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1 # Development and validation of a gene signature for patients with head and
2 neck carcinomas treated by postoperative radio(chemo)therapy

3

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93 **Abbreviations**

| | |
|---|---|
| 94 ci | concordance index |
| 95 CI | confidence interval |
| 96 CSC | cancer stem cells |
| 97 DM | freedom from distant metastases |
| 98 dLL | difference in log-likelihood |
| 99 DKTK | German Cancer Consortium |
| 100 DKTK-ROG | German Cancer Consortium Radiation Oncology Group |
| 101 FFPE | formalin-fixed paraffin-embedded |
| 102 HNSCC | head and neck squamous cell carcinomas |
| 103 HPV | human papilloma virus |
| 104 HR | high risk group |
| 105 LRC | loco-regional tumor control |
| 106 LR | low risk group |
| 107 oob | out-of-the-bag |
| 108 OS | overall survival |
| 109 PORT | postoperative radiotherapy |
| 110 PORT-C | postoperative radiochemotherapy |

111



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112 **Translational relevance:**

113 Patients with HPV-positive, locally advanced head and neck squamous cell carcinomas
114 (HNSCC) show a very good loco-regional tumor control (LRC) after postoperative radiochemo-
115 therapy (PORT-C) and are therefore candidates for trials on treatment de-escalation to reduce
116 toxicity. For patients with HPV-negative HNSCC, additional biomarkers are urgently needed to
117 identify subgroups of patients, (I) who are unlikely to respond to standard PORT-C and may
118 benefit from treatment escalation, or (II) who will likely not develop loco-regional recurrences.
119 We developed and independently validated a 7-gene signature prognostic for LRC of HPV-
120 negative tumors which is based on several radiobiological parameters or mechanisms. The prog-
121 nostic performance of this radiobiology-based signature combined with clinical parameters was
122 higher than that of a model containing hypoxia-associated genes and CSC markers only. After
123 additional prospective validation, the 7-gene signature may be applied in clinical trials for patient
124 stratification.



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125 **Abstract:**

126 Purpose: The aim of this study was to identify and independently validate a novel gene signature
127 predicting loco-regional tumor control (LRC) for treatment individualization of patients with
128 locally advanced HPV-negative head and neck squamous cell carcinomas (HNSCC) who are
129 treated with postoperative radio(chemo)therapy (PORT-C).

130 Experimental Design: Gene expression analyses were performed using nanoString technology on
131 a multicenter training cohort of 130 patients and an independent validation cohort of 121 pa-
132 tients. The analyzed gene set was composed by genes with previously reported association to
133 radio(chemo)sensitivity or resistance to radio(chemo)therapy. Gene selection and model building
134 were performed comparing several machine-learning algorithms.

135 Results: We identified a 7-gene signature consisting of the 3 individual genes *HILPDA*, *CD24,*
136 *TCF3* and one metagene combining the highly correlated genes *SERPINE1*, *INHBA*, *P4HA2*,
137 *ACTN1*. The 7-gene signature was used, in combination with clinical parameters, to fit a multi-
138 variable Cox model to the training data (concordance index, ci=0.82), which was successfully
139 validated (ci=0.71). The signature showed improved performance compared to clinical parame-
140 ters alone (ci=0.66) and to a previously published model including hypoxia-associated genes and
141 cancer stem cell markers (ci=0.65). It was used to stratify patients into groups with low and high
142 risk of recurrence, leading to significant differences in LRC in training and validation ($p<0.001$).

143 Conclusions: We have identified and validated the first hypothesis-based gene signature for
144 HPV-negative HNSCC treated by PORT-C including genes related to several radiobiological
145 aspects. A prospective validation is planned in an ongoing prospective clinical trial before poten-
146 tial application in clinical trials for patient stratification.



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147 **Introduction**

148 Head and neck cancer is the 6th most frequently occurring tumor entity worldwide (1) with an
149 overall 5-year survival rate of about 50% (2). Patients with resectable, locally advanced head and
150 neck squamous cell carcinomas (HNSCC) who are at high risk for tumor recurrence are being
151 routinely treated with postoperative radiochemotherapy (PORT-C). According to the results of
152 three randomized clinical trials, concurrent chemotherapy leads to improved loco-regional tumor
153 control (LRC) and prolonged overall survival (OS) compared to postoperative radiotherapy
154 (PORT) alone (3–5). Within the last years, radiotherapy of locally advanced HNSCC has been
155 further improved through the development of new treatment techniques, such as intensity modu-
156 lated radiotherapy. Despite the increase in treatment efficacy, patients show a very heterogene-
157 ous treatment response. Therefore, the consideration of the individual tumor biology by appro-
158 priate biomarkers in addition to well-established clinical parameters may further improve patient
159 stratification for treatment escalation or de-escalation strategies.
160 Besides the consumption of alcohol and tobacco as well-known risk factors for the development
161 of HNSCC, infection with the human papilloma virus (HPV) has been identified as another inde-
162 pendent parameter. Also, the incidence of HPV infection in HNSCC has been increasing within
163 the last decade (6). Preclinical and clinical studies have shown that HPV-positive HNSCC are
164 more radiosensitive than HPV-negative tumors (7,8). To investigate the impact of HPV in pa-
165 tients who receive PORT-C and to identify additional biomarkers for patient selection, a retro-
166 spective, multicenter study of the German Cancer Consortium Radiation Oncology Group
167 (DKTK-ROG) was conducted (9–13). For this cohort, we have shown that patients with HPV16
168 DNA-positive tumors have superior LRC and OS compared to patients with HPV-negative tu-
169 mors (9). In particular, 98% of the HPV-positive and only 80% of the HPV-negative oropharyn-



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170 geal tumors were loco-regionally controlled. For patients with HPV DNA negative tumors, addi-
171 tional biomarkers are urgently needed to identify subgroups of patients, who are unlikely to re-
172 spond to PORT-C and may benefit from treatment escalation, or who are not anticipated to de-
173 velop loco-regional recurrences.

174 Tumor hypoxia has been shown to be correlated with increased radioresistance (14). For patients
175 with locally advanced HNSCC, pre-treatment hypoxia was significantly associated with low tu-
176 mor control and OS after primary radio(chemo)therapy compared to patients with highly oxy-
177 genated tumors (15,16). Several hypoxia gene classifiers have been developed in the last decade
178 to assess hypoxia or hypoxia-related changes on the transcriptional level using routinely taken
179 pre-treatment biopsies (17,18). We have recently shown their prognostic validity for patients at
180 high risk of loco-regional failure receiving PORT-C (12). The association of hypoxia and LRC
181 after PORT-C is unexpected since the gross tumor has been removed and subsequently remain-
182 ing tumor cells are very unlikely to differ in hypoxia (12). This suggests that hypoxia impacts
183 LRC not only by a direct biochemical effect on cellular radioresistance but also by other radiobi-
184 ological mechanisms (12,19). Recent studies reported that hypoxia as an external factor also fa-
185 vors increased radioresistance of cancer stem cells (CSC) and invasive tumor growth (reviewed
186 in (20,21)), and CSCs are known to play a major role in radioresistance and tumor recurrence
187 (reviewed in (22)). The putative CSC markers *CD44*, *SLC3A2* and *MET* were shown to be prog-
188 nostic for LRC in patients who received PORT-C (12). The combined application of hypoxia-
189 associated gene panels and CSC markers further improved patient stratification regarding their
190 risk of loco-regional treatment failure (23,24), which was independently validated (25).

191 A gene signature including additional radiobiological aspects may predict patient outcome with
192 even higher accuracy. In the literature, the number of gene panels for stratification of patients



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193 with HNSCC is steadily growing. However, to the best of our knowledge they have been devel-
194 oped for patients who received primary radiotherapy (26,27) or have not been linked to a specific
195 treatment (28–30). Gene signatures prognostic for the response of patients with locally advanced
196 HNSCC to PORT-C covering a broad spectrum of radiobiological aspects are still missing.
197 Therefore, the major aim of this study was to develop and validate a gene signature and corre-
198 sponding statistical model for patient stratification beyond HPV infection status to improve the
199 risk assessment for patients with locally advanced HNSCC who receive PORT-C. For the devel-
200 opment of this gene signature, a gene set was composed in-house using a hypothesis-driven ap-
201 proach. The gene set incorporated genes that cover many radiobiologically important aspects
202 such as DNA repair, cell cycle, epithelial-mesenchymal transition, CSC markers, hypoxia, pro-
203 liferation, invasion, metastasis, as well as genes that were reported to be involved in cisplatinum-
204 resistance. The signature was developed and independently validated on two large patient co-
205 horts for the primary endpoint LRC and the secondary endpoints OS and freedom from distant
206 metastases (DM). To find the optimal results, internal validation methods were applied, and sev-
207 eral statistical methods were compared, including advanced machine learning techniques.



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208 **Materials and Methods**

209 **Patients**

210 Two different cohorts of patients with locally advanced HNSCC were being considered for this
211 study. The training cohort consisted of 221 patients who were treated with PORT-C between
212 2004 and 2012 within the 9 partner sites of the DKTK-ROG. Inclusion criteria, data collection,
213 handling and analyses of biomaterial were previously described (9,12). Briefly, all patients re-
214 ceived curatively intended cisplatinum-based PORT-C according to standard protocols with a
215 minimum follow-up of 24 months and presented with a tumor stage pT4 and/or >3 positive
216 lymph nodes and/or positive microscopic resection margins and/or extracapsular spread. The
217 validation cohort consisted of 152 patients who were enrolled by the following criteria: not in-
218 cluded in the previous DKTK-ROG training cohort, histologically proven HNSCC, treatment
219 between 1999 and 2006 with PORT or PORT-C according to standard radiotherapy protocols
220 with curative intention (25).

221

222 **Preparation of biomaterials and biomarker analyses**

223 Formalin-fixed paraffin-embedded (FFPE) blocks of the primary tumor specimens (removed by
224 surgery) were first subjected to haematoxylin and eosin staining to histologically confirm the
225 presence of squamous cell carcinoma. Afterwards, they were processed under standardized pro-
226 cedures for biomarker investigations. DNA extraction and PCR-array based analyses of HPV
227 status have been performed as described previously (9). Briefly, genomic DNA was extracted
228 from 5-µm FFPE sections using the QIAamp DNA FFPE tissue kit (Qiagen). HPV DNA anal-
229 yses including genotyping were performed using the LCD-Array HPV 3.5 kit (CHIPRON
230 GmbH, Berlin, DE) according to the manufacturer's instruction.



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231 For both cohorts, gene expression analyses were performed consecutively using nanoString ele-
232 ments technology (nanoString Technologies, Seattle, WA, USA) as described in (12,25). Briefly,
233 total RNA as well as reporter and capture probes specific to the genes of interest were mixed and
234 incubated at 62 °C for 22 hours. Samples were then kept at 4 °C for a maximum of 18 hours and
235 subjected to the nCounter system. Raw counts were logarithmized and then normalized by sub-
236 tracting the mean of the log-transformed counts of the reference genes *ACTR3*, *B2M*, *GNB2L1*,
237 *NDFIP1*, *POLR2A*, *RPL11* and *RPL37A*. Due to insufficient tumor material or too low RNA
238 yield, some of the samples had to be omitted from the analysis. In the training and validation
239 cohort, nanoString and HPV analyses could be performed for 195 and 142 samples, respectively.
240 The expression levels of 178 genes were evaluated by nanoString analyses for both cohorts. The
241 genes were selected by a literature search on a hypothesis-driven basis. Genes were included that
242 have previously been reported to be associated with sensitivity or resistance to ra-
243 dio(chemo)therapy, i.e. genes involved in proliferation, invasion and metastasis; tumor hypoxia-
244 associated genes, genes encoding for putative CSC markers and DNA repair as well as genes that
245 have been associated with cisplatinum-resistance, see Supplementary Table S1.
246

247 **Study design**

248 The aim of the present study was to develop and validate a gene signature for patient stratifica-
249 tion beyond HPV infection status to further improve the risk assessment for patients with locally
250 advanced HNSCC who receive PORT-C. Therefore, only patients with HPV16 DNA negative
251 tumors and available nanoString gene expressions were included (N=130/221 training,
252 N=121/152 validation). The study design is presented in Figure 1. Prognostic models including



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253 the following parameters should be compared: the identified gene signature alone, clinical pa-
254 rameters alone and the combination of clinical parameters and the identified gene signature.
255

256 **Statistics and clinical endpoints**

257 The primary endpoint was loco-regional tumor control (LRC). Secondary endpoints were free-
258 dom from distant metastases (DM) and overall survival (OS). All endpoints were calculated from
259 the first day of radiotherapy to the date of event or censoring. Death was considered a competing
260 risk for loco-regional recurrence and DM, while loco-regional recurrence and DM did not cause
261 censoring. Survival curves were estimated by the Kaplan-Meier method and compared by Log-
262 rank tests. Differences between the training and validation cohort were evaluated by Mann-
263 Whitney-U tests for continuous variables and by chi-squared tests for categorical variables. De-
264 scriptive analyses and the described statistical tests were performed using SPSS 23 (IBM Corpo-
265 ration, Armonk, NY, USA). A statistical framework was developed to identify gene signatures
266 and corresponding prognostic models in order to optimally and robustly predict the primary and
267 secondary endpoints. This framework is described in the Supplementary Materials in detail. To
268 evaluate the prognostic performance of the developed models, the concordance index (ci) was
269 calculated (31). While ci=0.5 is obtained for a non-informative model, ci=1.0 represents a per-
270 fectly predicting model. To compare the performance between nested multivariable Cox models,
271 the likelihood-ratio test was applied. The framework to determine gene signatures and corre-
272 sponding prognostic models was implemented in R Statistics version 3.3.2 and Python 2.7. An
273 overview of the used programs and packages is given in Supplementary Table S2. For all anal-
274 yses, two-sided tests were performed and p-values below 0.05 were considered as statistically
275 significant.



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276

277 **Statistical framework to identify gene signatures and perform model predictions**

278 The statistical framework to identify gene signatures consists of four main steps, which are de-
279 scribed in detail in Supplementary Sections S1-S3: (i) The gene expression data are prepro-
280 cessed. Genes are removed from analysis, if their median expression is below twice the median
281 control in the training cohort. The expression of each gene is z-transformed to mean 0 and stand-
282 ard deviation 1 on the training cohort, which is favorable for most machine-learning algorithms.
283 The gene expressions of the validation cohort are transformed based on the means and standard
284 deviations of the training cohort. (ii) A feature selection method is applied to select the most rel-
285 evant genes using internal 3-fold cross validation on the training cohort (repeated 333 times).
286 The genes are combined to an ensemble signature based on their frequency of occurrence and
287 their importance (Figure 2). The resulting signature is then used to build prognostic models on
288 1000 bootstrap samples of the training cohort to predict the considered outcome. Several feature
289 selection methods, prognostic models and different signature sizes (1-10) are compared and the
290 best signature is chosen using the out of the bag data of the bootstrap samples. (iii) To increase
291 the robustness of the signature, genes which are highly correlated to one of the signature genes in
292 the training cohort are combined with this gene to create a new metagene (median expression of
293 the highly correlated genes). The resulting metagene replaces the original gene within the gene
294 signature. (iv) Finally, the model is validated using the independent validation cohort. The 95%
295 confidence interval (CI) of the ci is estimated from 1000 bootstrap samples of the validation co-
296 hort. Finally, the validation is declared successful if the 95% CI does not contain 0.5.



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297 **Results**

298 **Patient cohorts**

299 In this retrospective study, a multicenter training cohort of 130 patients and an independent, mo-
300 nocenter validation cohort of 121 patients with HPV16 DNA negative locally advanced HNSCC
301 were available for the development of a gene signature to predict the clinical endpoints LRC, OS
302 and DM. Patient data, treatment parameters and tumor characteristics of both patient cohorts
303 were published previously (9,34) and are summarized in Table 1. Patients in the validation co-
304 hort were treated with PORT (N=90) or PORT-C (N=31), while all patients of the training cohort
305 received PORT-C as the standard treatment. The training cohort included 44.6% patients with
306 oropharyngeal and 37.7% patients with oral cavity carcinomas. In the validation cohort, 21.5%
307 of the patients have been diagnosed with oropharyngeal and 62.0% with oral cavity carcinomas.
308 Patients in the validation cohort showed lower LRC (statistical trend) and OS, while the inci-
309 dence of DM was not significantly different. Actuarial rates of LRC, freedom from DM and OS
310 two years after radiotherapy for the training and validation cohort were 83.8% vs 75.0%
311 (p=0.096), 79.0% vs 82.8% (p=0.72) and 76.4% vs 64.9% (p=0.042), respectively.

312

313 **7-gene signature predicts LRC for HPV16 DNA negative tumors**

314 In order to identify a prognostic gene signature for the primary endpoint LRC, the four steps (i)-
315 (iv) of the statistical framework outlined in materials and methods were performed.

316 During the preprocessing step (i) the genes *FGFR2*, *PROM1* and *TAF7L* were removed from the
317 analysis. The mean validation ci from the 3-fold internal cross validation of step (ii) ranged be-
318 tween 0.57 and 0.68 and was similar between different feature selection methods and statistical
319 models, see Supplementary Figure S1 for signature size 4. An ensemble gene signature was de-



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320 termined for each combination of feature selection method and prognostic model, as described in
321 Supplementary Section S4. The performance of these signatures was evaluated using 1000 boot-
322 strap samples of the whole training cohort, see Figure 3. The highest mean ci of 0.78 was ob-
323 tained for a signature, which contained the genes *SERPINE1*, *CD24*, *HILPDA* and *TCF3*. For the
324 final prediction model, Cox regression was chosen, as it is the most simple of the well perform-
325 ing models. Signature size 4 was chosen based on the mean ci and the signature score of the
326 genes (Supplementary Section S4, Supplementary Figure S2). The signature score was highest
327 for *SERPINE1*, followed by *CD24*, *HILPDA* and *TCF3,* which showed a similar score, see Sup-
328 plementary Figure S3. To improve the robustness of the identified 4-gene ensemble signature, it
329 was extended by other highly correlated genes in step (iii), as described in Supplementary Sec-
330 tion S4. *INHBA*, *ACTN1* and *P4HA2* were found to be highly correlated with *SERPINE1*, while
331 for *CD24, TCF3* and *HILPDA* no additional correlated genes were found (Supplementary Table
332 S3). Thus, our final 7-gene signature for LRC consisted of the genes *SERPINE1*, *INHBA*,
333 *ACTN1*, *P4HA2*, *CD24, TCF3* and *HILPDA*. For evaluation, the median of the z-transformed,
334 reference-gene normalized expression of *SERPINE1*, *INHBA*, *ACTN1* and *P4HA2* was consid-
335 ered as a new metagene variable (Supplementary Section S4). The whole training cohort was
336 used to fit the final Cox regression model, leading to a training ci of 0.81 (95% CI: 0.75-0.88).
337 The resulting model parameters are shown in Table 2. In the last step (iv) the validation of the
338 final model was performed on the independent validation cohort. A validation ci of 0.69 (0.60-
339 0.77) was obtained, which represents a successful validation of the gene signature for the end-
340 point LRC.

341 Patient stratification into groups of low and high risk of recurrence was performed for the final
342 Cox model depending on the risk score, which is given by the linear predictor of the model. The



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343 optimal cut-off was chosen based on the training cohort at a risk score of 0.10. This cut-off led to
344 the highest fraction of patient stratifications with a significant difference in LRC, based on 1000
345 bootstrap samples of the training cohort (992/1000). Patients in the high risk group showed sig-
346 nificantly lower LRC than patients in the low risk group, both for the training (p<0.001) and the
347 validation cohort (p=0.001), see Supplementary Figure S4.
348

349 **Inclusion of clinical parameters to the 7-gene LRC signature**

350 For the training cohort, it was shown that the established clinical parameters tumor localization
351 and ECE status were significantly correlated with LRC or the secondary endpoints (9,12). Using
352 only these two parameters in a multivariable Cox model resulted in a lower performance (train-
353 ing: ci=0.61 (0.53-0.74), validation: ci=0.66 (0.57-0.74)) compared to the 7-gene signature. Fi-
354 nally, a multivariable Cox model including both, the clinical parameters ECE status and tumor
355 localization (oral cavity vs others) as well as the 7-gene signature, increased the training ci to
356 0.82 (0.77-0.89) and the validation ci to 0.71 (0.62-0.78), see Table 2. While in training the clini-
357 cal Cox model was significantly improved by adding the 7-gene signature (p<0.001), adding the
358 clinical parameters to the 7-gene signature resulted in only small improvements (p=0.53). The
359 difference in validation ci was not statistically significant. An additional validation was per-
360 formed using only those patients who received concurrent chemotherapy, leading to similar re-
361 sults (validation ci 0.72).

362 The extended model was used to stratify the patients into two risk groups (cut-off 0.37), leading
363 to highly significant differences in LRC for the training (p<0.001) and the validation cohort
364 (p<0.001). The corresponding Kaplan Meier curves are presented in Figure 4 together with a
365 heatmap of the signature for the training cohort (see also Supplementary Figure S5).



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366

367 **Comparison to models based on CSC markers and hypoxia classifiers**

368 In a previous study, it was shown that the expression of CSC markers and hypoxia-related genes
369 were prognostic in patients with locally advanced HPV16 DNA negative HNSCC, who were
370 treated by PORT-C (12). These results were validated in (25). Here, the performance of these
371 models was compared to the 7-gene signature. While in (25) the best performing model, consist-
372 ing of ECE status, tumor localization, *CD44*>0.2 and the 15-gene hypoxia classifier (17), showed
373 a validation ci of 0.65 (0.54-0.74), the identified 7-gene signature combined with the clinical
374 parameters led to a validation ci of 0.71 (0.62-0.78).

375

376 **7-gene signature predicts for secondary endpoints**

377 As secondary endpoints, overall survival (OS) and freedom from distant metastases (DM) were
378 considered. The 7-gene signature determined for LRC, combined with the clinical features ECE
379 status and tumor localization, was trained and validated for OS and DM (Supplementary Tables
380 S4 and S5). For OS, training and validation led to a ci of 0.71 (0.65-0.79) and 0.64 (0.57-0.70),
381 respectively. For DM, the ci was 0.69 (0.64-0.80) for training and 0.63 (0.52-0.73) for validation.
382 Cox models including only the clinical features ECE status and tumor localization led to a vali-
383 dation ci of 0.60 (0.54-0.66) for OS and of 0.61 (0.52-0.71) for DM, respectively. Hence, the 7-
384 gene signature could improve the prognostic performance also for the secondary endpoints OS
385 and DM compared to clinical parameters alone. Validation on the subgroup of patients receiving
386 concurrent chemotherapy led to a higher ci for the 7-gene signature (OS: 0.72 (0.56-0.84), DM:
387 0.74 (0.52-0.90)). Kaplan-Meier survival analyses are presented in Supplementary Figures S6
388 and S7.



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389 **Discussion**

390 The overall aim of this study was to identify and validate a gene signature for the stratification of
391 patients with HPV-negative, locally advanced HNSCC who are treated by PORT-C based on the
392 clinical endpoint LRC. We identified a 7-gene signature, which contains genes from an extended
393 in-house gene set compared to previous work, which showed that patients with HPV-negative
394 tumors could be further stratified by the expression of CSC markers and hypoxia-associated
395 genes (12). In addition to the HPV infection status, CSC marker expression levels and tumor
396 hypoxia-associated genes, this gene set included genes related to DNA repair, cell cycle regula-
397 tion, epithelial-mesenchymal transition, proliferation or invasion. A statistical framework was
398 developed with the objective of identifying a gene signature which accurately and robustly pre-
399 dicts the risk of loco-regional failure. The framework contains data preprocessing, internal cross
400 validation, signature selection, model building and independent validation.

401 The identified 7-gene signature contained the genes *SERPINE1*, *INHBA*, *ACTN1* and *P4HA2*
402 (which were combined into a single metagene due to high mutual correlation) as well as the
403 genes *CD24, TCF3* and *HILPDA*. *SERPINE1 (*also known as *PAI-1)*, *HILPDA*, *INHBA* and
404 *P4HA2* are being induced by the hypoxia-inducible factor *HIF1* leading to extracellular matrix
405 remodeling (35–37). *SERPINE1* plays a role in enhanced migration and cell proliferation as well
406 as decreased cisplatinum induced apoptosis (38,39). In a prospective clinical study including 190
407 patients, high expression of *SERPINE1* has been shown to be associated with poor local recur-
408 rence-free, progression-free and cancer-specific survival (38). In a panel of head and neck xeno-
409 graft tumors, *SERPINE1* expression levels was over-expressed prior to treatment mainly in hy-
410 poxic tumors (40). After fractionated irradiation, a correlation between *SERPINE1* expression
411 levels and local tumor control was found *in vivo* (40). In addition, mild hypoxia has been shown



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412 to induce SERPINE1 expression via the hypoxia-inducible factor HIF1 (37). SERPINE1 is func-
413 tionally associated with *INHBA* (41), and *ACTN1* (42). In the hypoxia-associated signatures,
414 *HILPDA* (also known as *HIG2*) and *P4HA2* have also been included (17,43). *HILPDA* has been
415 shown to promote proliferation and invasion (44). In the literature, conflicting data exist for
416 *CD24*, which is expressed in different tumor entities such as breast cancer and cervical cancer
417 and has shown to be associated with increased tumor growth and progression (45). *CD24* has
418 also been shown to be involved in cisplatinum resistance (46) and a shortened progression free
419 survival was observed for several tumor entities with higher expression (45,47). In contrast,
420 *CD24* over-expression has been shown to be correlated with better survival in patients with oral
421 carcinoma (48). They further showed that CD24-/- mice are able to develop progressive oral
422 cancer. Lack of the surface protein CD24 resulted in the expansion of a highly immunosuppres-
423 sive CD11b$^{+}$Gr1$^{+}$ myeloid cell population leading to oral cancer progression. To date, very little
424 is known about the transcription factor 3 (*TCF3*) and its potential role in cancer. *TCF3* is a criti-
425 cal cell signaling molecule (49) and has been shown to promote cell migration and wound repair
426 (50). In contrast, *TCF3* was found to be a cell-intrinsic inhibitor of pluripotent self-renewal
427 through limiting the steady-state levels of self-renewal factors such as Oct-4, Sox2 and Nanog in
428 mouse embryonic stem cells (51). Lack of TCF3 leads to increasing levels of Nanog and other
429 self-renewal genes, minimizing the response to differentiation stimuli (51). According to the fac-
430 tors of the final Cox model, overexpression of *SERPINE1* (as well as the highly correlated genes
431 *INHBA*, *ACTN1* and *P4HA2*) and *HILPDA* increased the risk for loco-regional failure, which is
432 in line with the literature (38,39). In contrast, a high expression of *CD24* led to decreased risk of
433 recurrence. For oral cavity cancer it has been shown that *CD24* dampens the functional expan-
434 sion of myeloid-derived suppressor cells and gives rise to a more favorable prognosis as de-



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435 scribed above (48), which is in line with our findings. The final Cox model also predicted that a
436 high expression of *TCF3* is related to improved LRC, which may be due to its role in the sup-
437 pression of self-renewal genes (51). However, the functional role of *TCF3* in HNSCC needs to
438 be explored in further mechanistic studies.

439 The identified 7-gene signature showed a good prognostic ability for the endpoint LRC on the
440 validation cohort (ci=0.69). When combined with the clinical parameters ECE status and tumor
441 localization, its performance could be further improved (ci=0.71). This indicates that the combi-
442 nation of well-established clinical parameters and prognostic biomarkers may lead to a more
443 accurate prognosis than each of them alone. The model including only the clinical parameters
444 showed the lowest validation performance (ci=0.66). In the Cox model combining clinical pa-
445 rameters with the 7-gene signature, most signature genes were significantly associated with LRC
446 which explains its good performance. While this may be expected on the training cohort, the
447 impact of the 7-gene signature in validation is less clear, since the relevant improvement in ci by
448 0.05 was not statistically significant. Evaluating the signature combined with HPV status for all
449 patients increased the validation ci to 0.74, which is similar to or even higher than in other stud-
450 ies (30,52).

451 The final Cox model showed a better performance on the training cohort (ci=0.82) than on the
452 validation cohort (ci=0.71). This difference is expected, since the final Cox model is adjusted to
453 the training cohort and potential overfitting might occur. In addition, the validation of the pro-
454 posed 7-gene signature might be impeded by the significant differences between both patient
455 cohorts. Patients in the validation cohort were clinically characterized by a higher percentage of
456 prognostically favorable R0-resections of primary tumors and less lymph nodes with ECE. On
457 the other hand, the validation cohort had a higher percentage of prognostically unfavorable oral



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458 cavity tumors, much less concurrent chemotherapy (31/121) than the training cohort and was
459 treated with outdated radiation technologies (53). These negative prognostic factors outbalanced
460 the positive ones resulting in worse outcome in terms of LRC (p=0.096) and OS (p=0.042) (25).
461 Lack of concurrent chemotherapy may impede the validation of genes related to cisplatin re-
462 sistance. For the 7-gene signature, however, only *CD24* has been reported to be strongly in-
463 volved in resistance to cisplatin (46), but also in other mechanisms (48).
464 Based on the final Cox model, a risk score was calculated for each patient, which allowed strati-
465 fication into groups of low and high risk of recurrence. However, mean gene expressions (Sup-
466 plementary Table S6) as well as clinical parameters were significantly different between the
467 training and validation cohort. These differences caused a shift in the risk score, such that the
468 stratification cut-off, which was based on the training cohort, led to imbalanced patient risk
469 groups for the validation cohort. While in training approximately 45% of the patients were strati-
470 fied in the low risk group and 55% in the high-risk group, for the validation cohort only about
471 12% of the patients were classified as high risk. Such imbalances may be caused by the differing
472 tumor and treatment characteristics between the cohorts. In addition to clinical reasons, differ-
473 ences in gene expression might also be caused by several biomaterial-related factors such as
474 storage time of FFPE-material (3 to 18 years) or batch effects and stability of reagents and con-
475 sumables (Supplementary Table S7). Renormalizing the validation data to the training data, as
476 described in (12,54), gives the same fraction of patients in the low and high risk group and simi-
477 lar LRC rates for both cohorts (Supplementary Figure S5). However, to apply this renormaliza-
478 tion method for individual patient prognosis within clinical trials, the inclusion of reference sam-
479 ples may be required, for which the expected gene expression levels are known. This methodolo-
480 gy will be applied to the planned prospective validation of the 7-gene signature. In addition, the



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481 application of broadly available and cost-effective PCR-based methods may further improve
482 biomarker stability.

483 In this study, several algorithms for gene selection and risk prediction were compared. Feature
484 selection algorithms based on mutual information, such as MIFS and MRMR, typically led to a
485 higher ci than simple univariable methods such as Pearson or Spearman correlations (Figure 3).
486 This behavior can be expected, since the more complex algorithms do not only account for the
487 correlation of the gene expressions to outcome but also consider correlations between the select-
488 ed genes. Therefore, each gene in the signature represents additional information, which increas-
489 es the performance of the signature. The performance of prediction models, ranging from the
490 well-known Cox model to complex random forests, was similar on the training cohort. There-
491 fore, the performance of the signature was finally assessed by multivariable Cox regression,
492 which allows easy interpretation. Most of the considered models require additional hyper-
493 parameters, such as the regularization parameters $\lambda_1$ and $\lambda_2$ for penalized Cox models or node
494 size and node depth for random forests (see Supplementary Section S3). In an initial experiment,
495 these parameters were chosen based on their default values given in the used software packages
496 and then tuned by a grid search using 2-fold internal cross validation on the training cohort. The
497 resulting parameters were applied in this study and are reported in Supplementary Table S8.
498 While random forests did not outperform simple Cox regression in this study, this may not hold
499 in other situations (55).

500 The presented 7-gene signature was identified for patients with HPV16 DNA negative tumors
501 and the primary endpoint LRC. However, it also improved the prognostic value of the clinical
502 parameters for the secondary endpoint OS, while for DM no significant difference was observed.
503 In particular for patients receiving concurrent chemotherapy, the validation performance of the



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504 7-gene signature was improved by 10%. This may further enhance the clinical potential of this
505 signature.
506 A limitation of this study might also be the limited number of genes contained in the initial gene
507 set. Although this has been composed on a hypothesis-driven basis and comprehensive literature
508 search, it may not include all genes of radiobiological relevance. For example CD44, which has
509 been shown to be a prognosticator for LRC in patients with locally advanced HNSCC who re-
510 ceived PORT-C (12), had to be omitted from the nanoString analysis due to incorrect probe de-
511 sign. Since the set-up of our gene set other genes have been shown to be prognostic for outcome
512 in HNSCC. For example, TCGA analyses (56) suggested several genes, related to HPV status.
513 Of these genes *CCND1*, *NOTCH1*, *YAP1* and *SOX2* were found to overlap with our gene set. In
514 the TCGA dataset, patients with *CCND1* overexpressing tumors, who received surgery with or
515 without postoperative radiochemotherapy showed worse prognosis. In our study, *CCND1* had no
516 impact on the primary endpoint LRC (p=0.72). Therefore, it was not selected in the gene signa-
517 ture. However, *CCND1* showed a significant correlation to the secondary endpoints OS and DM
518 using univariable Cox-regression for all 195 patients. For the subgroup of patients with HPV-
519 negative tumors, *CCND1* neither correlated with OS nor with DM. This could be explained by
520 the strong correlation of *CCND1* with the HPV status in our cohort. In contrast, *YAP1* was signif-
521 icantly associated with LRC in our study, but was rated only at rank 14 such that it was not in-
522 cluded in the 7-gene signature. *NOTCH1* and *SOX2* were not related to LRC. Another example is
523 PD-L1, which was strongly associated with local failure in HPV-negative HNSCC (13,57), but
524 not included in our gene set. In order to consider these novel developments and identify further
525 biomarkers, whole transcriptome analyses supplemented by whole methylome analyses might be
526 performed and potentially further improve patient stratification.



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527 Currently, an adaptive clinical biomarker matrix trial is set-up within the DKTK-ROG for dose
528 escalation and de-escalation in HNSCC. In the first stage, patients with HPV-positive tumors
529 treated by PORT-C will receive a 10% lower radiation dose of the standard concurrent radi-
530 ochemotherapy schedule. In the second stage, the 7-gene signature is one candidate biomarker
531 for selecting patients with high-risk HPV-negative tumors for dose escalation. To reduce toxici-
532 ties, especially at higher doses, proton therapy will be considered (58).
533 In conclusion, this study introduces a novel 7-gene signature predicting LRC for patients with
534 locally advanced HNSCC treated by PORT-C. A prognostic Cox model was trained on a large
535 multicenter patient cohort and independently validated. Although the validation cohort differed
536 in many aspects from the training cohort, a successful validation was achieved, which indicates
537 the robustness of the signature. Prospective validation of the signature is planned within an ongo-
538 ing prospective clinical trial of the DKTK-ROG before regular application in clinical trials for
539 patient stratification.
540

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550 A. Linge, C. Krenn, C. von Neubeck and F. Buchholz performed or supervised NanoString anal-
551 yses. S. Schmidt, A. Zwanenburg, S. Leger and S. Löck performed the statistical analyses and
552 interpretation. S. Schmidt and S. Löck created the figures. All authors contributed in writing the
553 manuscript.

554

555 **Data and materials availability**: The final models and the raw genomic data used for creating
556 the models are available upon request.



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729 **Figures & Tables:**

730

731 **Figure 1.** Study design.

732

733 **Figure 2.** Cross-validation scheme for identifying the ensemble gene signature. The training co-
734 hort was randomly split into 3 equal parts. Each part was used for internal validation and the re-
735 maining patients for internal training. This was repeated 333 times. Feature selection was per-
736 formed on each internal training sample and a prognostic model was trained using the selected
737 genes. This model was subsequently internally validated. Finally, the occurrence and importance
738 of the genes as well as the validation ci of all cross-validation experiments were used to define
739 the ensemble gene signature

740

741 **Figure 3.** Performance of ensemble gene signatures for loco-regional tumor control on the train-
742 ing cohort. For each combination of feature selection algorithm and statistical model the mean
743 out-of-the-bag (oob) validation ci of the training cohort and its 95%-confidence interval is
744 shown. Performance for the endpoint loco-regional tumor control was estimated using 1000
745 bootstrap samples of the entire training cohort with signature size 4.

746

747 **Figure 4.** Patient stratification by the 7-gene signature and clinical parameters for loco-regional
748 tumor control. Kaplan-Meier estimates of loco-regional tumor control (LRC) are shown for (A)
749 the training cohort and (B) the validation cohort. Patients were stratified into a low risk group
750 (LR) or a high risk group (HR) by the linear predictor of the multivariable Cox model which
751 included the 7-gene signature and the clinical parameters ECE status and tumor localization. The
752 cut-off risk score (0.37) was determined on the training cohort and applied to the validation co-
753 hort. (C) Heatmap of the 7-gene signature as well as ECE status (0: light, 1: dark), localization
754 (oral cavity: dark, others: light), risk group (low: light, high: dark) and LRC during follow-up
755 (yes: light, no: dark) for the training cohort.



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756 **Table 1.** Patient characteristics for the training and validation cohort. * Log-rank test; + 95%
757 confidence interval

| | **Training cohort** (2004-2011) | | **Validation cohort** (1999-2006) | | |
|---|---|---|---|---|---|
| | **HPV16 DNA negative tumors** | | | | |
| **Characteristics** | **Median (range)** | | **Median (range)** | | **p-value** |
| Follow-up (months) | 57.4 (11.5 – 94.5)+ | | 62.1 (24.7 – 153.0)+ | | <**0.001***|
| Age (years) | 56.5 (32.0 – 74.0) | | 52.3 (36.3 – 70.6) | | **0.005** |
| Dose (Gy) | 64.0 (56.0 – 68.0) | | 64.0 (60.0 – 66.0) | | **0.006** |
| | **Number of pts** | **(%)** | **Number of pts** | **(%)** | |
| Gender Male/Female | 101/29 | 77.6/22.3 | 105/16 | 86.8/13.2 | 0.061 |
| ECE status no/yes/unknown | 62/68/0 | 47.7/52.3 | 82/39/0 | 67.8/32.2/0 | **0.001** |
| Localization Oropharynx/Oral cavity/ Hypopharynx/Larynx | 58/49/ 23/0 | 44.6/37.7/ 17.7/0 | 26/75/ 13/7 | 21.5/62.0/ 10.7/5.8 | **<0.001** |
| Grading 1/2/3/unknown | 4/84/ 42/0 | 3.1/64.6/ 32.3/0 | 3/67/ 51/0 | 2.5/55.4/ 42.1/0 | 0.27 |
| Chemotherapy yes/no | 130/0 | 100/0 | 31/90 | 25.6/74.4 | **<0.001** |
| Loco-regional recurrences | 26 | 20.0 | 35 | 28.9 | 0.096* |
| Distant metastases | 31 | 23.8 | 29 | 24.0 | 0.72* |
| Deaths | 54 | 41.5 | 73 | 60.3 | **0.042*** |

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759 **Table 2.** Multivariable Cox regression of loco-regional tumor control. Three multivariable Cox
760 regression models were built using the training cohort: a model consisting of only the 7-gene
761 signature (top); a model consisting only of the clinical ECE status and tumor localization (cen-
762 ter); and a model combining both the 7-gene signature and clinical parameters (bottom). Hazard
763 ratios (HR) are given with their 95% confidence intervals (CI) and the corresponding p-values.
764 For each model, the concordance index (ci) is given for the training and validation cohort as well
765 as for the patients of the validation cohort who received concurrent chemotherapy. Its 95% CI is
766 determined from 1000 bootstrap samples of the respective cohort. The improvement of the com-
767 bined model, including the 7-gene signature and the clinical parameters, compared to the 7-gene
768 signature and clinical parameters alone is shown (bottom) based on the difference in log-
769 likelihood (dLL).

| **Parameter** | **HR (95% CI)** | **p-value** | **ci training (95% CI)** | **ci validation (95% CI)** | **ci validation, chemotherapy (95% CI)** |
|---|---|---|---|---|---|
| **7-gene signature** | | | | | |
| Metagene from *SERPINE1*, *INHBA*, *ACTN1* and *P4HA2* | 2.13 (1.18-3.88) | **0.012** | | | |
| *HILPDA* | 1.48 (1.00-2.18) | 0.049 | | | |
| *CD24* | 0.71 (0.48-1.04) | 0.072 | | | |
| *TCF3* | 0.54 (0.32-0.88) | **0.017** | 0.81 (0.75-0.88) | 0.69 (0.60-0.77) | 0.69 (0.39-0.87) |
| **Clinical parameters** | | | | | |
| ECE status | 1.26 (0.57-2.82) | 0.57 | | | |
| Localization oral cavity | 2.07 (0.95-4.56) | 0.069 | 0.61 (0.53-0.74) | 0.66 (0.57-0.74) | 0.65 (0.30-0.84) |
| **7-gene signature and clinical parameters** | | | | | |
| Metagene from *SERPINE1*, *INHBA*, *ACTN1* and *P4HA2* | 1.98 (1.09-3.83) | **0.026** | | | |
| *HILPDA* | 1.52 (1.02-2.26) | **0.041** | | | |
| *CD24* | 0.69 (0.46-1.05) | 0.083 | | | |
| *TCF3* | 0.55 (0.32-0.94) | **0.031** | | | |
| ECE status | 1.40 (0.62-3.24) | 0.43 | | | |
| Localization oral cavity | 1.27 (0.51-3.19) | 0.61 | 0.82 (0.77-0.88) | 0.71 (0.62-0.78) | 0.72 (0.43-0.90) |

| **Improvement of combined model compared to** | **dLL** | **degrees of freedom** | **p-value** |
|---|---|---|---|
| 7-gene signature only | 1.26 | 2 | 0.53 |
| Clinical parameters only | 24.19 | 4 | **<0.001** |

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Figure 1

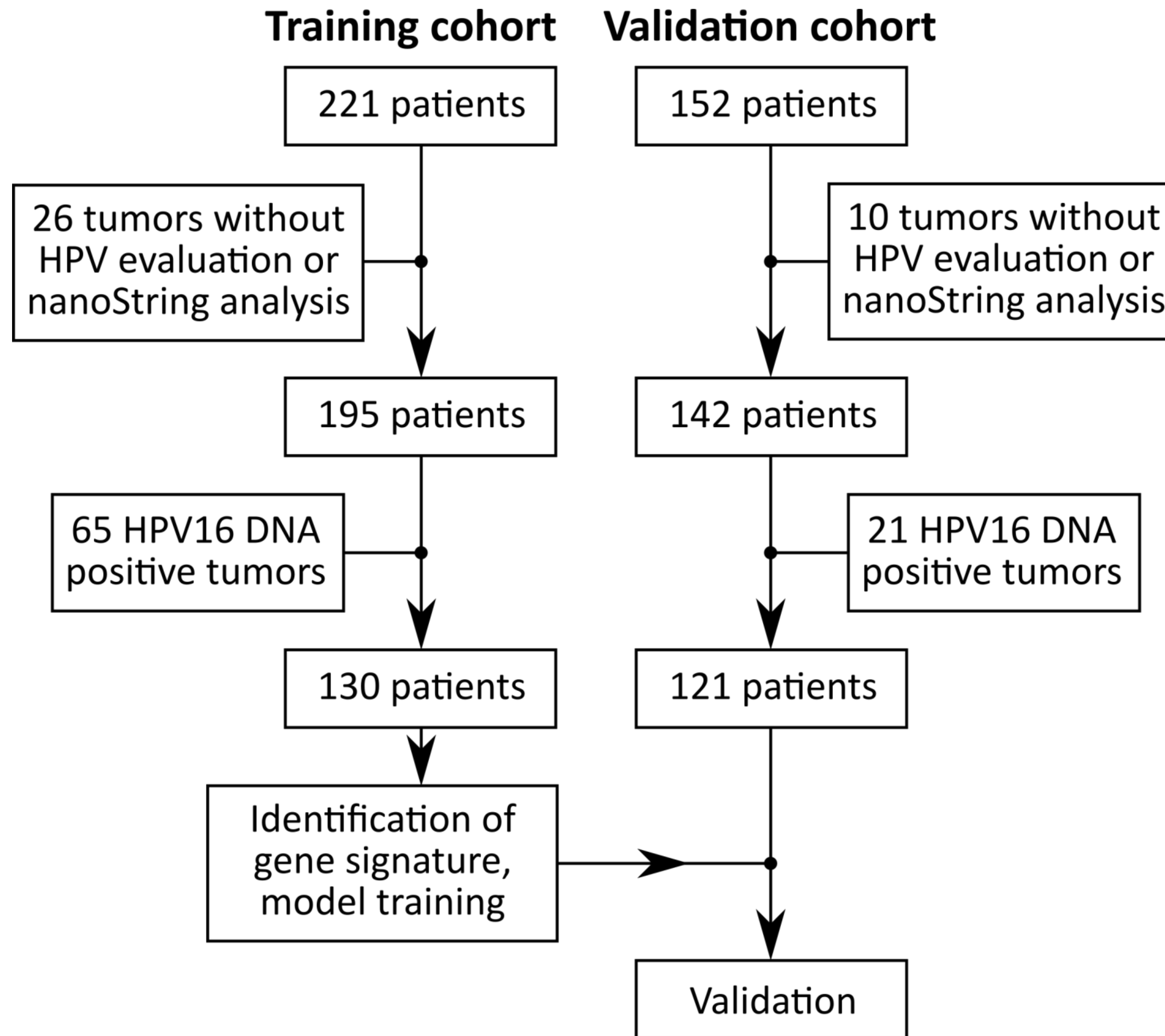


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Figure 2

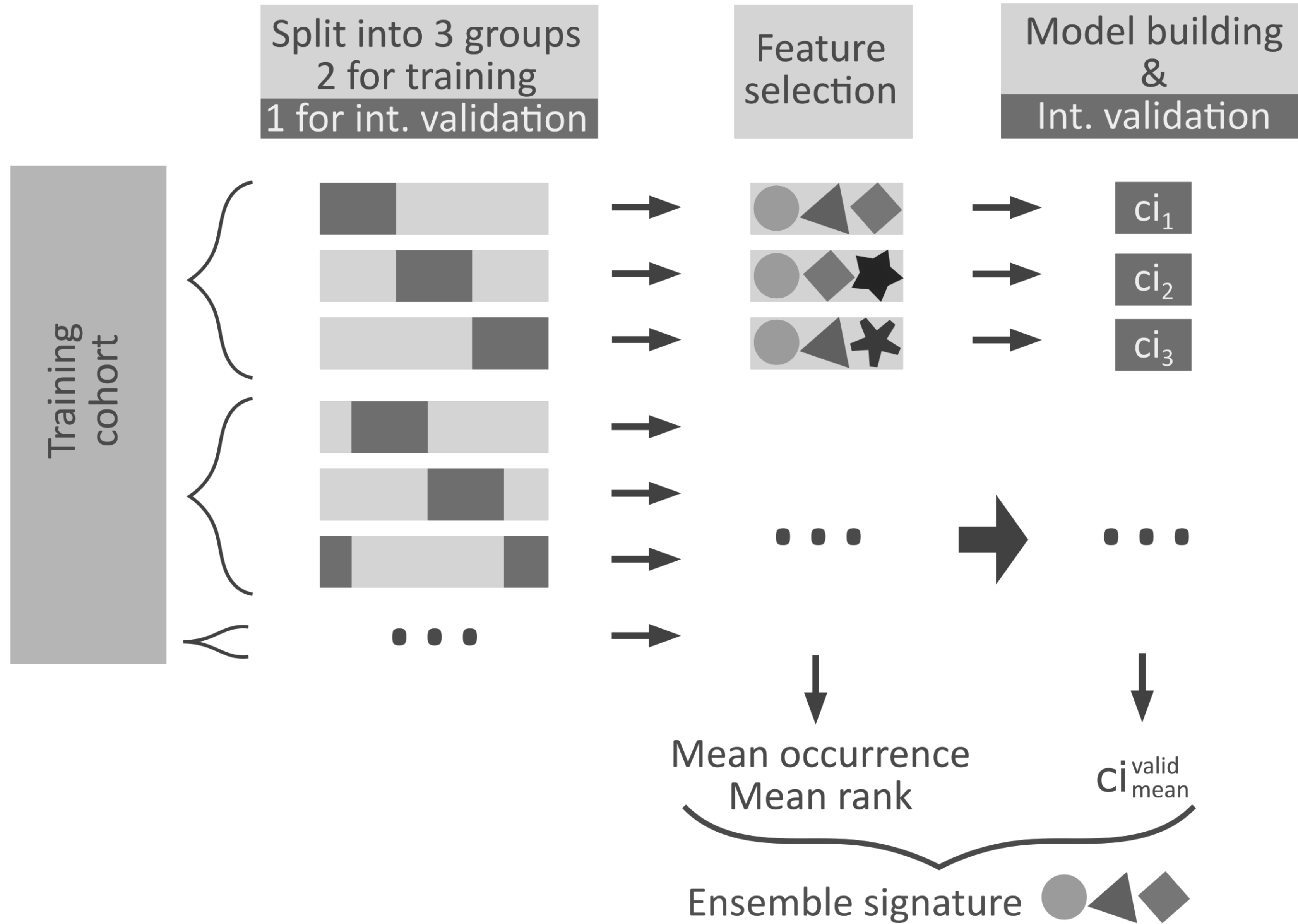


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Figure 3

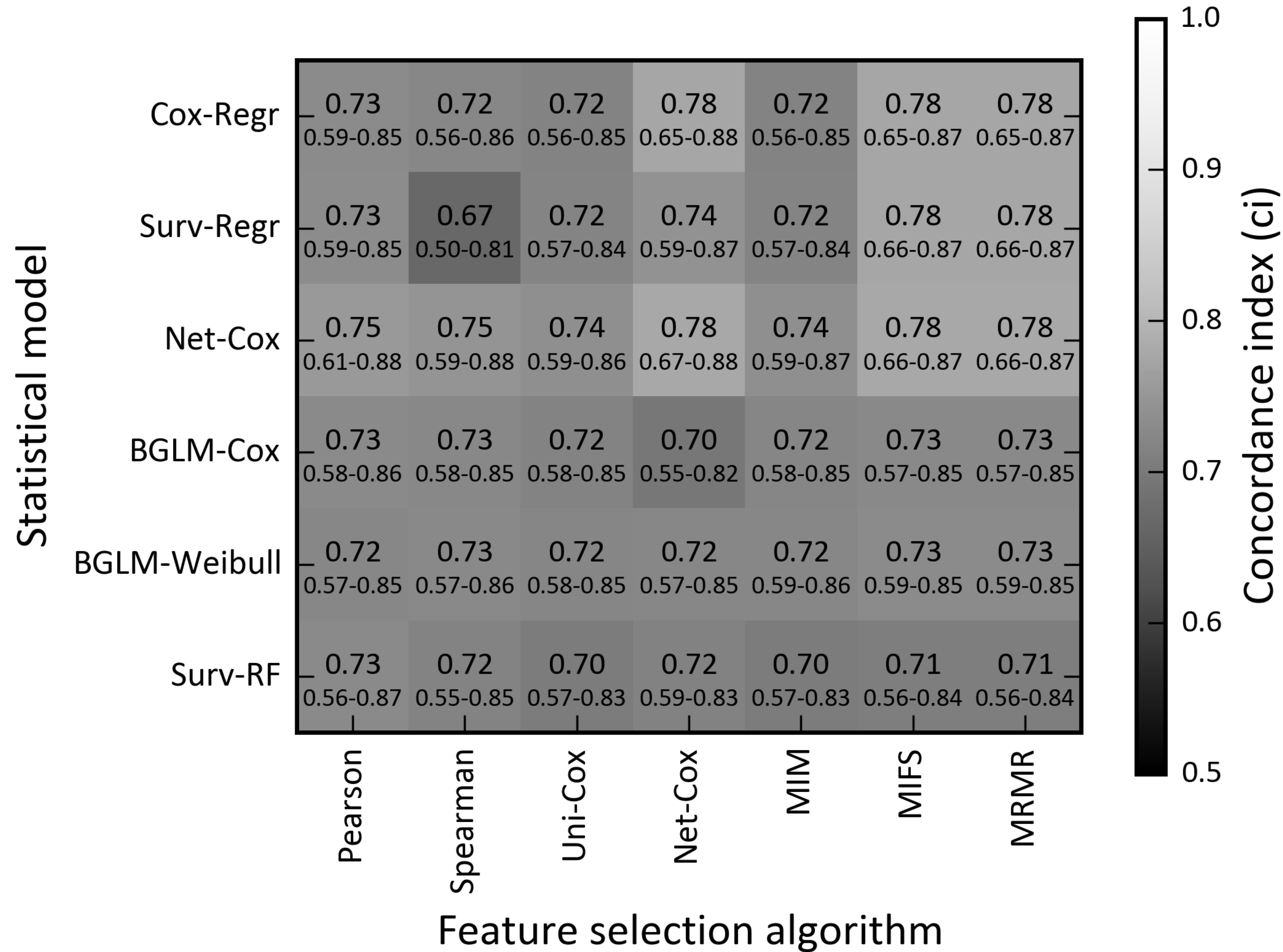


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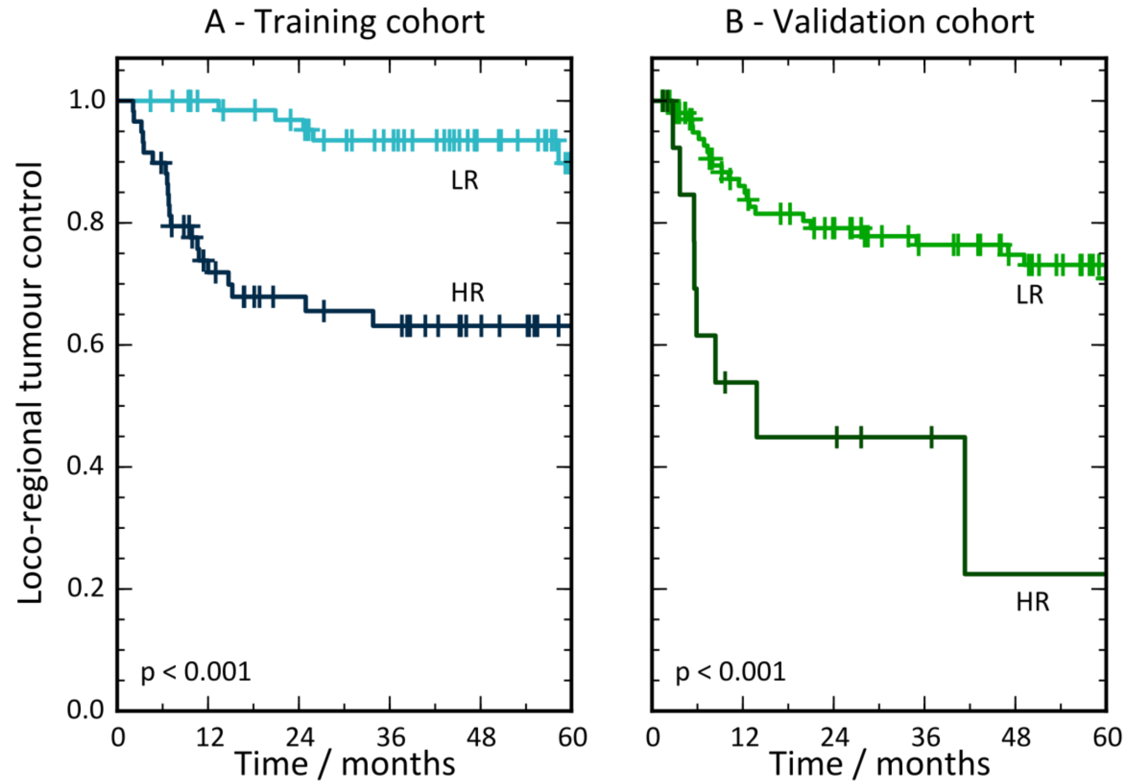
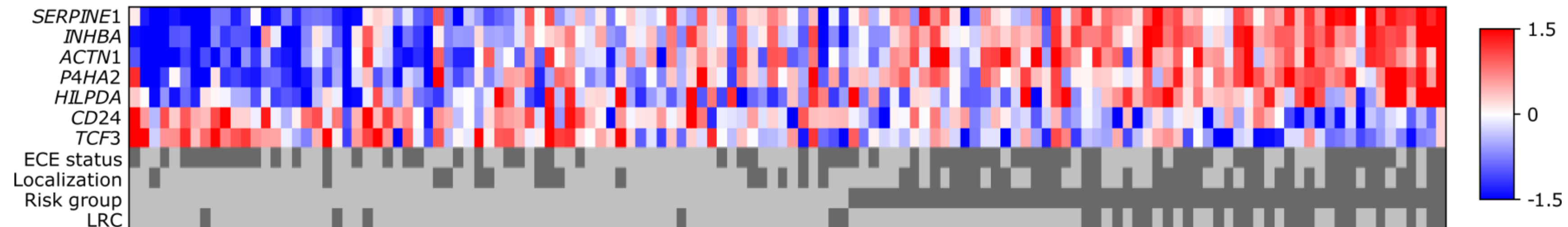
Figure 4




Patients at risk

| | 0 | 12 | 24 | 36 | 48 | 60 | 0 | 12 | 24 | 36 | 48 | 60 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LR | 71 | 65 | 60 | 48 | 35 | 22 | 106 | 76 | 64 | 53 | 45 | 33 |
| HR | 59 | 37 | 29 | 26 | 17 | 10 | 15 | 6 | 5 | 3 | 1 | 1 |

C

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Clinical Cancer Research

# Development and validation of a gene signature for patients with head and neck squamous cell carcinomas treated by postoperative radio(chemo)therapy

Stefan Schmidt, Annett Linge, Alex Zwanenburg, et al.

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