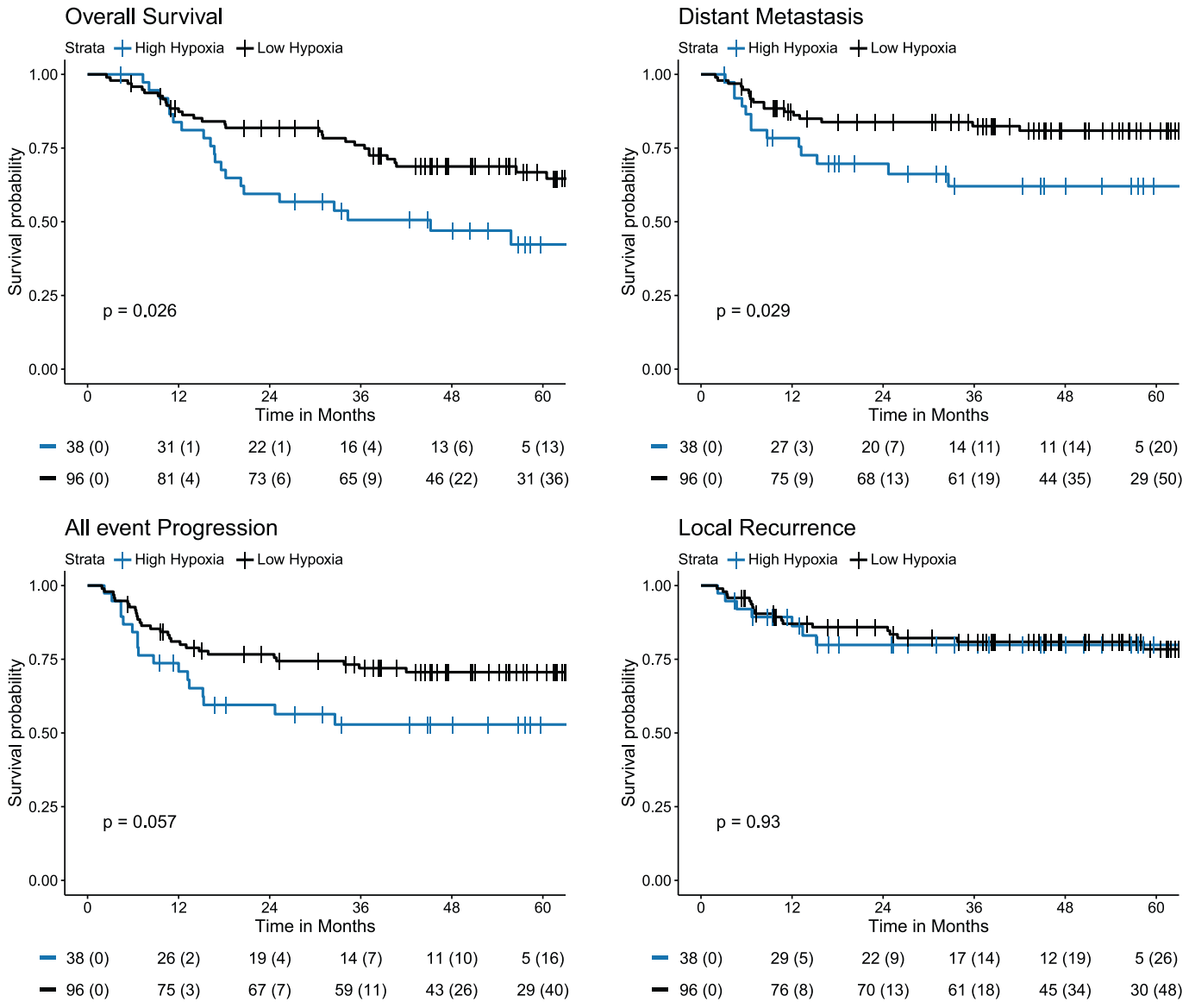

Fig. 1. Validation of Hypoxia-M in patients with HNSCCs treated with surgery and postoperative RCHT in a retrospective multicentric cohort from DKTK-ROG. Hypoxia-M is prognostic for worsened OS, PFS and increased rates of DM but not associated with increased rates of LR ($p < 0.05$).

respectively, $p < 0.02$) and GARD-GES high assignment was associated with worsened OS (HR = 3.6, $p < 0.02$).

In the Hypoxia-M low group, HICR, 5-miRNA signature and 12-gene SIG were prognostic for OS (HRs = 7.71–2.63–5.69 respectively, $p < 0.03$), PD (HRs = 7.6–2.6–2.5, $p < 0.02$ respectively) and DM (HRs = 11.0–4.41–2.66, $p < 0.0005$ respectively). Additionally, EMT GES was prognostic for OS (HR = 2.39, $p < 0.02$) and PD (HR = 2.26, $p < 0.02$) but not DM (HR = 1.87, $p = 0.22$). The hypoxia-15 GES and 7-gene SIG were prognostic for PD only (HR = 2.0–2.2, $p < 0.053$).

Given the independent prognostic ability of HICR both in hypoxia-M high and hypoxia-M low groups, multivariate analysis was conducted adjusting for Hypoxia-M status, HICR and clinical characteristics. Both Hypoxia-M and HICR were independently prognostic of worsened OS, DM and PD (Fig. 4). Dual stratification by Hypoxia-M and HICR identified 49 patients (37%) at high risk for disease recurrence, with eight tumors (6%) identified as both Hypoxia-M high and HICR high risk.

Discussion

This study reports the validation of a DNA methylation-based classifier of Hypoxia, Hypoxia-M, in an independent well-characterized cohort of patients with HNSCC after PORT-C treatment. Hypoxia-M was an independent prognostic factor of OS, PD and DM, after adjusting for clinical parameters but not for LR. Interestingly, Hypoxia-M was first validated in a retrospective cohort of patients treated with primary definitive RCHT from the DKTK-ROG (Supplementary Table S3), where an association between Hypoxia-M and LR but not DM could be demonstrated. Paradoxically, in this current report, hypoxia-M was prognostic of DM but not LR in patients whose tumors were resected. Thus, the prognostic utility of Hypoxia-M may be modulated by treatment type. In the PORT-C cohort, there was in total 30 distant metastases (22%) and 26 local recurrences (19%) during follow-up. Distant metastasis was the primary pattern of failure in 19 patients and the first type of recurrence in five patients (in total 18%). LR was the primary pattern of failure in 15 patients and the first to occur in three patients (in total 13%). Additionally, three patients (2%) were annotated to have both simultaneously DM and LRs. In contrast, for the primary RCHT cohort, local recurrence was the main pattern of failure in 31 patients and the first to occur in three patients (total 34/88 or 39%). Distant metastasis was the main pattern of failure in nine patients and the first to

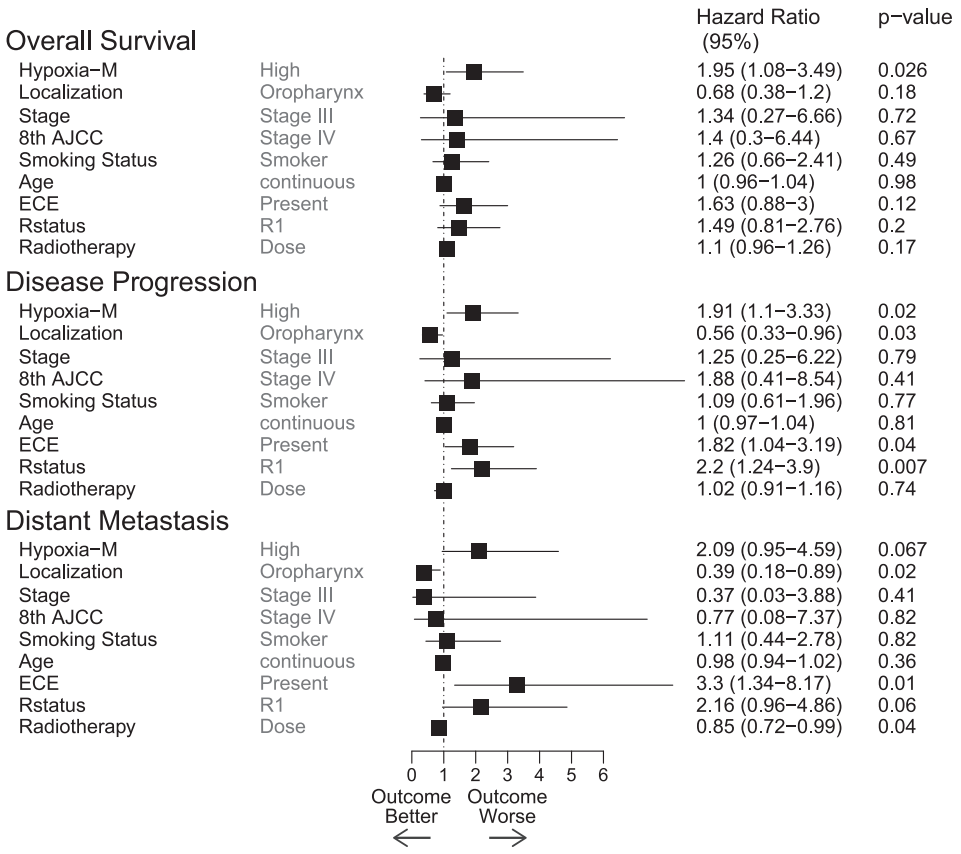

Fig. 2. Hypoxia-M is independently prognostic for OS and progression after adjusting for clinical characteristics. Hypoxia-M is associated with higher hazard rates for OS (HR = 1.95, $p < 0.026$), PFS (HR = 1.91, $p < 0.02$) and showed a trend towards increased rates of DM (HR = 2.09, $p = 0.067$). Additionally, oropharyngeal localization was associated with lower rates of disease progression (HR = 0.56, $p < 0.03$) and distant metastasis (HR = 0.39, $p < 0.02$). Additionally, presence of risk factors such as ECE or R1 resection was independently prognostic of disease progression (HRs = 1.82–2.2, $p < 0.04$) and distant metastasis (ECE: HR = 3.3, $p < 0.01$, R1: HR = 2.16, $p < 0.06$). Increased radiotherapy dose was favorable for lower rates of distant metastasis (HR = 0.85, $p < 0.04$).

occur in one patient (total 10/88 or 11%). Two patients had simultaneous DM and LRs (2%). These results demonstrate the importance of detailed clinical annotation in translational clinical research to decipher the interaction between tumor biology, treatment type and patient outcomes.

Additionally, a "one-biomarker stratifies all patients" approach may not suffice to stratify patients for personalized therapies based on the tumor biology since locoregional control is a priority for patients with HNSCC, treated in the adjuvant setting. Hence, it is worthwhile to integrate additional biomarkers for patient stratification. Practically, Hypoxia-M high risk tumors may benefit from additional stratification using HICR classifier. Patients with low Hypoxia-M may benefit from additional stratification using the HICR, 5-miR-signature or 12-gene SIG for identification of patients at risk for disease recurrence.

Testing for HPV driven HNSCC was performed using the previously reported HPVM classifier, instead of p16 immunohistochemistry or HPVDNA PCR because HPVM was the superior prognosticator of OS and correlated best with HPV oncoproteins in the retrospective PORT-C cohort of the DKTK-ROG[24].Nevertheless, Hypoxia-M remained prognostic after adjusting for p16-IHC in this cohort.

Within the context of tumor biology, we had previously reported on the correlation of Hypoxia-M with the Hypoxia-15 GES, the RNA molecular subtypes (classical, atypical, basal and mesenchymal) and with immune cell infiltration[5]. In this manuscript, these findings could be validated. Most importantly, consistent with the reported evidence of the interplay between hypoxia and immune cell depletion in cancer [5,9]once again we could demonstrate that Hypoxia-M high tumors had significantly reduced T-cell infiltration. The correlation of Hypoxia-M with the Hypoxia-15 GES $r = 0.3$ ($p < 0.05$) was comparable with the previously demonstrated correlation rates of (0.34–0.46) [5]. Similarly, Hypoxia-M high tumors were also more enriched for the classical molecular subtype (associated with xenobiotic metabolism and heavy smoking history) showed an inverse association with the mesenchyma subtype and atypical subtypes in the PORT-C cohort [5,17,19].

The correlation of hypoxia-M with GES of radiosensitivity/radioresistance ($r = 0.4$) may be attributed to sharing key signature-genes that are differentially regulated in hypoxia (example, *ADM, DDIT4 and ENO2* are part of the 12-Sig GES and the Hypoxia-15 GES) and the prognostic impact of these GES varied as a function of Hypoxia-M risk vs low groups. (Supplementary Table S2).

Regarding the interplay of Hypoxia-M and HICR, no significant association or correlation could be found between both classifiers. Combining both classifiers for stratification resulted in the classification of 37% in the cohort as having a higher risk of disease recurrence, with 6% patients assigned as high risk by both signatures, 18% having Hypoxia-M high tumors and 13% having HICR high risk status. Both classifiers were independently prognostic of OS; DM and PD. Additionally, HICR was prognostic of LR.

One limitation of this study is lack of imaging biomarkers for hypoxia to correlate with the Hypoxia-M. In clinical practice, tumor hypoxia can be non-invasively quantified via Positron emission tomography (PET) imaging of fluorine-18 fluoromisonidazoke (FMISO) tracer uptake[36] and has been prognostic and/or predictive in patients with locally advanced head and neck cancer treated with primary radiochemotherapy[37–39]. Tumor hypoxia on FMISO imaging was prognostic in the Trans-Tasman Radiation Oncology Group (TROG 98.02) randomized phase II trial, which randomized patients with locally advanced HNSCC to receive primary RCHT of 70 Gy in 35 fractions

Table 2
Association of Hypoxia-M with previously reported molecular parameters.

Variable	All Patients n = 134		Hypoxia-M V2 High n = 38		Hypoxia-M V2 Low n = 96		p-value
HICR							0.45
High	25	19%	8	21%	17	18%	
Low	96	72%	24	63%	72	75%	
Missing	13	10%	6	16%	7	7%	
Hypoxia 15							**0.056**
High	73	54%	24	63%	49	51%	
Low	48	36%	8	21%	40	42%	
Missing	13	10%	6	16%	7	7%	
Hypoxia 26							0.47
High	89	66%	26	68%	63	66%	
Low	29	22%	6	16%	23	24%	
Missing	16	12%	6	16%	10	10%	
5-miRNA							**0.053**
High	40	30%	12	32%	28	29%	
Low	36	27%	4	11%	32	33%	
Missing	58	43%	22	58%	36	38%	
CD8 total score	4 (2–9)		4 (2–8)		5 (2–9)		**0.031**
CD3 total score	4 (0–10)		3 (0–6)		5 (0–10)		**0.0002**
PD-L1 total score	0 (0–1)		0 (0–1)		0 (0–1)		0.232
PD-1 Total Score	4 (3–11)		4 (3–10)		4 (3–11)		0.37
SLC3A2 (median)	−2.78		−2.5		−2.88		**0.002**
Molecular Subtypes							**0.0005**
Atypical	17	13%	0	0%	17	18%	17
Basal	24	18%	7	18%	17	18%	24
Classical	17	13%	9	24%	8	8%	17
Mesenchymal	24	18%	1	3%	23	24%	24
Not Classified	36	27%	14	37%	21	22%	36
Missing	16	12%	6	16%	10	10%	16
GARD							0.41
High	58	43%	18	47%	40	41%	
Low	60	45%	14	37%	46	47%	
Missing	16	12%	6	16%	10	12%	
Radiosensitivity							**0.0005**
High	43	32%	21	55%	22	22%	
Low	75	56%	11	29%	64	65%	
Missing	16	12%	6	16%	10	13%	
EMT							0.15
High	59	44%	20	53%	39	40%	
Low	59	44%	12	31%	47	49%	
Missing	16	12%	6	16%	10	11%	
SIG							**0.0005**
High	62	46%	28	73%	34	35%	
Low	56	42%	4	11%	52	52%	
Missing	16	12%	6	16%	10	11%	
7-gene signature							**0.04**
High	62	46%	22	42%	40	42%	
Low	56	42%	10	26%	46	48%	
Missing	16	12%	6	16%	10	11%	

combined with either concurrent cisplatin plus hypoxia sensitizer tirapamazine (TPZ/CIS arm) or Cisplatin and Fluorouarcil (Chemoboost arm) [38]. In a subgroup analysis of 45 patients, tumor hypoxia was detectable on FMISO-PET imaging in 71% of patients at baseline and 19% of patients at weeks 4–5 of RCHT[40]. Among patients with hypoxic tumors, locoregional failure as well as risk of failure or death was significantly higher in those who received chemoboost vs those who received hypoxic modification using tirazapime (HR = 7.1, $p < 0.038$ and HR = 4.7, $p < 0.004$ respectively) [40]. Distant Metastasis was the first type of treatment failure in 9 out of 45 patients (20%). Interestingly, 8 of 9 DM events (89%) occurred in patients with baseline hypoxia vs 1 in patients without baseline hypoxia (HR = 3.4, $p = 0.29$)[40].

More recently, Nancy Lee et al have utilized FMISO-PET imaging to guide treatment de-escalation in patients with T0-T2/N1-N2c HPV driven oropharyngeal cancer, receiving definitive cisplatin-based RCHT after resection of the primary tumor sites but not the involved lymph nodes (NCT03323463, [39]). FMISO-PET imaging was performed at baseline and 2 weeks after RT start. Patients without baseline tumor hypoxia or with resolution of tumor hypoxia on their intreatment FMISO-PET were de-escalated to receive RT of 30 Gy/2Gy. Patients with persistent hypoxia on intreatment FMISO PET received a total 70 Gy dose in 2 Gy fractions[39]. Patients in the de-escalated cohort had 2-year PFS and OS rates of 94% and 100% and significantly lower rates of acute grade 3–4 adverse events (32% vs 58%, $p = 0.02$) [39]. Interestingly, a subsequent cohort study from MSKCC of 281 patients with HPV driven oropharyngeal cancer identified tumor hypoxia on FMISO PET as a potential biomarker of distant metastasis[41]. In this study, patients with HPV driven oropharyngeal cancer with persistent intratreatment hypoxia on FMISO PET had significantly DM rates (HR = 3.51, $p < 0.04$) and worsened OS (HR = 2.66, $p = 0.02$)[41]. No distant failures were seen in patients with hypoxia-negative disease before RCHT[41].

To our understanding, our study is the first to report on an association between tumoral hypoxia and distant metastasis in patients with HPV negative HNSCC following surgical resection and postoperative radiochemotherapy. Until KEYNOTE-689, randomized phase III clinical trials in resectable HPV negative HNSCC have consistently demonstrated that treatment intensification corresponds to improved locoregional reductions which translate into overall survival benefit, specifically with the addition of cisplatin to radiotherapy in patients with extracapsular extension (ECE) or positive resection margins[42].

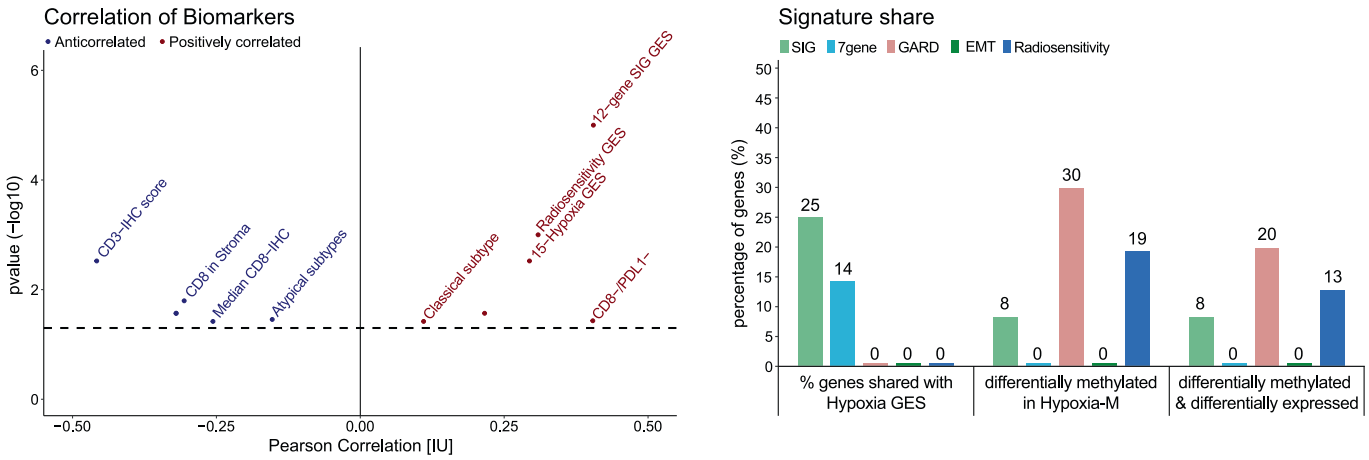

Fig. 3. (Top) Correlation (pearson's) between Hypoxia-M and previously reported biomarkers for the DKTK-ROG retrospective cohort treated with PORT-C. Hypoxia-M showed a strong negative correlation with immune cell stainings (CD3, CD8) and with the atypical and mesenchymal subtypes. Hypoxia-M was positively correlated with the Hypoxia-15 GES (which was used for patient assignment for training Hypoxia-M in the TCGA-HNSCC cohort), with the 12-gene SIG GES, the radiosensitvity GES, SLC3A2 expression and the classical subtype. All correlations were significant with adjusted pFDR < 0.05(Bottom). The 12-gene SIG GES, 7-gene GES and GARD signature share 22.5–33% genes in common with either the Hypoxia-15 GES or with differentially methylated probes/differentially methylated genes in Hypoxia-M.

However, historically, there were no improvements in rates of distant metastasis. KEYNOTE-689 tested the addition of perioperative pembrolizumab to the standard of care and is the first trial to demonstrate a significant improvement in distant metastasis free survival rates (median DMFS was 51.8 months in the pembrolizumab + standard of care (SOC) arm vs 35.7 months with SOC, HR = 0.71, 95%CI 0.56–0.90)[43]. Although KEYNOTE-689 did not mention tumoral hypoxia, the correlation between hypoxia, immune cell suppression and tumor stemness (EMT reprogramming resulting in distant metastasis) is well known [9,44,45]. Specifically, it is known that hypoxia drives immune suppression, which may be reactivated following treatment with immune checkpoint blockade[44,45]. This link needs to be investigated in future trials, but it may be worth postulating that Hypoxia-M could help refine patient selection by identifying those who are likely to benefit the most from treatment escalation with perioperative immune checkpoint blockade.

Conclusion

Bridging the gap between knowledge about tumor hypoxia and clinical consequences for radiation oncology treatment has been limited by the lack of robust and reliable hypoxia biomarkers that could be implemented in the clinical routine.

In this study, Hypoxia-M, a random forest-based DNA methylation signature of hypoxia was validated in an additional independent multicentric retrospective cohort of patients with HNSCC treated with postoperative RCHT. Hypoxia-M was independently prognostic of OS, PD, DM but not LR, hinting at the influence of treatment modality on its prognostic utility. Hypoxia-M maintained its association with the classical molecular subtype of HNSCC and the link between Hypoxia-M and decreased immune cell infiltration in Hypoxia-M high tumors was validated.

The complementary prognostic utility of Hypoxia-M with another DNA-methylation based classifier, HICR, reaffirmed the potential of tumor DNA methylation pattern in prognosticating radiotherapy-specific outcomes in HPV negative HNSCC and identifying patients at high risk for treatment failure who may be candidates for therapy escalation or individualization in well-designed clinical trials. [46–48]. Validation of Hypoxia-M and HICR is planned in the prospective HNprädBio study of DKTK-ROG (https://www.clinicaltrials.gov, NCT02059668).

Ethics declarations

Local ethics committees at all eight DKTK partner sites granted approval for retrospective data collection and the analysis of clinical and biological data.

Data Availability

DNA Methylome data were deposited in the ArrayExpress database at EMBL-EBI (https://www.ebi.ac.uk/arrayexpress) under accession number (E-MTAB-10577).

All authors conflicts of interests

I Tinhofer: Research Grant; Merck Serono

S.E. Combs. Honoraria; BMS Brazil, BrainLab. Consultant: Daiichi Sankyo, BMS Brazil, BrainLAB. Speaker's Bureau, BMS Brazil, BrainLab. Travel Expenses; BMS Brazil, BrainLAB, Daiichi Sankyo.

M. Baumann: Michael Baumann, CEO and Scientific Chair of the German Cancer Research Center (DKFZ, Heidelberg) is responsible for collaborations with a large number of companies and institutions worldwide. In this capacity, he has signed contracts for research funding and/or collaborations, including commercial transfers, with industry and academia on behalf of his institute(s) and staff. He is a member of several supervisory boards, advisory boards and boards of trustees. Michael Baumann confirms that he has no conflict of interest with respect to this paper.

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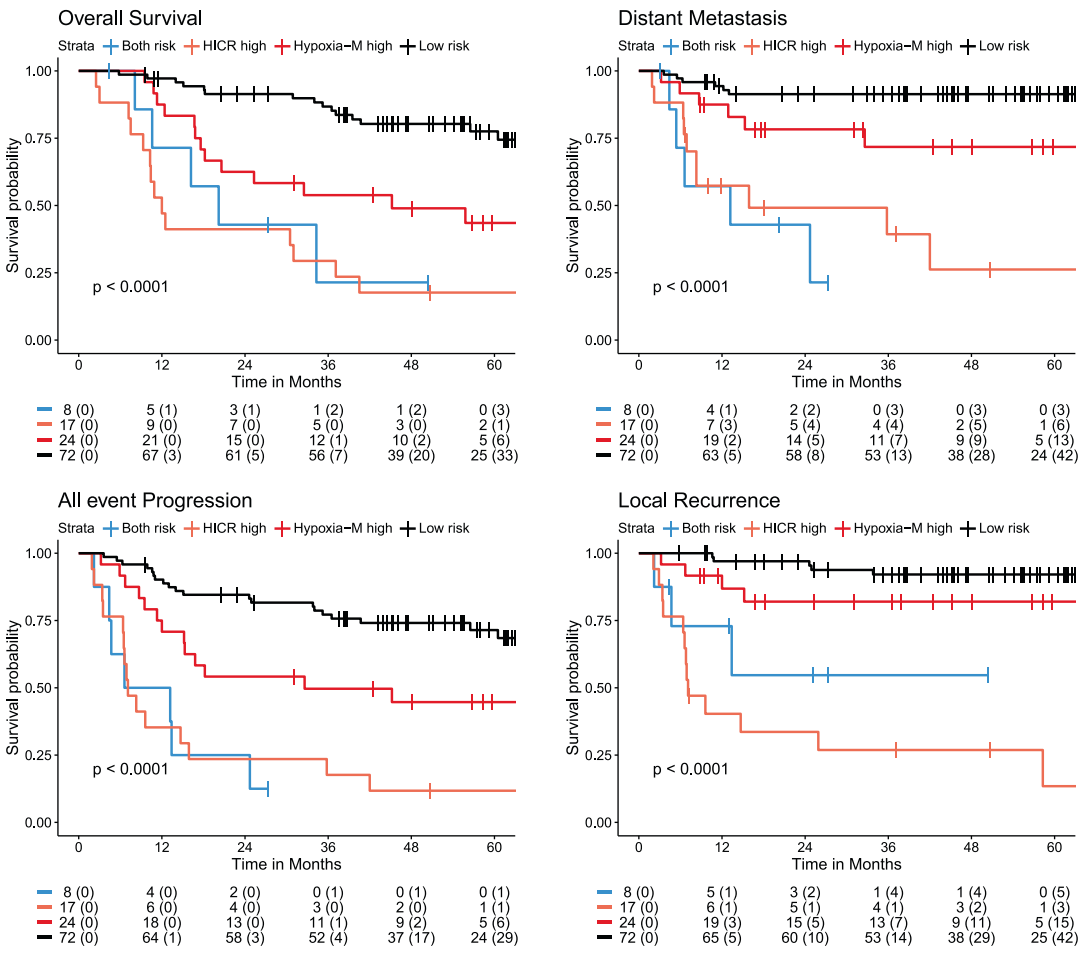
M. Krause: In the past five years, Dr. Krause received funding for her research projects from Merck KGaA (2014–2018 for preclinical study; 2018–2020 for clinical study) and Medipan GmbH (2014–2018). She is involved in an ongoing publicly funded (German Federal Ministry of Education and Research) project with the companies Medipan, Attomol GmbH, GA Generic Assays GmbH, Gesellschaft für medizinische und wissenschaftliche genetische Analysen, Lipotype GmbH, and PolyAn



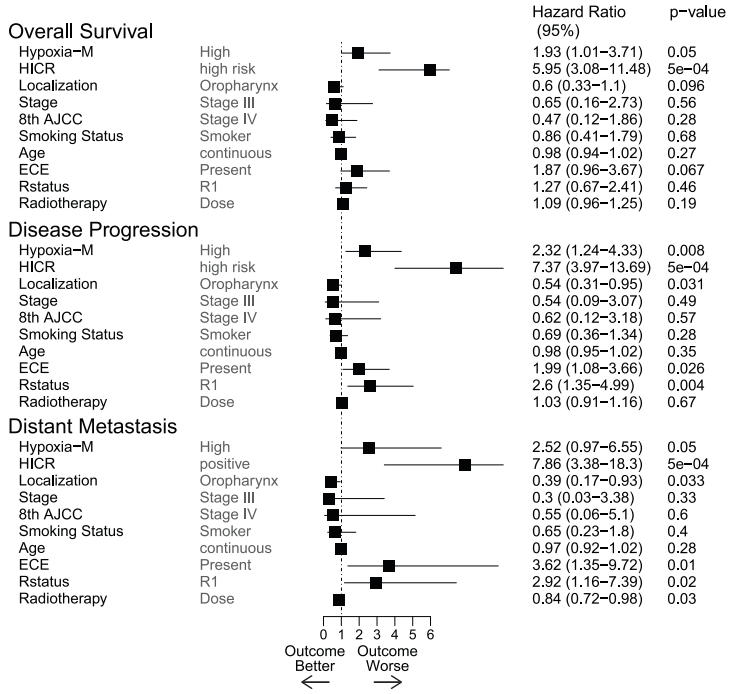


Fig. 4. Patient stratification using DNA-methylation classifiers of hypoxia (Hypoxia-M) and disease recurrence (HICR) identify complementary groups of patients at high risk for worsened outcomes. Hypoxia-M and HICR identify patients at high risk of death, disease progression and DM. Additionally, HICR is an independent prognosticator of LR.

GmbH. In the present study, Dr. Krause confirmed that none of the aforementioned funding sources were involved.

A. Linge: Dr. Linge is involved in an ongoing publicly funded (German Federal Ministry of Education and Research) project with the companies Medipan, Attomol GmbH, GA Generic Assays GmbH, Gesellschaft für medizinische und wissenschaftliche genetische Analysen, Lipotype GmbH, and PolyAn GmbH. In the present study, Dr. Linge confirmed that none of the aforementioned funding sources were involved.

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All other co-authors declare that they have no competing interests.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.radonc.2026.111433.

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