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Original article

A Five-MicroRNA Signature Predicts Survival and Disease Control of Patients with Head and Neck Cancer Negative for HPV-infection

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Running title:
A prognostic 5-miRNA-signature in HPV-negative HNSCC

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Statement of translational relevance (word count: 150 words)

HPV-negative HNSCC cancer is currently treated with a set of standard-of-care therapeutic approaches which in total result in approx. 50% overall survival for locally advanced HNSCC demonstrating that substantial subgroups are not likely to profit from state-of-the-art therapy. The most relevant clinical event limiting success of HNSCC therapy is recurrence of the disease after surgical tumor resection followed by radio(chemo)therapeutic treatment. The presented HNSCC HPV-negative five-miRNA-signature predicts the risk for recurrence in HNSCC and allows, in combination with the clinically established risk factors, the definition of four prognostically distinct groups. This provides the first prerequisite for the consideration of personalized treatment approaches in HPV-negative HNSCC. Possible personalized treatment options include consideration of adjusting therapy intensity according to the overall risk for therapy failure in the first line. Further, and most importantly, it represents the basis for a more focused search for molecular therapeutic targets improving therapy success for appropriate patients.

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Abstract (word count: 249 words)

Purpose:

HPV-negative head and neck squamous cell carcinoma (HNSCC) associates with unfavorable prognosis while independent prognostic markers remain to be defined.

Experimental Design:

We retrospectively performed miRNA expression profiling. Patients were operated for locally advanced HPV-negative HNSCC and had received radiochemotherapy in eight different hospitals (DKTK-ROG; *n*=85). Selection fulfilled comparable demographic, treatment and follow-up characteristics. Findings were validated in an independent single-center patient sample (LMU-KKG; *n*=77). A prognostic miRNA-signature was developed for freedom from recurrence and tested for other endpoints. Recursive-partitioning analysis was performed on the miRNA-signature, tumor and nodal stage, and extracapsular nodal spread. Technical validation used qRT-PCR. A miRNA-mRNA target network was generated and analyzed.

Results:

For DKTK-ROG and LMU-KKG patients, the median follow-up was 5.1 and 5.3 years, the 5-year freedom from recurrence rate was 63.5% and 75.3%, respectively. A five-miRNA-signature (hsa-let-7g-3p, hsa-miR-6508-5p, hsa-miR-210-5p, hsa-miR-4306 and hsa-miR-7161-3p) predicted freedom from recurrence in DKTK-ROG (HR 4.42, 95% CI 1.98−9.88, $P<0.001$), which was confirmed in LMU-KKG (HR 4.24, 95% CI 1.40−12.81, $P=0.005$). The signature also predicted overall survival (HR 3.03, 95% CI 1.50−6.12, $P=0.001$), recurrence-free survival (HR 3.16, 95% CI 1.65−6.04, $P<0.001$) and disease-specific survival (HR 5.12, 95% CI 1.88−13.92, $P<0.001$), all confirmed in LMU-KKG data. Adjustment for relevant covariates maintained the miRNA-signature predicting all endpoints. Recursive-partitioning analysis of both samples combined classified patients into low (*n=17*), low-intermediate (*n=80*), high-intermediate (*n=48*) or high risk (*n=17*) for recurrence ($P<0.001$).

Conclusions:

The five-miRNA-signature is a strong and independent prognostic factor for disease recurrence and survival of patients with HPV-negative HNSCC.

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Introduction

Prognosis of patients with locally advanced head and neck squamous cell carcinoma (HNSCC) generally remains poor. Whereas patients with high-risk human papillomavirus (HPV) associated HNSCC have a considerably more favorable outcome, HPV-negative patients still have to expect limited disease control and survival (1,2). From the biologic perspective, intrinsic resistance of tumor cells to radiochemotherapy or therapy failure caused by metastatic spread are possible underlying factors. Consequently, research aims at altering radiation dose and fractionation or - more recently - at the additional administration of targeted drugs and/or immune checkpoint inhibitors (3,4). However, biomarkers to predict which patients potentially would profit from these approaches are missing.

Complex and heterogeneous genomic aberrations and mutation patterns molecularly control initiation and progression of HNSCC (5-7). MicroRNAs (miRNAs), involved in posttranscriptional regulation, have been shown to be highly deregulated in most cancers and might well be of prognostic relevance (8,9). In HNSCC aberrantly expressed miRNAs were described (10-12). However, so far no study has investigated the prognostic role of miRNAs by comprehensive miRNA-profiling in well-characterized HPV-negative HNSCC cohorts.

Here we analyzed miRNA expression profiles in cancer tissue of locally advanced HNSCC (*n=162*). We hypothesized that we can develop a miRNA-based molecular signature, which allows to stratify HPV-negative HNSCC patients according to risk of recurrence following adjuvant radio(chemo)therapy.

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Materials and Methods

Patient specimens and study design

In the present study, we analyzed two independent samples of HNSCC patients who had undergone surgical resection followed by adjuvant radio(chemo)therapy: the DKTK-ROG (German Consortium for Translational Cancer Research, Radiation Oncology Group) and the LMU-KKG (Ludwig-Maximilians-University of Munich, Clinical Cooperation Group "Personalized Radiotherapy in Head and Neck Cancer") samples. For both of which, clinical data and treatment-naive patient tissue specimens were collected retrospectively. All patients were diagnosed with histologically proven HNSCC of the hypopharynx, oropharynx or the oral cavity. Only HPV-negative HNSCC were included (Supplementary Methods). Ethical approval (EA) for this retrospective study, carried out in accordance with the Declaration of Helsinki, was obtained by the ethics committees of all DKTK-ROG partners including the LMU (EA 312-12, 448-13, 17-116). Tumor stage was assessed using the UICC TNM Classification of Malignant Tumors, 7th edition.

The multicentric study sample DKTK-ROG originally included 221 HNSCC patients treated at one of the eight different DKTK partner sites (13). This study reports on 85 out of 143 patients with HPV-negative tumors who were treated between 2005 and 2011. 58 patients had to be omitted due to insufficient tumor material. All patients received postoperative radiotherapy covering the previous tumor region and regional lymph nodes with concurrent cisplatin(CDDP)-based chemotherapy according to standard protocols. Inclusion criteria were positive microscopic resection margins and/or extracapsular extension (ECE) of lymph nodes and/or tumor stage pT4 and/or or more than three positive lymph nodes. The median overall treatment time was 44 days (interquartile range IQR 43-46 days). Adjuvant radiotherapy including elective irradiation of cervical lymph nodes was applied with a median dose of 50 Gy (median dose 2 Gy/fraction) and a boost to the former tumor region and to microscopic disease (if any) to a median dose of 66 Gy (median dose 2 Gy/fraction). Cisplatin was applied weekly with a median cumulative dose of 200 mg/m² body surface area (BSA) (range 100-300 mg/m² BSA).

The monocentric study sample LMU-KKG included originally all HNSCC patients with at least UICC TNM stage III or close/positive microscopic resection margins (resection margins were considered "close margin" when declared R0, but less than 5 mm by the local pathologist) who were treated with adjuvant radiotherapy between 06/2008 and 01/2013 at the LMU Department of Radiation Oncology (14). The median overall treatment time was 45 days (IQR 43-47 days)

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with five fractions per week. A median radiation dose of 64 Gy (median dose 2 Gy/fraction) was applied to the former tumor bed or regions of ECE, elective lymph node regions have been covered according to tumor stage and localization with a median dose of 50 Gy (median dose 2 Gy/fraction), 56 Gy (median dose 2 Gy/fraction) were applied to involved lymph node regions.

In the case of close/positive microscopic resection margins and/or ECE, patients received concurrent chemotherapy. The majority (76 %) of the patients received CDDP/5-fluorouracil (5-FU) (CDDP: 20 mg/m² BSA day 1–5/29–33; 5-FU: 600 mg/m² BSA day 1–5/29–33). In selected cases, Mitomycin C (MMC) or 5-FU/MMC replaced platin-based chemotherapy. This study reports on the HPV-negative tumor subset (n=77) of all patients with available tumor tissue specimens (n=115).

After histopathological review of haematoxylin and eosin stained tissue sections from available blocks with formalin-fixed and paraffin-embedded (FFPE) tumor tissue by a pathologist (DKTK-ROG: KW; LMU-KKG: CW/AW), the tumor area was annotated. If necessary, microdissection was performed prior nucleic acids extraction in order to ensure a tumor cellularity (i.e., the percentage of tumor cells in analyzed tissue) of at least 60% (DKTK-ROG: median 60%, IQR 60-70%; LMU-KKG: median 70%, IQR 70-80%).

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Procedures

Total RNA, including the small RNA fraction, was extracted using the Qiagen miRNeasy FFPE- (DKTK-ROG) or the AllPrep DNA/RNA FFPE-Kit (LMU-KKG) according to the manufacturer's protocols (Qiagen, Hilden, Germany). Isolated RNA was quantified with the Qubit-Fluorometer and integrity of small RNAs was assessed (Supplementary Methods).

miRNA expression was profiled using SurePrint G3 8x60K Human miRNA Microarrays (AMADID 70156; Agilent Technologies, Santa Clara, CA, USA) representing 2,549 human miRNAs (content sourced from miRBase database, Release 21.0; Supplementary Methods). Microarray raw data were uploaded to the publicly available database ArrayExpress (accession no. E-MTAB-5793). miRNA expression microarray profiling resulted in a data set of 162 HNSCC samples (DKTK-ROG: n=85; LMU-KKG: n=77).

Data analysis was performed using the R statistical software (version 3.3.1) in combination with R-Bioconductor/CRAN packages (Supplementary Methods)(15).

For the purpose of building a Cox proportional hazards model predicting disease recurrence in combination with miRNA expression, we used a robust likelihood-based survival modelling approach deploying an iterative forward-selection algorithm implemented in the R package rbsurv (16). We recently built a miRNA-signature predicting outcome in glioblastoma using the same approach (Supplementary Methods)(17).

Experimentally validated miRNA-target genes of the signature miRNAs were obtained from the miRTarBase database (Release 6.0). The Cytoscape software (version 3.2.1) with the Reactome FI plugin (version 4.0.0) was used to generate a miRNA-mRNA target regulatory network and to conduct pathway enrichment analysis of the target genes. Pathways with P-values < 0.05 were considered as significantly enriched with target genes (18).

For technical validation of microarray data quantitative real-time RT-PCR (qRT-PCR) analysis was performed (Supplementary Methods).

Clinical endpoints and statistical analysis

As the main objective of the study was the identification of a miRNA-signature that allows separation of patients according to risk of recurrence, the primary endpoint was freedom from recurrence. Freedom from recurrence was defined as the time (days) from the start of radiotherapy treatment to the time of the first observation of confirmed locoregional or distant recurrence. Data for recurrence-free patients were right-censored either at the date of death or last follow-up visit. Additional endpoints included were recurrence-free survival, overall survival,

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disease-specific survival, disease-unspecific survival, distant control, and locoregional control. We calculated recurrence-free survival (days) from the date of radiotherapy treatment start to the first observation of locoregional/distant recurrence or death due to any cause; overall survival from the date of radiotherapy treatment start to the date of death from any cause; disease-specific survival from the date of radiotherapy treatment start to the date of tumor related death; non-tumor related survival from the date of radiotherapy treatment start to the date of non-tumor related death; distant control from the date of radiotherapy treatment start to the date of distant recurrence; locoregional control from the date of radiotherapy treatment start to the date of local recurrence. In the absence of an event, patients were censored at the date of the last follow-up visit (or the date of death).

Kaplan-Meier curves were compared for statistical difference using the log-rank test using the R-package survival. Median time-to-event estimates and Hazard ratios (HR) with 95% confidence intervals (CI) were determined. Univariate Cox proportional hazard analysis was performed to evaluate the association of clinicopathological variables with outcome (Supplementary Methods). We used multivariate Cox proportional hazards analysis to assess the prognostic value of the identified miRNA-signature after adjustment for other prognostic clinical parameters as covariates.

The clinical endpoint prediction performance of the five-miRNA-signature and clinicopathological variables in terms of sensitivity and specificity, represented by the corresponding areas under the curve (AUCs), was determined for follow-up times from 1 to 5 years (Supplementary Methods).

Recursive partitioning analysis (RPA) for the generation of a decision tree considering the clinical parameters ECE status, TNM T stage, TNM N stage and resection margin status with or without the five-miRNA-signature defined risk groups was conducted using the R-package rpart (Supplementary Methods).

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Results

The clinicopathological characteristics of the HNSCC patients included in our study (median follow-up: DKTK-ROG 5.1 years, IQR 3.7-5.6; LMU-KKG 5.3 years, IQR 4.4-6.4) are listed in Table 1. Compared to the DKTK-ROG sample, which exclusively contained patients treated by postoperative radiotherapy with concurrent cisplatin-based chemotherapy, only 63.6% of the LMU-KKG sample received concurrent radiochemotherapy. Accordingly, the LMU-KKG sample contained fewer patients with UICC TNM stage IV, advanced nodal stage, ECE or positive microscopic resection margins. 31.5% of all patients (51/162) developed disease recurrence within the observed follow-up time while the two samples did not differ with regard to the endpoints freedom from recurrence and recurrence-free survival (Figure S1). The 5-year freedom from recurrence rate was 63.5% and 75.3% for DKTK-ROG and LMU-KKG patients, respectively.

The miRNA expression profiling of 162 tumor specimens identified 1,031 expressed miRNAs. After univariate preselection 524 miRNAs remained for feature selection using a robust likelihood-based survival modeling forward-selection approach (Table S1). The best model according to the Akaike Information Criterion (AIC) contained the five miRNAs hsa-let-7g-3p, hsa-miR-6508-5p, hsa-miR-210-5p, hsa-miR-4306 and hsa-miR-7161-3p with the following Cox proportional hazard coefficients: -0.5214183, -0.5254865, 0.6461524, -0.3678727 and -0.8165854, respectively. The coefficients were subsequently used for individual risk score calculation after linear combination with appropriate expressions of the signature miRNAs. Using the median risk score as a cut-off, 43 patients of the DKTK-ROG sample (training set) were assigned to the low-risk group (median time to event not reached (NR), 95% CI 2047−not estimable (NE); eight events) and 42 to the high-risk group (median time to event 748 days, 95% CI 459–NE; 24 events). As expected, the groups differed significantly in their risk of recurrence (HR 4.42, 95% CI 1.98−9.88; log-rank $P<0.001$; Figures 1A, S2).

We applied the five-miRNA-based signature prediction model to the miRNA expression data set of the LMU-KKG sample (validation set) using the cut-off as calculated from the training sample data (0.03629712) and assigned 38 patients to the low-risk (median NR, 95% CI NE−NE; four events) and 39 patients to the high-risk group (median NR, 95% CI 708−NE; 15 events). The risk for recurrence of the high-risk patients was significantly increased compared to that of the low-risk patients (HR 4.24, 95% CI 1.40−12.81; $P=0.005$) confirming the prognostic value of the five-miRNA-signature (Figures 1A, S2). miRNA-based risk group classification was not

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associated with simultaneous chemotherapy treatment (Table 1), which was further supported after stratification to LMU-KKG patients treated by concurrent radiochemotherapy (n=49; HR 3.85, 95% CI 1.09-13.58, P=0.024; Figure S3).

Moreover, high-risk patients of both samples showed significantly reduced recurrence-free survival, overall survival and disease-specific survival rates (Figure 1B). We could also demonstrate an impact of both failure sites (locoregional and distant) on the risk stratification, while low- and high-risk patients did not differ in non-tumor related death (Figure S4).

In order to assess whether the five-miRNA-signature was an independent prognosticator, associations of known clinicopathological factors with the miRNA-defined risk groups were tested. TNM T stage, ECE and tumor localization were associated with the miRNA risk groups (Table 1). In the subsequent univariate Cox proportional hazard analysis, TNM T stage and lymphovascular invasion (LVI) were significantly associated with freedom from recurrence in both samples, ECE was identified as a significant parameter in the DKTK-ROG sample only, whereas no differences between the three tumor localizations were observed (Table S2; Figures S5-S7). After adjustment for these parameters in multivariate Cox regression analysis, the five-miRNA-signature retained its independent and exclusive prognostic role in both samples (training set: HR 5.55, 95% CI 2.09-14.79, P<0.001; validation set: HR 3.94, 95% CI 1.23-12.59, P=0.021; Table 2).

We analyzed the sensitivity and specificity of the five-miRNA-signature in the prediction of different clinical endpoints in comparison to the clinical prognostic parameters TNM T stage, LVI and ECE. At 5 years follow-up, the five-miRNA-signature demonstrated a superior prediction of all endpoints analyzed (Figures 2A, S8). Furthermore, in time-dependent analysis (follow-up years 1 to 5), the five-miRNA-signature superiorly predicted all endpoints from 2 to 5 years compared to the clinicopathological parameters. After one year follow-up, higher AUCs for the miRNA-signature compared with the analyzed endpoints were only observed in the training set for the endpoints disease-specific survival and overall survival (Figures 2B, S9, S10). After combining the five-miRNA-signature with the clinicopathological parameters (TNM T stage, LVI, ECE) an even better prediction of all endpoints from 2 to 5 years was achieved for both HNSCC samples, also when compared to combinations of the clinicopathological risk factors (Figures 2C, S11). This was also the case after one year follow-up in the DKTK-ROG sample.

In order to obtain deeper insights into the biological regulatory function of the signature miRNAs, we generated a miRNA-mRNA target regulatory network comprising experimentally

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validated miRNA-target interactions, whereby twelve target genes were found to be shared by the signature miRNAs (Table S3, Figure S12). Pathway enrichment analysis of the target genes revealed 36 pathways including *p53, ATM*, and *FoxO signalling, DNA double-strand break response, pre-NOTCH expression and processing, mitosis* and *senescence* associated pathways (Table S4).

For technical validation of the five-miRNA-signature and potential clinical diagnostic application, we measured the expression of the signature miRNAs in the validation set (n=71) by qRT-PCR confirming the microarray-derived results as the miRNA-classified risk groups significantly differed in freedom of recurrence (HR 5.07, 95% CI 1.17−21.94, P=0.016; Figure S13)

In a Kaplan-Meier analysis in which the samples were pooled (n=162) and stratified according to resection margin status, TNM T stage, TNM N stage, ECE and tumor localization the resulting five-miRNA-signature risk groups significantly differed in clinical outcome (Figures S14, S15). This motivated us to further combine the five-miRNA-signature with clinically relevant parameters. RPA identified four different risk groups for recurrence ("low-risk", "low-intermediate-risk", "high-intermediate-risk" and "high-risk") including the five-miRNA-signature as strongest parameter together with TNM T stage, ECE and TNM N stage (Figure 3 and extended Figure version S16). The worst prognostic group included miRNA-signature-high-risk patients with ECE-positive T3/T4 tumors (median freedom from recurrence 438 days), while miRNA-signature-low-risk patients with T1/T2 N0/N1 HNSCC had the best prognosis (no event). The four risk groups also significantly differed with regard to locoregional and distant control, recurrence-free survival, overall survival and disease-specific survival (Figures S17, S18). RPA considering only the clinical parameters identified three risk groups for recurrence with T stage as the strongest parameter together with ECE and N stage (Figure S19A). Combining the three RPA derived risk groups with the risk factor of our five-miRNA-signature revealed patient subgroups significantly differing in clinical outcome ("RPA intermediate-risk": HR 2.71, 95% CI 1.21-6.06, P=0.012; "RPA high-risk": HR 12.20, 95% CI 1.54-96.90, P=0.004; Figure S19B).

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Discussion

Here we report, for the first time a five-miRNA-signature in HPV-negative patients that predicts decreased cancer control following adjuvant radiochemotherapy. Freedom from recurrence was the chosen primary endpoint to better estimate treatment effects, as HNSCC patients often suffer from multiple comorbidities that affect overall survival (19). Overall, baseline and treatment characteristics of our patients were balanced and compare well to reports on HPV-negative HNSCC. Remarkably our identified five-miRNA-signature predicts survival as well. Of note: its prognostic significance is independent from known clinical parameters

A potential limitation of the study is the fact that clinical data for both samples were obtained retrospectively. We thus cannot fully exclude certain selection bias. Heterogeneity due to inclusion of a multicenter HNSCC patient sample minimized and potentially excluded selection bias. In addition, the signature's robustness and potential clinical applicability was underlined by identification in a multicenter sample and validation in an independent monocentric sample. Most other studies introducing prognostic miRNA-signatures (e.g. ovarian, nasopharyngeal and colon cancer) followed a comparable strategy (8,20,21).

The fact that the DKTK-ROG sample exclusively included HNSCC patients treated by post-operative radiochemotherapy, whereas the LMU-KKG sample comprised both adjuvant treatment groups – radiotherapy with simultaneous chemotherapy and radiotherapy alone – might be seen as another limitation of our study. However, from our point of view, the independence of the five-miRNA-signature from the addition of simultaneous chemotherapy even strengthens the potential of our five-miRNA-signature.

A further potential shortcoming of our study is that the final RPA was limited by small numbers of patients. In order to achieve the highest possible number of cases and the maximum statistical power, we pooled both HNSCC samples for this analysis (n=162). In all clinical endpoints a significant separation of risk groups defined by clinical risk factors combined with the five-miRNA-signature was achieved.

To substantiate our findings on patient stratification into risk groups, further validation of our five-miRNA-signature in independent retrospective and in particular prospective patient populations with fully annotated clinical data will be important future steps.

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Previous studies have identified multiple deregulated miRNAs in HNSCC partly with prognostic relevance for patients (10-12,22-26). A meta-analysis revealed that in particular overexpression of miR-21, one of the most frequently studied cancer-related miRNAs, predicts poor prognosis in HNSCC (10). However, in general, the overlap of prognostic miRNAs across different HNSCC studies is small. This can be potentially explained by differences in demography, treatment parameters, composition of patient subgroups (e.g. subsite and HPV-status) as well as by methodological issues like the lack of independent validation, limitations due to small sample size, the analysis of different endpoints, the number of miRNAs screened and the non-availability of thorough clinical information including HPV-status (27). Our comprehensive miRNA profiling approach deliberately and exclusively focused on HPV-negative patients based on the fact that all current data indicate a completely distinct molecular pathogenesis of HPV-associated cancer, which, meanwhile, is regarded a distinct clinical entity (2,6).

Nevertheless, in our study we were able to confirm previously reported prognostic miRNAs in HNSCC such as hsa-miR-21-3p, hsa-let-7g-3p, hsa-miR-210-5p and hsa-miR-210-3p (Figure S20) underlining the validity of our miRNA analysis (10,22,26,28,29). In addition, hsa-mir-210-5p and hsa-let-7g-3p form part of our five-miRNA-signature. hsa-let-7g was shown to predict prognosis in oral cavity squamous cell carcinoma (29) and breast cancer patients (30) via inhibition of cell invasion and metastasis. Besides head and neck cancer (28), hsa-mir-210 was already reported as prognostic factor in breast cancer (31-34), soft-tissue sarcoma (35), osteosarcoma (36), pancreatic cancer (37), non-small cell lung cancer (38), renal cancer (39) and glioblastoma (40). Multiple functions of hsa-miR-210 are described including hypoxic response, regulation of mitochondrial metabolism, cell cycle, cell survival, differentiation DNA repair and immune response (41). To the best of our knowledge, the remaining three signature miRNAs (hsa-miR-6508-5p, hsa-miR-4306 and hsa-miR-7161-3p) have not yet been associated with HNSCC or cancer in general.

miRNAs are integrative regulator molecules with a highly promiscuous nature thereby interfering with multiple pathways. Thus, it is not possible to deduce a definitive functional role of a given miRNA within a signaling network. Nevertheless, studying the miRNA-mRNA-target network our five-miRNA-signature suggests enrichment of specific signaling pathways: *p53, ATM, FoxO signaling,* and *DNA double strand break response, pre-NOTCH expression and processing*, as well as *mitosis* and *senescence* associated pathways. Several of the pathways and miRNA target genes were already shown to be relevant for the pathogenesis and radiation response of HNSCC

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(5-7,42-47). Mutations of IGF1R and ARID1A and the involvement of CADM1 and SOD2 in HNSCC have been reported (6,43,46,47).

Gene expression relates to prognosis of HNSCC (48) as does a seven-gene signature, recently also described in our patients (49); this signature, however, predicts freedom from recurrence independently from the above mentioned five-miRNA-signature (unpublished). Analogous to their prognostic independence the molecular impact of the Schmidt et al. seven-gene signature shows no obvious overlap with that of our five-miRNA-signature (49). However, to pin down mechanisms and pathogenic relevance of the five-miRNA-signature further studies are required.

At present, treatment decisions for patients with HNSCC are guided predominantly by clinical findings. The only relevant biological marker with yet limited influence on treatment decisions is HPV-status (1). A key prerequisite for the potential clinical application of a molecular signature is a robust, fast and easy to perform laboratory assay. Our qRT-PCR validation of the high-throughput omics data is a first step in this direction.

The five-miRNA-signature's potential is particularly exemplified by the fact that, when combined with the clinically relevant prognostic parameters TNM T stage, ECE and TNM N stage, it allowed the significant stratification of patients into four risk groups for recurrence. Strikingly, in this context, the five-miRNA-signature was the strongest factor for patient stratification. Furthermore, the integration of the molecular signature with clinical factors not only improved the prediction of outcome but also allowed a more detailed, clinically meaningful stratification of patients, which, in turn, could be used as a clinical patient stratification tool.

Possible personalized treatment options include consideration of adjusting therapy intensity according to the overall risk for therapy failure. In particular patients with the highest risk of recurrence, for whom the standard treatment is not sufficient, might be candidates for more personalized treatment options such as the addition of targeted drugs or immune checkpoint inhibitors to radio(chemo)therapy, dose escalation or further (neo)adjuvant chemotherapy. On the other hand, for patients with the lowest risk of recurrence de-escalation strategies for the reduction of therapy-associated toxicity could be considered. Here dose de-escalation and the omission of chemotherapy would be options, as the long-term benefit from the addition of simultaneous chemotherapy to radiotherapy is not given for all patients (50). Further, the five-miRNA-signature represents the basis for a more focused search for molecular therapeutic targets improving therapy success for appropriate patients.

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In order to evaluate the predictive value of the five-miRNA-signature for the guidance of treatment decisions, prospective validation studies and clinical trials considering treatment stratification are required in future.

In summary, the herein identified prognostic five-miRNA-signature independently predicts disease control and survival of HPV-negative patients. The target gene network of the signature miRNAs is well in line with known mechanisms driving HNSCC pathogenesis. In combination with established prognostic clinical parameters the ability of the signature to predict disease control and survival even improves and allows the definition of four prognostically distinct groups. These may provide an important step towards personalized HNSCC treatment.

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Tables

Table 1: Clinical and pathological characteristics of HNSCC patients included in the DKTK-ROG and the LMU-KKG sample and stratified according to the five-miRNA-signature

	Training set DKTK-ROG (*n*=85)				Validation set LMU-KKG (*n*=77)			
	Number of all patients	**low-risk (*n*=43)**	**high-risk (*n*=42)**	**p-value***	**Number of all patients**	**low-risk (*n*=38)**	**high-risk (*n*=39)**	**p-value***
Age (years)				0.77				0.86
<45	7 (8%)	2 (5%)	5 (12%)		3 (4%)	1 (3%)	2 (5%)	
45-54	26 (31%)	13 (30%)	13 (31%)		17 (22%)	7 (18%)	10 (26%)	
55-64	35 (41%)	18 (42%)	17 (40%)		28 (36%)	15 (39%)	13 (33%)	
65-74	17 (20%)	10 (23%)	7 (17%)		26 (34%)	13 (34%)	13 (33%)	
>75	0	0	0		3 (4%)	2 (5%)	1 (3%)	
Sex				1.0				1.0
Male	67 (79%)	34 (79%)	33 (79%)		52 (68%)	26 (68%)	26 (67%)	
Female	18 (21%)	9 (21%)	9 (21%)		25 (32%)	12 (32%)	13 (33%)	
Tumor Localization				0.12				0.022
Hypopharynx	13 (15%)	9 (21%)	4 (10%)		15 (19%)	4 (11%)	11 (28%)	
Oral cavity	32 (38%)	12 (28%)	20 (48%)		27 (35%)	11 (29%)	16 (41%)	
Oropharynx	40 (47%)	22 (51%)	18 (43%)		35 (45%)	23 (61%)	12 (31%)	
UICC TNM Stage				0.56				0.79
I	0	0	0		2 (3%)	1 (3%)	1 (3%)	
II	3 (4%)	2 (5%)	1 (2%)		6 (8%)	4 (11%)	2 (5%)	
III	13 (15%)	5 (12%)	8 (19%)		23 (30%)	12 (32%)	11 (28%)	
IV	69 (81%)	36 (84%)	33 (79%)		46 (60%)	21 (55%)	25 (64%)	
T stage				0.33				0.042
T1	12 (14%)	9 (21%)	3 (7%)		17 (22%)	9 (24%)	8 (21%)	
T2	35 (41%)	17 (40%)	18 (43%)		29 (38%)	18 (47%)	11 (28%)	
T3	22 (26%)	10 (23%)	12 (29%)		21 (27%)	10 (26%)	11 (28%)	
T4	16 (19%)	7 (16%)	9 (21%)		10 (13%)	1 (3%)	9 (23%)	
N stage				0.14				0.41
N0	10 (12%)	5 (12%)	5 (12%)		19 (25%)	8 (21%)	11 (28%)	
N1	10 (12%)	2 (5%)	8 (19%)		20 (26%)	10 (26%)	10 (26%)	
N2	57 (67%)	33 (77%)	24 (57%)		36 (47%)	20 (53%)	16 (41%)	
N3	8 (9%)	3 (7%)	5 (12%)		2 (3%)	0	2 (5%)	
Lymphovascular invasion (LVI)				0.46				1.0
0	42 (49%)	25 (58%)	17 (40%)		50 (65%)	26 (68%)	24 (62%)	
1	27 (32%)	13 (30%)	14 (33%)		17 (22%)	9 (24%)	8 (21%)	
Missing information	16 (19%)	5 (12%)	11 (26%)		10 (13%)	3 (8%)	7 (18%)	
Venous tumor invasion (VTI)				1.0				1.0
0	62 (73%)	33 (77%)	29 (69%)		66 (86%)	34 (89%)	32 (82%)	
1	7 (8%)	4 (9%)	3 (7%)		3 (4%)	2 (5%)	1 (3%)	
Missing information	16 (19%)	6 (14%)	10 (24%)		8 (10%)	0	6 (15%)	
Perineural invasion (PNI)				1.0				0.55
0	0	0	0		37 (48%)	19 (50%)	18 (46%)	
1	0	0	0		15 (19%)	6 (16%)	9 (23%)	
Missing information	85 (100%)	43 (100%)	42 (100%)		25 (32%)	13 (34%)	12 (31%)	
Resection margin status				0.52				0.49
0	45 (53%)	21 (49%)	24 (57%)		57 (74%)	28 (74%)	29 (74%)	
1	40 (47%)	22 (51%)	18 (43%)		17 (22%)	7 (18%)	10 (26%)	
2	0	0	0		1 (1%)	1 (3%)	0	
Missing information	0	0	0		2 (3%)	2 (5%)	0	
Extracapsular extension (ECE)				0.007				0.38
yes	41 (48%)	14 (33%)	27 (64%)		25 (32%)	11 (29%)	14 (36%)	
no	34 (40%)	24 (56%)	10 (24%)		32 (42%)	19 (50%)	13 (33%)	
not applicable (N0)	10 (12%)	5 (12%)	5 (12%)		19 (25%)	8 (21%)	11 (28%)	
Missing information	0	0	0		1 (1%)	0	1 (3%)	
Grading				0.56				0.29
1 (well differentiated)	3 (4%)	2 (5%)	1 (2%)		2 (3%)	2 (5%)	0	
2 (moderately differentiated)	50 (59%)	23 (53%)	27 (64%)		34 (44%)	15 (39%)	19 (49%)	
3 (poorly differentiated)	32 (38%)	18 (42%)	14 (33%)		41 (53%)	21 (55%)	20 (51%)	
ECOG performance status				0.64				0.20
0	18 (21%)	8 (19%)	10 (24%)		13 (17%)	4 (11%)	9 (23%)	
1	33 (39%)	17 (40%)	16 (38%)		40 (52%)	21 (55%)	19 (49%)	
2	6 (7%)	4 (9%)	2 (5%)		5 (6%)	1 (3%)	4 (10%)	
Missing information	28 (33%)	14 (33%)	14 (33%)		19 (25%)	12 (32%)	7 (18%)	
Smoking status				0.18				0.68
Non-smoker	5 (6%)	4 (9%)	1 (2%)		6 (8%)	2 (5%)	4 (10%)	
Smoker	52 (61%)	23 (53%)	29 (69%)		52 (68%)	25 (66%)	27 (69%)	
Missing information	28 (33%)	16 (37%)	12 (29%)		19 (25%)	11 (29%)	8 (21%)	
Smoking history – pack-years				0.20				0.67
≤10 (including non-smokers)	7 (8%)	5 (12%)	2 (5%)		6 (8%)	2 (5%)	4 (10%)	
>10	23 (27%)	9 (21%)	14 (33%)		48 (62%)	25 (66%)	23 (59%)	
Missing information	55 (65%)	29 (67%)	26 (62%)		23 (30%)	11 (29%)	12 (31%)	
Simultaneous Chemotherapy				1.0				0.16
Yes	85 (100%)	43 (100%)	42 (100%)		49 (64%)	21 (55%)	28 (72%)	
No	0	0	0		28 (36%)	17 (45%)	11 (28%)	

Data are numbers (%). *Chi-square test or Fisher's exact test.

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Table 2: Multivariate Cox regression analysis of the five-miRNA-signature and clinicopathological parameters with freedom from recurrence (training and validation set)

	Training set DKTK-ROG		Validation set LMU-KKG	
Parameter	**HR (95% CI)**	**p-value**	**HR (95% CI)**	**p-value**
Five-miRNA-signature (high-risk *vs* low-risk)	5.55 (2.09-14.79)	<0.001	3.94 (1.23-12.59)	0.021
TNM T stage (T3/T4 *vs* T1/T2)	2.19 (0.96-5.02)	0.064	2.71 (0.99-7.44)	0.052
Lymphovascular invasion (yes *vs* no)	2.22 (0.99-4.97)	0.053	2.50 (0.84-7.45)	0.099
Extracapsular extension (yes *vs* no*)	1.45 (0.61-3.48)	0.40	2.29 (0.77-6.78)	0.13

*N0 tumors were included in the group of extracapsular extension negative tumors

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Figure Legends

Figure 1: **Freedom from recurrence stratified by risk according to the five-miRNA-signature: miRNA expression and Kaplan-Meier curves in the DKTK-ROG (training set) and the LMU-KKG (validation set) sample**

(A) Upper panel: Heat map colors indicate scaled miRNA log2 expression values multiplied by the Cox proportional hazard coefficients (coxph) from low (blue) to high (red) on a scale from -3 to 3 for each of the five signature miRNAs in the DKTK-ROG (left panel) and the LMU-KKG sample (right panel). Lower panel: Kaplan-Meier curves for the endpoint freedom from recurrence for HNSCC patients of the training (DKTK-ROG sample; left panel) and validation set (LMU-KKG sample; right panel) stratified into low- and high-risk patients according to the five-miRNA-signature. *P*-values are derived by log-rank test. **(B)** Kaplan-Meier curves for recurrence-free survival (upper panel), overall survival (middle panel) and disease-specific survival (lower panel) in patients of the training (DKTK-ROG sample; left) and validation set (LMU-KKG sample; right) stratified according to their risk (low- and high-risk group) by the five-miRNA-signature.

Figure 2: **Performance of the prediction of freedom from recurrence comparing the five-miRNA-signature with clinicopathological risk factors**

(A) Sensitivity and specificity derived areas under the curve (AUCs) for the prediction of freedom from recurrence in the DKTK-ROG (training set; left panel) and the LMU-KKG sample (validation set; right panel) at five follow-up years. The AUCs and the 95% CI of the five-miRNA-signature derived risk factor (black dashed curve), TNM T stage, lymphovascular invasion (LVI) and extracapsular extension (ECE) are shown.

Time-dependent sensitivity and specificity derived AUCs for the prediction of freedom from recurrence in the DKTK-ROG t (left panel) and the LMU-KKG sample (right panel) at follow-up years 1-5: **(B)** AUCs of the five-miRNA-signature derived risk factor (black dashed curve), TNM T stage, LVI and ECE. **(C)** AUCs for the five-miRNA-signature derived risk factor alone (black dashed curve), the five-miRNA-signature combined with TNM T stage, LVI and ECE (purple and greenish curves) and combinations of the clinicopathological risk factors TNM T stage, LVI and ECE (bluish curves).

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Figure 3: **Risk groups for recurrence identified by recursive partitioning analysis (RPA)**

RPA tree and risk groups for recurrence combining the parameters five-miRNA-signature (high-risk, low-risk), ECE (negative - including N0 tumors, positive), T stage (T1/T2, T3/T4) and N stage (N0/N1, N2/N3) in the pooled HNSCC data set (n=162). Each node shows the predicted probability of recurrence (locoregional or distant failure; color code low to high: blue-red), the number of events for the total number of patients and the percentage of observations in the node. Kaplan-Meier curves for the endpoint freedom from recurrence for the four identified risk groups "low-risk", "low-intermediate-risk", "high-intermediate-risk" and "high-risk". Multivariate and pairwise comparisons are shown. *P*-values are derived by log-rank test. See extended Figure version Supplementary Figure S16.

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Figure 1

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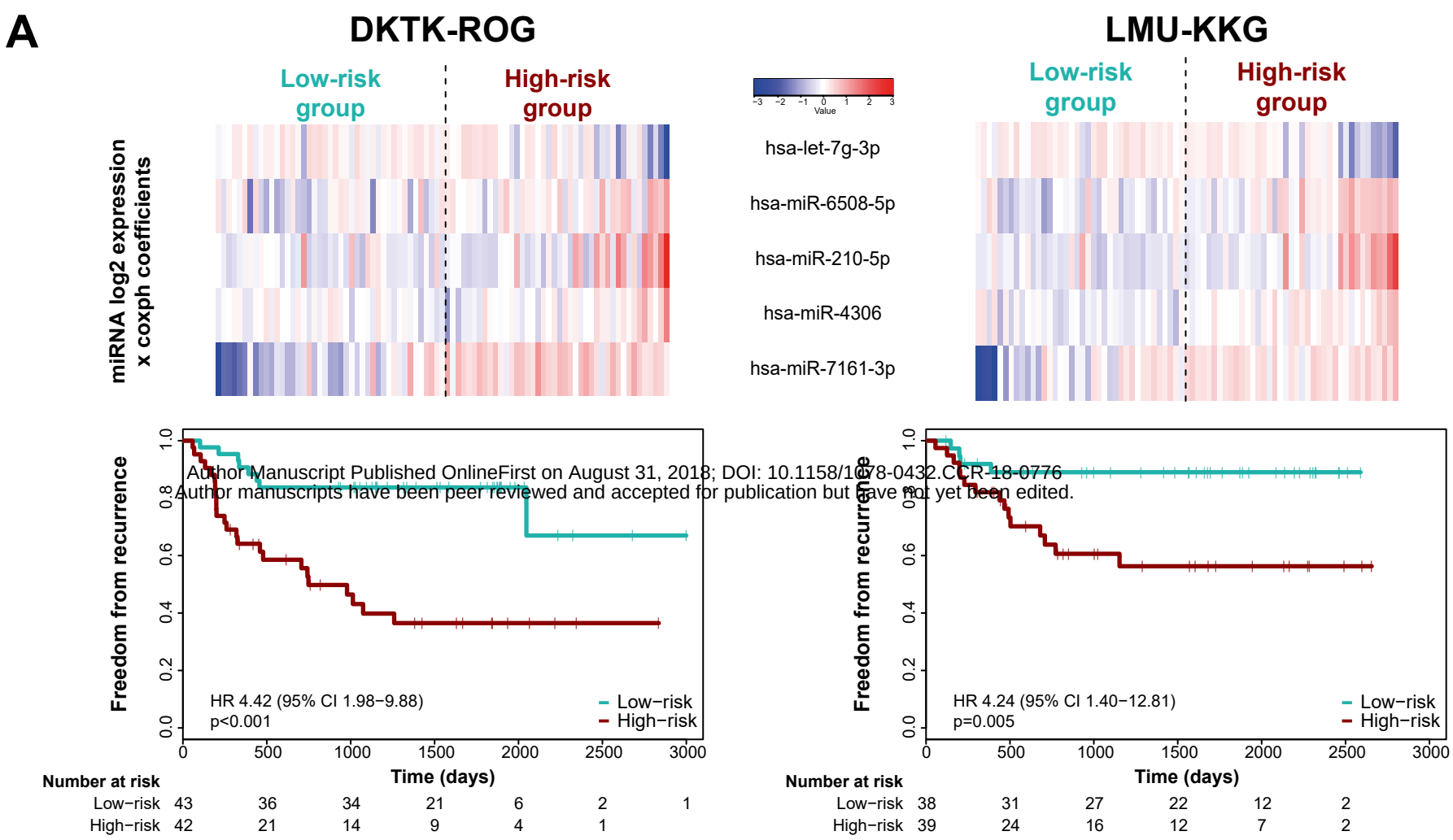

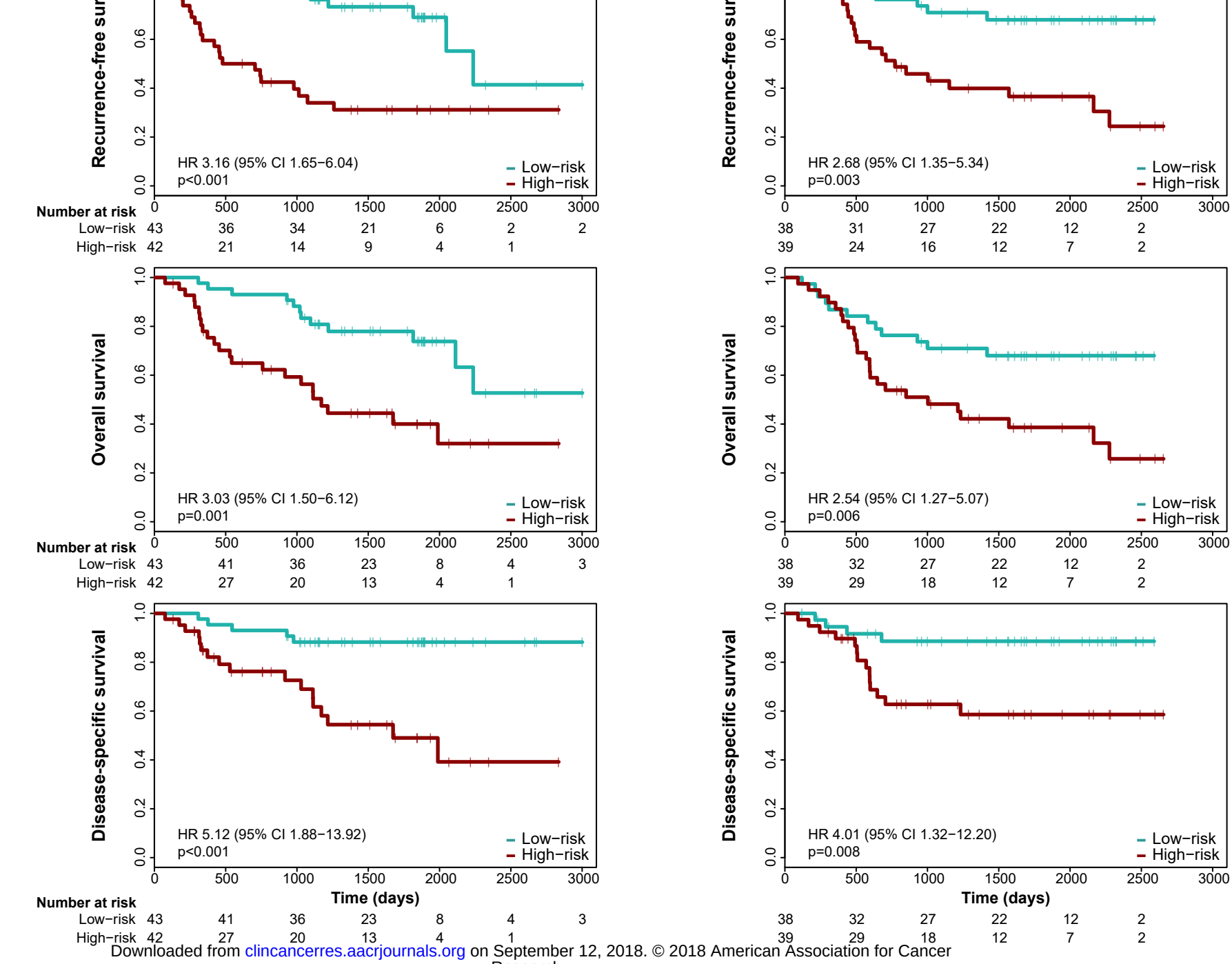

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Figure 2

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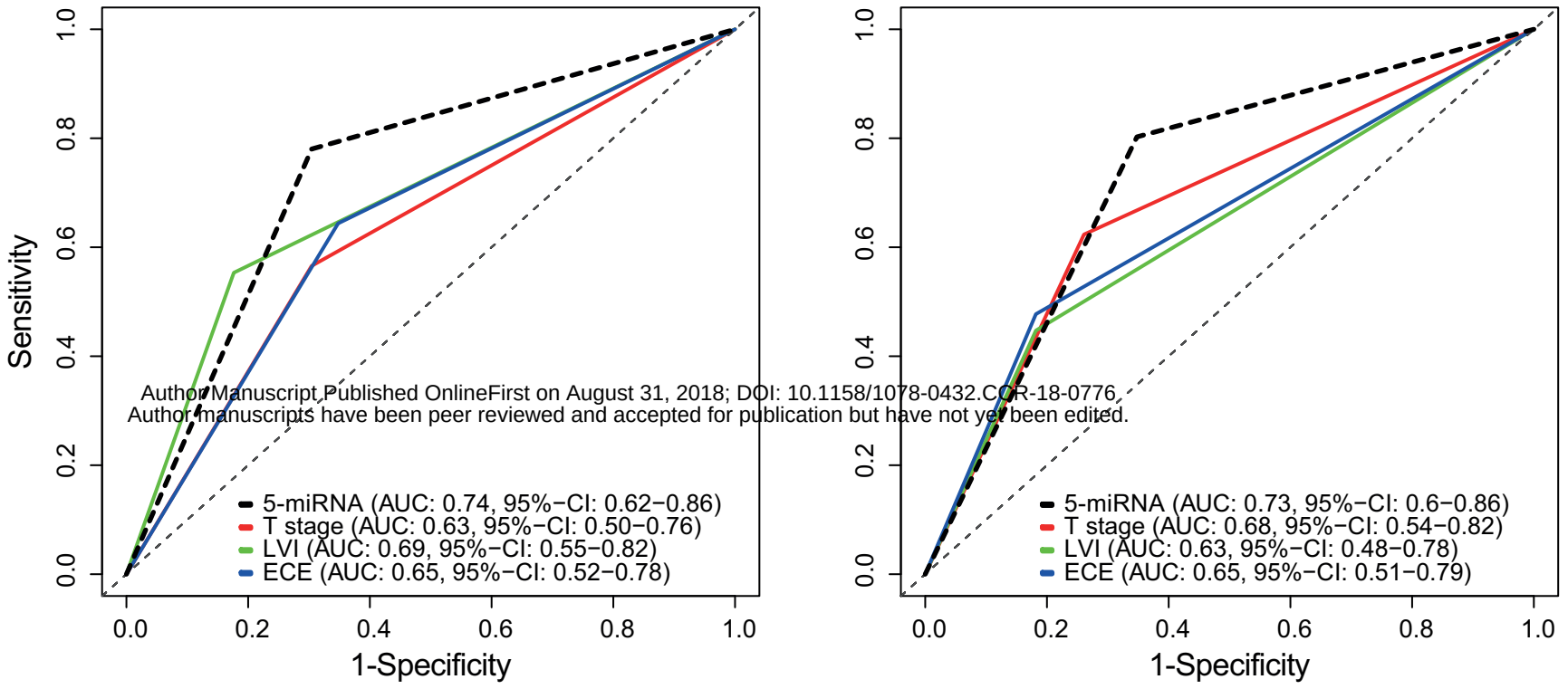
A



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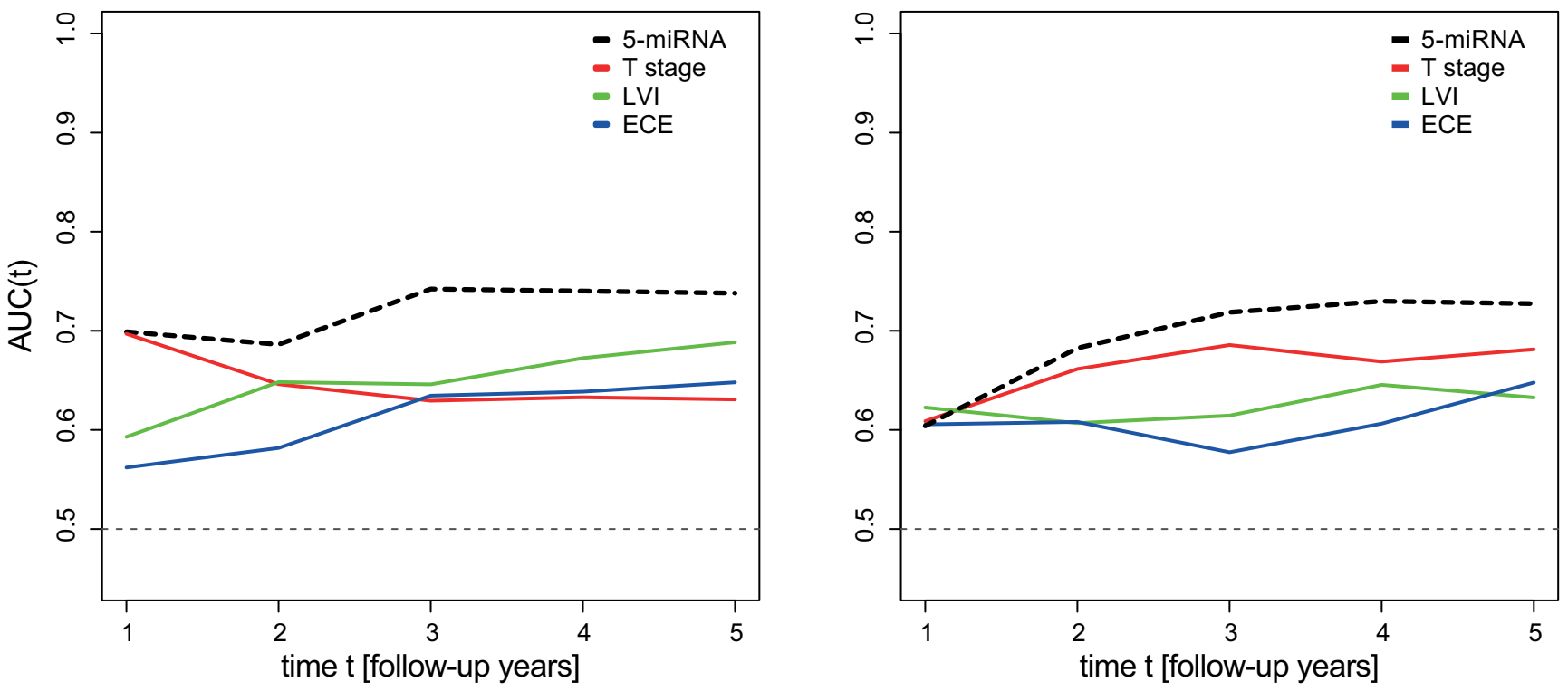

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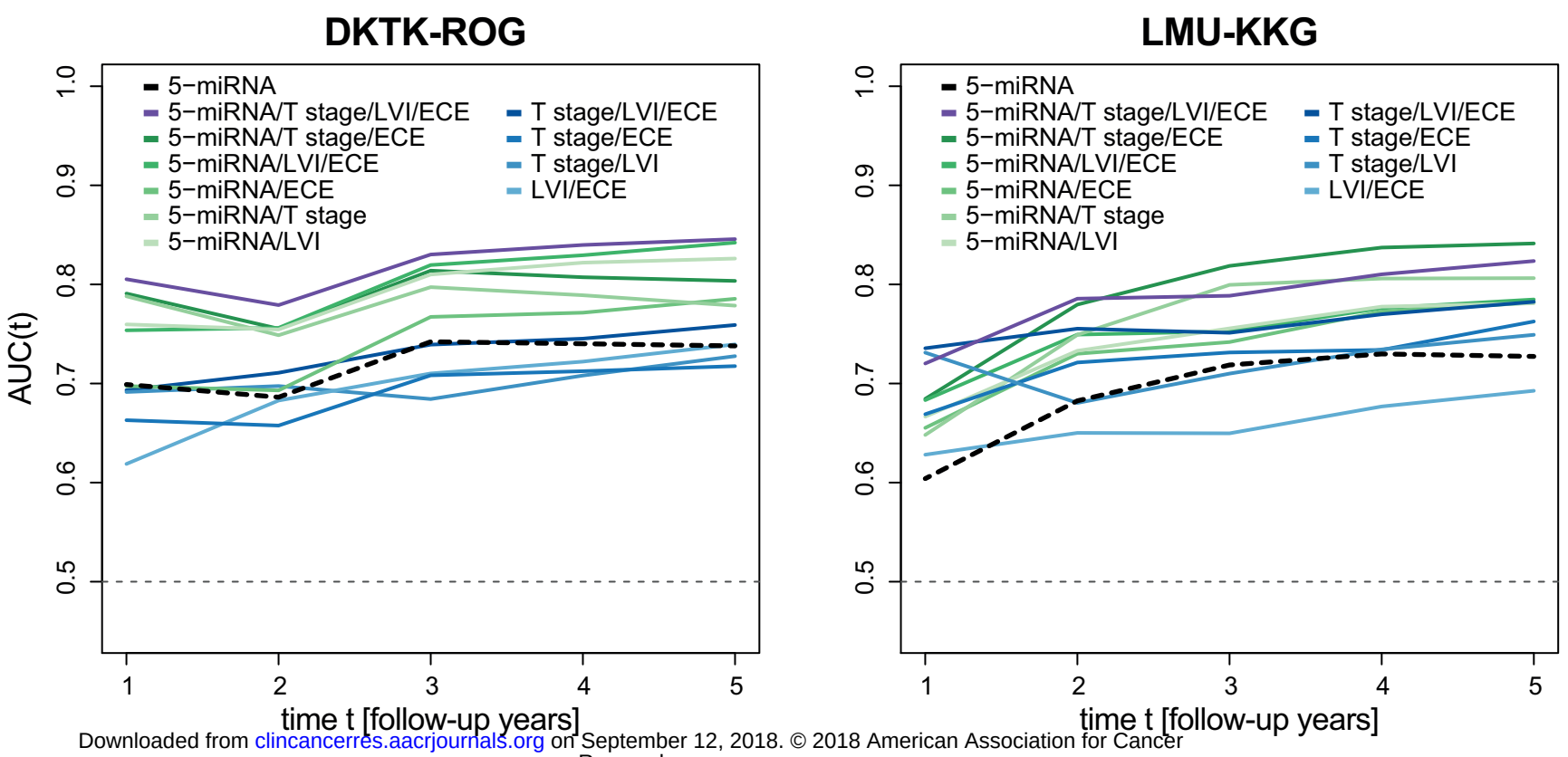

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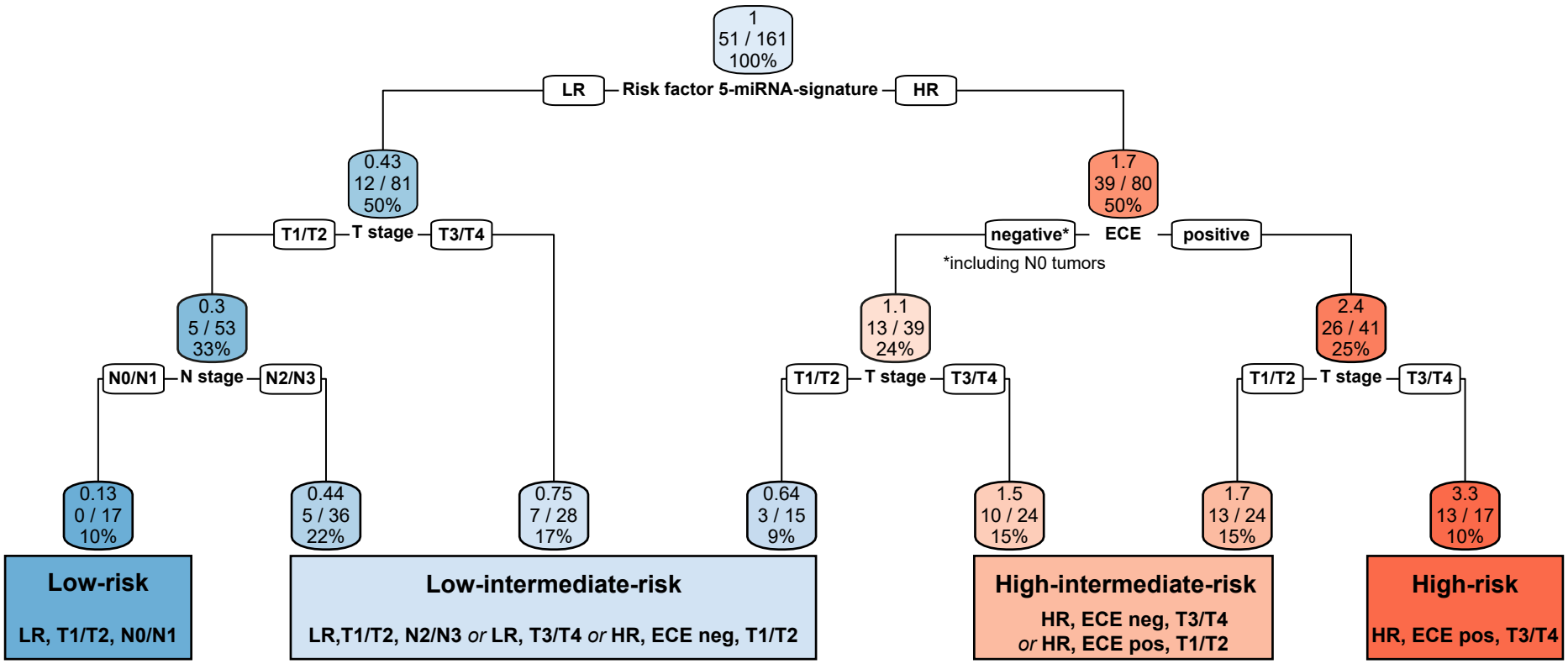
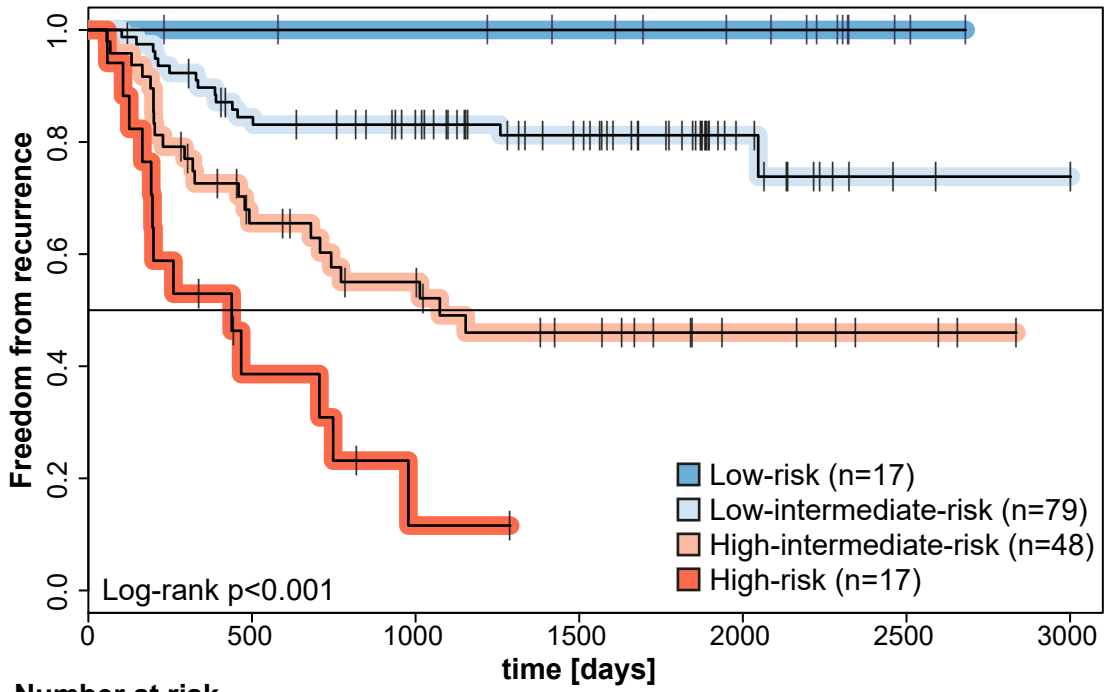
Figure 3





Number at risk

Low-risk (n=17)	17	16	15	13	10	2	
Low-intermediate-risk (n=79)	79	63	54	37	12	2	1
High-intermediate-risk (n=48)	48	27	20	13	6	3	
High-risk (n=17)	17	8	1				

Low-risk *vs.* low-intermediate-risk
p=0.042
HR NA*
*not estimable because no event in the low-risk group

Low-intermediate-risk *vs.* high-intermediate-risk
p<0.001
HR 3.17 (95% CI 1.65-6.09)

High-intermediate-risk *vs.* high-risk
p=0.006
HR 2.55 (95% CI 1.28-5.11)

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Clinical Cancer Research

A Five-MicroRNA Signature Predicts Survival and Disease Control of Patients with Head and Neck Cancer Negative for HPV-infection

Julia Hess, Kristian Unger, Cornelius Maihoefer, et al.

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