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Prediction of hypoxia

Gene expression classifier predicts for hypoxic modification of radiotherapy with nimorazole in squamous cell carcinomas of the head and neck

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ABSTRACT

Purpose: To validate the predictive impact of a hypoxia gene expression classifier in identifying patients with head and neck squamous cell carcinoma (HNSCC) having benefit from hypoxic modification of radiotherapy.

Patients and methods: Gene expressions were quantified from formalin-fixed, paraffin-embedded tumour biopsies of 323 HNSCC patients randomized for placebo or nimorazole in conjunction with radiotherapy in the DAHANCA 5 study. Tumours were classified as either "more" or "less" hypoxic with a classifier constituting of 15 hypoxia responsive genes. The predictive impact was evaluated by analysing the response to nimorazole vs. placebo in terms of loco-regional tumour control (LRC) and disease-specific survival (DSS) in the two classified groups.

Results: Hundred and fourteen patients (35%) were classified as having "more" hypoxic tumours. These patients had a significant benefit of hypoxic modification with nimorazole compared with placebo in terms of LRC (5-year actuarial values 49% vs. 18%; $p = 0.001$) and DSS (48% vs. 30%; $p = 0.04$). "Less" hypoxic tumours had no significant effect of hypoxic modification (LRC: 50% vs. 44%; $p = 0.39$, DSS: 57% vs. 51%; $p = 0.49$) and generally an outcome, which was similar to "more" hypoxic tumours treated with nimorazole. In contrast to HPV-negative tumours, HPV-positive tumours had a substantially better outcome in response to radiotherapy, which was irrespective of hypoxic modification.

Conclusions: A predictive 15-gene hypoxia classifier could identify patients associated with improved outcome after combining radiotherapy with hypoxic modification and underlines the relevance of such therapy. The impact of the classifier was limited to HPV-negative tumours.

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Tumour hypoxia represents a considerable problem in the radiotherapeutic management of malignant head and neck squamous cell carcinomas (HNSCCs) [1,2]. The hypoxic microenvironment causes a reduced formation of radiation induced damaging free radicals, and thus the overall therapeutic response to radiotherapy is compromised and reduced. To overcome this obstacle, hypoxic modification of radiotherapy with various modalities has been applied [3]. One of the most promising modifiers is the hypoxic radiosenzitizer nimorazole, which in the DAHANCA 5 study has been shown to improve loco-regional tumour control in HNSCC when applied in conjunction with radiotherapy [4].

Tumours are heterogeneous with respect to the degree and extent of hypoxia [5], and this has been studied extensively in relation to the prognostic impact [1,6]. Less is known about the relevance of hypoxia assessing methods in relation to the prediction of response to hypoxic modification of radiotherapy. Although the importance of hypoxic heterogeneity for selection of patients for hypoxia-modifying or -targeting therapies has been clearly recognized [7], there are at present no clinically useful markers that have been properly standardized and validated for use in clinical trials.

One class of potential biomarkers is based on evaluating the expression of hypoxia responsive genes in tumour biopsies [6]. Hypoxia gene expression signatures have been developed [8–11], that can separate tumours from clinical datasets of the head and neck, breast, lung and ovarian cancer, into "more" and "less" hypoxic groups correlating with poor and good prognosis, respectively. None of these signatures have yet been verified as predictors of hypoxic modification of radiotherapy. With the aim of developing such a classifier, we have generated a novel 15-gene expression signature that can classify head and neck cancer patients as having either "more" or "less" hypoxic tumours [12]. The classifier is based on routine formalin-fixed paraffin-embedded (FFPE) sections

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and can be used to classify patients individually. Briefly, hypoxia responsive genes were identified through in vitro experiments with human squamous cell carcinoma cell lines [13,14]. The initial gene list contained genes that were upregulated by hypoxia, independently of pH, across different cell lines. These genes were first validated in xenograft models, and the final classifier was developed from an independent training set of 58 patients with HNSCC, in which the hypoxia status had previously been determined and ranked using oxygen electrode measurements [15,16]. The classifier is therefore based on the association with oxygen levels in tumours, and has been optimized to work robustly on FFPE sections.

Here, we explore and validate the clinical relevance and predictive impact of this novel hypoxia classifier in the DAHANCA 5 study, where patients were randomized between hypoxic modification (nimorazole) or placebo in combination with radiotherapy [4]. As the aetiologic background and HPV-status has a significant impact on the radiotherapeutic response and prognosis of the individual patient [17–19], especially regarding hypoxic modification [20], it was furthermore evaluated whether HPV-status, assessed by p16 immunohistochemistry, was of importance in addition to pre-therapeutic hypoxic evaluation.

Materials and methods

Patients and tissues

In the DAHANCA 5 study (January 1986 to September 1990), patients with advanced cancer of the supraglottic larynx or pharynx treated with conventional radiotherapy (62–68 Gy in 33–34 fx, 5 fx/wk) were randomized to placebo or the hypoxic sensitizer nimorazole (Fig. 1A) [4]. Neither received surgery or chemotherapy. From the 414 patients enrolled in the DAHANCA 5 study we had access to formalin fixed, paraffin embedded (FFPE) pre-treatment tumour biopsies from 323 patients in sufficient amounts for gene expression classification. Clinical characteristics of patients with available material were similar to patients without available material (Supplementary Table 1). Tumours were previously evaluated for HPV-status by p16 immunohistochemistry [20].

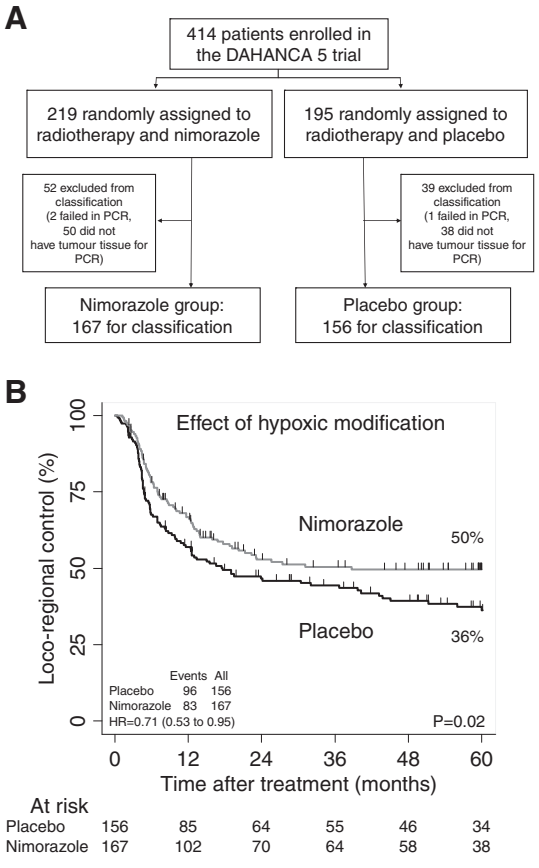

Fig. 1. DAHANCA 5 study design (A). Effect of hypoxic modification with nimorazole vs. placebo in conjunction with radiotherapy on loco-regional control (B).

Gene expression quantification

Gene expression analyses were performed as previously described [12]. Total RNA was extracted from a 7 μm section of FFPE-biopsies with a silica bead-based, fully automated isolation method for RNA on a robotic Tissue Preparation System using VERSANT Tissue Preparation Reagents (Siemens Healthcare Diagnostics, Tarrytown, NY) [21,22]. cDNA was generated using the High Capacity cDNA Archive kit and pre-amplified using the TaqMan PreAmp Master Mix Kit (Applied Biosystems; ABI). Quantitative PCR was performed on an ABI Prism 7900 HT Sequence Detector using TaqMan Gene Expression PCR mastermix (Applied Biosystems; ABI). Gene expression levels were calculated using RealTime Statminer (Intergromics).

Classification into "more" and "less" hypoxic groups

Classifications into "more" or "less" hypoxic groups were performed as previously described [12]. In short, 58 HNSCC cancer patients from an independent test study, who were previously hypoxia evaluated with oxygen electrodes [15,16], were divided into two groups. Tumours were ranked according to the relative number of measurements below or equal to 2.5 mm Hg in their metastatic lymph nodes, and divided into two groups where the largest possible gene expression discrimination was observed. The 15 genes in the classifier were characterised by their mean expression (and estimated variance) in each of the two groups, "more hypoxic" or "less hypoxic". For each patient in the DAHANCA 5 trial, the expression level of each gene was compared to the mean expression levels of the genes in the two pre-defined groups from the independent test study. Each patient was then assigned individually to the group where the sum of the distance to mean expressions (weighed by the estimated variance) was the lowest. As the classifier was developed in an independent data set, no attempts were made to optimize the number of genes or expression levels prior to the classification of patients in the DAHANCA 5 cohort. Not all genes could be measured in all patients, but the 15 genes represent a robust signature and only failed to classify 3 out of 326 patients where FFPE material was available for RNA extraction (Fig. 1A). The investigator performing the classification procedure (KT) was blinded to patient treatment and clinical outcome data until the classification had been performed.

Statistical analysis

Patient and tumour characteristics were compared with the chi-squared test. Primary endpoint was loco-regional tumour control after radiotherapy and the secondary endpoints were disease-specific and overall survival. Loco-regional tumour control was defined as complete and persistent disappearance of disease in the primary (T-site) and regional lymph nodes (N-site). Failure was registered in case of recurrent or persistent tumour. Disease-specific mortality was defined as death from or with the primary HNSCC cancer. Overall mortality was death from any cause. Follow-up of the patients was completed in connection with the original publication of the study [4]. All patients were observed for loco-regional tumour control for at least 5 years or until death.

Survival status has subsequently been tracked until September 2009. Loco-regional tumour control and survival were described with the Kaplan Meier method, compared using the log-rank test, and also expressed as hazard ratios (HR) using a univariate Cox proportional hazards model. A Cox multivariate proportional-hazard analysis was applied under the assumption of proportional hazards. A test for interaction was performed to compare the response to nimorazole vs. placebo. Statistical analyses were performed with STATA 11. All *P*-values are two-sided with a 5% level of significance. HR's are presented with 95% CI.

Results

The effect of hypoxic modification with nimorazole among the 323 classified tumours is shown in Fig. 1B. Overall, patients assigned placebo had a significantly poorer loco-regional tumour control than those assigned hypoxic modification (5 year actuarial values 36% vs. 50%, HR = 0.71 [0.53–0.95]). Similar results were obtained in the original study including 414 patients (33% vs. 49%, HR = 0.66 [0.51–0.86]) [4]. Based on the 15-gene hypoxia classifier, 114 patients (35%) were classified as having "more" hypoxic tumours, whereas 209 patients (65%) were classified as having "less" hypoxic tumours. Patient and tumour characteristics from the "more" and "less" hypoxic groups are presented in Table 1. Apart from node-positive disease, which was significantly overrepresented in the "less" hypoxic group, there was no significant difference in the distribution of patient and tumour characteristics between the groups. The relative number of p16 positive tumours was equally distributed between the groups.

To evaluate the prognostic impact of the hypoxia classifier, the "more" and "less" hypoxic tumours were compared in the 156 patients from the placebo arm alone. Tumours classified as "more" hypoxic suffered a significantly poorer outcome both in terms of loco-regional tumour control (Fig. 2A, 18% vs. 44%, HR = 1.85 [1.21–2.82]) and disease-specific survival (Fig. 2B, 30% vs. 51%, HR = 1.57 [1.02–2.43]) compared to the "less" hypoxic tumours, whereas the association did not reach statistical significance for overall survival (26% vs. 40%, HR = 1.39 [0.97–2.00]).

The predictive impact of the classifier was evaluated by comparing the effect of nimorazole vs. placebo in the groups classified as "more" and "less" hypoxic tumours, respectively. In Fig. 3A–D, the response to nimorazole and the effect on both 5-year loco-regional tumour control and disease-specific survival is illustrated for the two groups. For loco-regional tumour control, a significant beneficial effect of nimorazole was observed in tumours classified as "more" hypoxic (Fig. 3A, 49% vs. 18%, HR = 0.46 [0.29–0.75]), whereas there was no significant effect in the "less" hypoxic tumours (Fig. 3B, 50% vs. 44%, HR = 0.85 [0.58–1.23]). A similar result was observed for disease-specific survival, with a beneficial effect of nimorazole in the more hypoxic group (Fig. 3C, 48% vs. 30%, HR = 0.60 [0.37–0.98]), and no significant effect in the "less" hypoxic group (Fig. 3D, 57% vs. 51%, HR = 0.87 [0.51–1.29]). For overall survival, no significantly beneficial effect from nimorazole was observed in neither the "more" (30% vs. 26%, HR = 0.88 [0.56–1.29]) nor the "less" (40% vs. 40%, HR = 1.01 [0.76–1.34]) hypoxic group.

The univariate findings were confirmed in a multivariate analysis (Table 2). Besides the hypoxic classification and p16 status, the covariates included gender, age, tumour classification (T34 vs. T12), nodal classification (N+ vs. N0), and nimorazole vs. placebo.

Table 1
Patient and tumour characteristics in the "more" and "less" hypoxic groups as categorised by the 15-gene hypoxia classifier.

Patient/tumour data	All patients (n = 323)		"More" hypoxic (n = 114)				"Less" hypoxic (n = 209)				P^*
			Placebo (n = 43)		Nimorazole (n = 71)		Placebo (n = 113)		Nimorazole (n = 96)		
	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)	
Age (years)											
Median	60		62		62		57		60		
Range	(24–84)		(36–79)		(34–83)		(24–84)		(28–83)		
<60 years	159	49	19	44	33	46	59	52	48	50	n.s.
>60 years	164	51	24	56	38	54	54	48	48	50	
Sex											
Female	92	28	11	26	18	25	30	27	33	34	n.s.
Male	231	72	32	74	53	75	83	73	63	66	
Tumour site											
Supraglottic larynx	100	31	13	30	24	35	32	28	31	32	
Hypopharynx	44	13	7	16	11	15	13	12	13	13	n.s.
Oropharynx	141	44	20	47	30	42	54	48	37	39	
Rhinopharynx	38	12	3	7	6	8	14	12	15	16	
Tumour stage											
T1–2	155	48	19	44	29	41	53	47	54	56	n.s.
T3–4	168	52	24	56	42	59	60	53	42	44	
Nodal stage											
N0	138	43	23	53	34	48	39	35	42	44	0.05
N1–3	185	57	20	47	37	52	74	65	54	56	
Disease stage											
I-II	66	20	11	26	16	23	18	16	21	22	n.s.
III-IV	257	80	32	74	55	77	95	84	75	78	
Tumour differentiation											
Well or moderate	124	38	18	42	32	45	45	40	29	30	n.s.
Poor	199	62	25	58	39	55	68	60	67	70	
P16-status/HPV											
Negative	239	74	33	77	49	69	88	78	69	72	n.s.
Positive	84	26	10	23	22	31	25	22	27	28	

n.s., Not significant.
* Chi-squared test for comparison between "more" and "less" hypoxic groups.

The latter covariates were all significant in a multivariate analysis on loco-regional failure in the original DAHANCA 5 cohort [4]. In patients with "more" hypoxic tumours, a significant difference in hazard ratio was observed for those assigned nimorazole vs. those assigned placebo for both loco-regional failure (HR = 0.42 [0.25–0.68]) and disease-specific death (HR = 0.61 [0.37–1.00]). The difference was not observed for patients with "less" hypoxic tumours, neither for loco-regional failure (HR = 0.98 [0.67–1.44]), nor disease-specific death (HR = 1.07 [0.72–1.58]). A test for interaction comparing the response to hypoxic modification in the two classified groups demonstrated a significantly different response to nimorazole in the "more" hypoxic group compared to the "less" hypoxic group concerning both loco-regional failure ($P = 0.003$) and disease-specific death ($P = 0.05$). No significant difference in hazard ratio was observed for overall death (Supplementary Table 2).

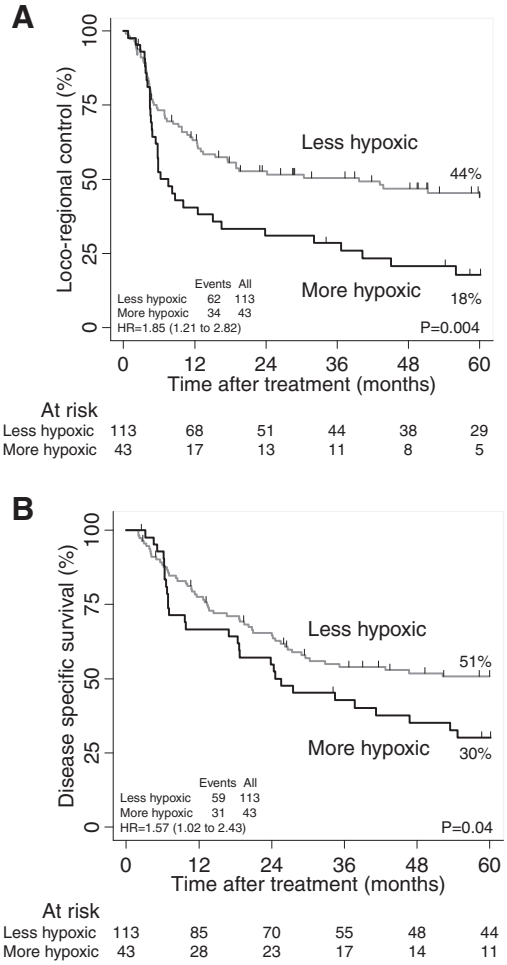

Fig. 2. Prognostic impact of the 15-gene hypoxia classifier in terms of loco-regional control (A) and disease-specific survival (B).

In a subgroup analysis, the predictive impact of the classifier was tested in HPV-positive and HPV-negative patients, respectively (Fig. 4A–D). In HPV-positive tumours, a generally improved prognosis was observed in terms of loco-regional tumour control. This was unaffected by hypoxic modification with nimorazole both among the "more" hypoxic tumours (Fig. 4A, 62% vs. 47%, HR = 0.72 [0.23–2.18]) and the "less" hypoxic tumours (Fig. 4B, 65% vs. 64%, HR = 1.03 [0.40–2.68]). In contrast, a beneficial effect of nimorazole was observed in HPV-negative tumours, but only among the "more" hypoxic tumours (Fig. 4C, 43% vs. 9%, HR = 0.44 [0.25–0.74]). No significant beneficial effect of nimorazole was observed among the HPV-negative/"less" hypoxic tumours (Fig. 4D, 44% vs. 38%, HR = 0.86 [0.57–1.30]). A similar trend was observed for disease-specific survival, although the beneficial effect of nimorazole among the subgroup of HPV-negative/"more" hypoxic tumours did not reach statistical significance (41% vs. 19%, HR = 0.61 [0.35–1.05], $P = 0.07$).

Discussion

The beneficial effect of combining radiotherapy of HNSCC with hypoxic modification demonstrated in the DAHANCA 5 study [4] has recently been confirmed in a large meta-analysis [3]. Although clinical trials exploring agents counteracting hypoxic radioresistance are ongoing [23], hypoxic modification does not have any impact on general clinical practice [7], except for the use of nimorazole in Denmark. As tumours display variable degrees of hypoxia [5], it is becoming increasingly clear that patient selection is an important factor in the evaluation and interpretation of clinical trials, and thus there is a need for standardized and validated biomarkers for hypoxia [7].

Table 2
Multivariate analysis of all patients and stratified in "more" and "less" hypoxic tumours, respectively. End points are loco-regional tumour failure and disease-specific death.

	Multivariate analysis[a]		
	All patients HR (95% CI)	"More" hypoxic HR (95% CI)	"Less" hypoxic HR (95% CI)
Risk of loco-regional tumour failure in the total cohort and stratified by the 15-gene hypoxia classifier			
Gender (male vs. female)	1.30 (0.92–1.84)	1.65 (0.87–3.10)	1.31 (0.85–2.02)
Age	1.01 (1.00–1.03)	1.01 (0.98–1.03)	1.02 (1.00–1.04)
Tumour classification (T3–4 vs. T1–2)	1.45 (1.07–1.96)	1.01 (0.60–1.71)	1.74 (1.18–2.55)
Nodal classification (N + vs. N0)	2.12 (1.55–2.90)	1.90 (1.16–3.12)	2.84 (1.85–4.36)
Nimorazole vs. placebo	0.69 (0.51–0.94)	0.42 (0.25–0.68)	0.98 (0.67–1.44)
p16-Positive vs. p16-negative	0.42 (0.28–0.63)	0.44 (0.24–0.82)	0.43 (0.26–0.74)
"More" hypoxic vs. "less" hypoxic	1.41 (1.03–1.94)		
Risk of disease specific death in the total cohort and stratified by the 15-gene hypoxia classifier			
Gender (male vs. female)	1.65 (1.14–2.39)	1.82 (0.96–3.45)	1.58 (0.99–2.52)
Age	1.01 (1.00–1.03)	1.01 (0.98–1.03)	1.02 (1.00–1.04)
Tumour classification (T3–4 vs. T1–2)	1.24 (0.90–1.69)	0.88 (0.53–1.49)	1.60 (1.08–2.37)
Nodal classification (N + vs. N0)	2.61 (1.87–3.64)	1.79 (1.08–2.99)	3.95 (2.45–6.37)
Nimorazole vs. placebo	0.83 (0.60–1.13)	0.61 (0.37–1.00)	1.07 (0.72–1.58)
p16-Positive vs. p16-negative	0.37 (0.24–0.56)	0.33 (0.17–0.64)	0.39 (0.23–0.68)
"More" hypoxic vs. "less" hypoxic	1.40 (1.01–1.95)		

[a] Parameters included in the Cox proportionate multivariate analysis: Tumour and Nodal classification, gender, age, nimorazole vs. placebo, p16-status and hypoxic status as categorised by the 15-gene hypoxia classifier [12].

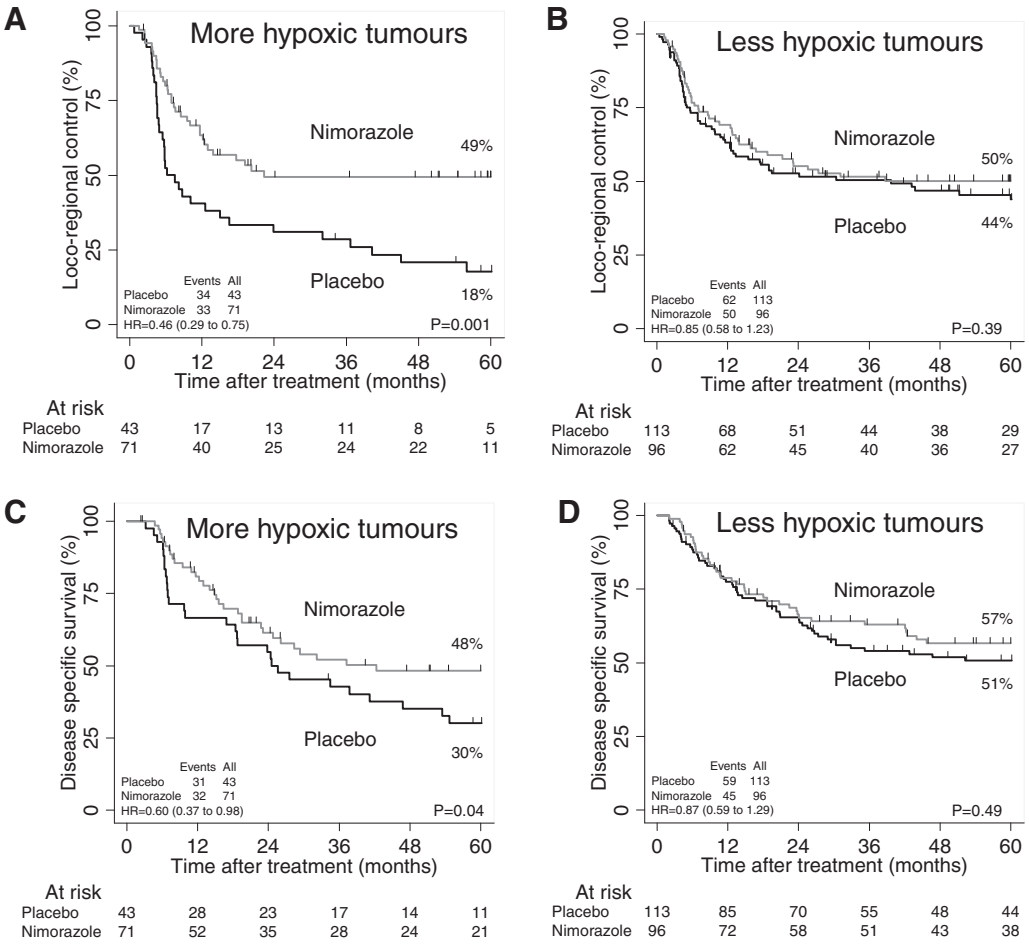

Fig. 3. Predictive impact of the 15-gene hypoxia classifier in "more" hypoxic tumours (A and C) vs. "less" hypoxic tumours (B and D) in terms of loco-regional control (A and B) and disease-specific survival (C and D).

The importance of biomarkers is highlighted by the last two phase III clinical trials on hypoxic modification. In the TROG 02.02 HeadSTART trial there was no significant effect of adding the hypoxic cytotoxin tirapazimine (TPZ) [24]. However, among patients who had no major QA deviations in their radiotherapy, a trend for better loco-regional control was observed in the TPZ arm for patients receiving $\geqslant$60 Gy and radiotherapy [25]. Studies on patients from the preceding phase I and II trials [26,27] demonstrated that both FMSIO-PET [28] and FAZA-PET [29] could identify a subset of patients with hypoxic tumours and that these patients did have a better outcome when receiving TPZ. In the ARCON phase III trial on accelerated radiotherapy combined with carbogen and nicotinamide, the early results showed a significant effect on regional control but no effect on local control [23]. In the subset of patients receiving the hypoxic marker pimonidazole, the effect on regional control was only found in patients with high hypoxic fraction whereas patients with low hypoxic fraction did not benefit from ARCON [30]. Similar observations were made in a subset of patients from the ARCON phase I and II trials [31], where high hypoxic fraction was significantly associated with decreased loco-regional control, but this association disappeared in patients treated with ARCON [32].

Of the several different methods for measuring hypoxia, FMISO-[28] and FAZA-PET [29] are examples of functional, non-invasive imaging techniques [33]. Other methods include oxygen electrode measurements [34,35], exogenous hypoxia markers [5,36–38], exemplified by pimonidazole [30,32], and endogenous hypoxia markers [39,40]. The 15-gene classifier presented here is an example of the latter class of biomarkers, and has certain unique characteristics. The initial gene list used to build the classifier was based on in vitro data, making it possible to avoid genes that were only induced by hypoxia at normal pH [13,14], and hypoxic upregulation has been validated in xenograft models [12]. In an attempt to ensure that the classifier detects clinically relevant hypoxia [41], the final classifier was developed based on an independent training set with known hypoxic status determined by the relative number of oxygen electrode measurements below or equal to 2.5 mm Hg [15,16]. Finally, the classifier has been developed specifically in order to be used on routine formalin-fixed, paraffin-embedded tumour sections. In the present study, we have demonstrated that the classifier can identify a subgroup of "more" hypoxic tumours, where hypoxic modification by nimorazole is associated with a significantly improved loco-regional control and disease-specific survival. The improved outcome in patients with "more" hypoxic tumours receiving hypoxic modification is similar to the outcome for patients with "less" hypoxic tumours, where there is no association with hypoxic modification. Thus, although nimorazole was observed to provide an overall benefit in the original study [4], the issue of tumour heterogeneity in terms of hypoxia was underlined, as we could identify a subgroup of patients where this benefit was very pronounced. Other hypoxia modifying strategies including a hypoxic cell cytotoxin [24] or ARCON therapy [23] may have different mechanisms of action. However, studies indicating that the effect of these strategies is limited to hypoxic patients have all applied nitroimidazole-based hypoxia detecting techniques like FMSIO-PET [28], FAZA-PET [29], and

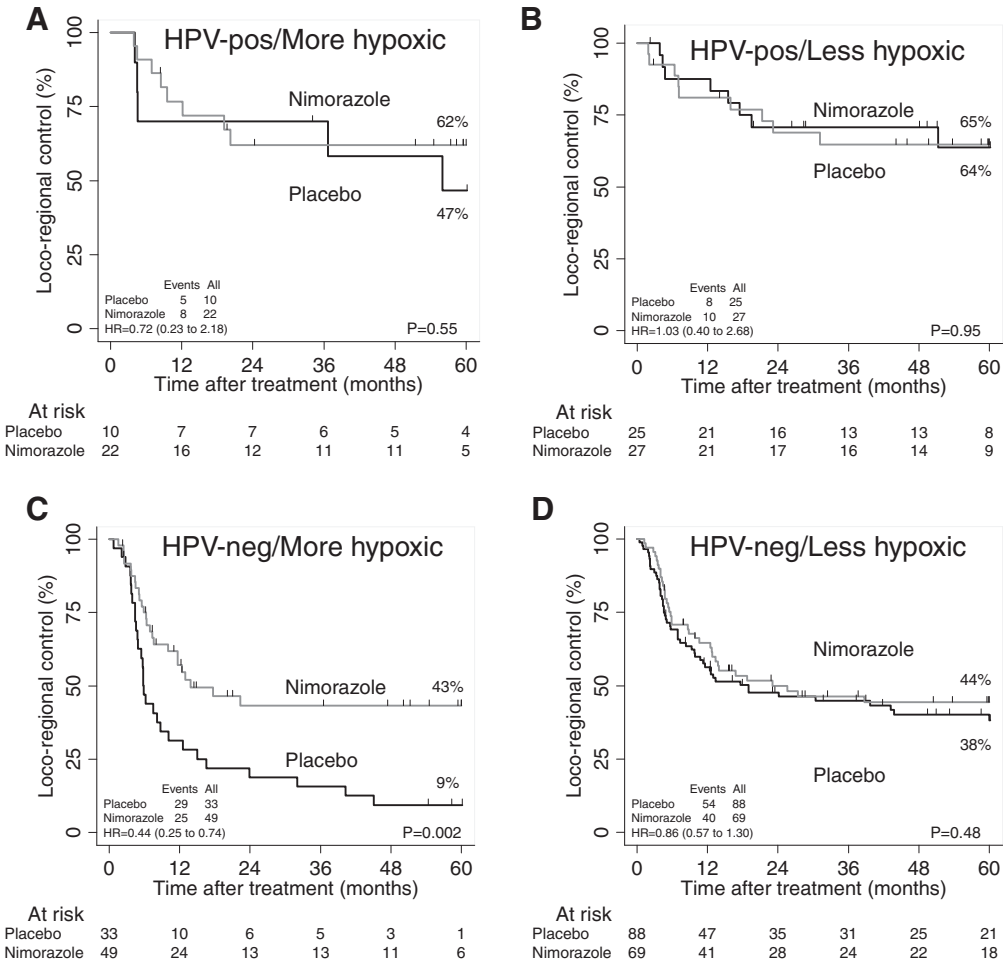

Fig. 4. Predictive impact of the 15-gene hypoxia classifier stratified by HPV status (evaluated by p16 immunohistochemistry) on loco-regional control. HPV-positive/"more" hypoxic tumours (A), HPV-positive/"less" hypoxic tumours (B), HPV-negative/"more" hypoxic tumours (C), and HPV-negative/"less" hypoxic tumours (D).

pimonidazole [30]. As the presented classifier can identify patients that obtain a beneficial effect of nimorazole, which is a 5-nitroimidazole compound, the classifier might also potentially identify candidate patients for these other treatment strategies. Furthermore, as the classifier should be applicable to any clinical studies where FFPE material is available, we believe that the classifier can be used in clinical trials as a biomarker for selection of patients that are likely to benefit from hypoxic modification, both retrospectively and prospectively.

Due to the increasing incidence of HPV associated HNSCC and the improved response to radiotherapy observed for these tumours [17–19], we have previously analysed the impact of HPV on hypoxic modification in the DAHANCA 5 study [20]. Loco-regional control was significantly associated with hypoxic modification in HPV-negative tumours (evaluated by p16 immunohistochemistry). In general, HPV-positive tumours were associated with a significantly better outcome, but this was not associated with hypoxic modification. A similar observation was made in a subset of patients from the TROG 02.02 trial, where there was a trend towards improved outcome in the TPZ arm among patients with HPV-negative tumours, but not among HPV-positive [42]. We have previously speculated that HPV-positive tumours might display less hypoxia [20], which could explain both the overall improved prognosis and the lack of association with hypoxic modification. However, here we observe the same frequencies of "more" vs. "less" hypoxic tumours in HPV-positive and HPV-negative tumours, respectively. It therefore appears that the improved outcome for HPV-positive tumours after radiotherapy is not linked primarily to differences in hypoxic status, but may be more associated with other factors like – hypothetically – the number or distribution of radio-resistant cancer stem cells [43,44]. Irrespective of the mechanism, the presented observations suggest hypoxic classification and HPV-assessment as important parameters that need to be taken into consideration when future clinical trials exploring the effect of hypoxic modification are planned.

Plasma osteopontin (OPN) level has been suggested as an endogenous biomarker for hypoxia [45]. We have previously evaluated plasma OPN in the DAHANCA 5 dataset, and high plasma OPN levels were associated with a significant benefit of hypoxic modification of radiotherapy by nimorazole [46]. Although there might be a correlation with OPN levels and hypoxia the findings have been ambiguous and we have also found that when using the end point of relative measurements below 2.5 mm Hg as an estimate of hypoxic fraction, there was no association between plasma OPN and oxygen electrode measurements. Neither was there any correlation between plasma OPN and tumour OPN as estimated with immunohistochemistry [47].

In the present study, we did not find a significant correlation between patients classified by the 15-gene classifier and patients classified by the previously reported plasma OPN levels (data not shown). Also, when evaluating the beneficial effect of nimorazole in the combined group of patients classified as "more" hypoxic by the 15-gene classifier and/or with high plasma OPN levels, we did not find an improved hazard ratio compared to patients

evaluated by the 15-gene classifier alone (data not shown). In comparison of the two predictive assays, the measurement of plasma OPN levels is complicated by the lack of standardized and validated methods [48] combined with previously contradictory results. Therefore, we consider the presented classifier is contributing with a more reliable estimate of the hypoxia fraction.

In conclusion, we tested and validated a 15-gene hypoxia classifier which proved to be a useful predictive biomarker for the selection of patients with HNSCC that benefit from hypoxic modification of radiotherapy with nimorazole. In patients classified as "more" hypoxic, nimorazole increased the outcome in terms of locoregional tumour control and disease-specific survival to the same level as patients with "less" hypoxic tumours. Although HPV-positive tumours do not appear to have the same benefit from hypoxic modification, they display the same degree of hypoxia in accordance to the classifier. Together, these observations highlight the importance of using biomarkers for both HPV and hypoxia status. The presented 15-gene classifier is a novel predictive hypoxia biomarker that can be applied both retrospectively and prospectively, and only requires access to routine formalin-fixed, paraffin-embedded tumour material.

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Appendix A

A.1. Gene expression quantification

Gene expression analysis were performed as previously described [49]. Total RNA was extracted from a 7 μm section of FFPE-biopsies with a silica bead-based, fully automated isolation method for RNA on a robotic Tissue Preparation System using VERSANT Tissue Preparation Reagents (Siemens Healthcare Diagnostics, Tarrytown, NY) [50,51]. cDNA was generated using the High Capacity cDNA Archive kit and pre-amplified using the TaqMan PreAmp Master Mix Kit (Applied Biosystems; ABI). Quantitative PCR was performed on an ABI Prism 7900 HT Sequence Detector using TaqMan Gene Expression PCR mastermix (Applied Biosystems; ABI). Gene expression levels were calculated using RealtTime Statminer (Intergromics).

A.2. Classification into "more" and "less" hypoxic groups

Classifications into "more" or "less" hypoxic groups were performed as previously described [49]. In short, 58 HNSCC cancer patients from an independent test study, whom where previously hypoxia evaluated with oxygen electrodes [52,53], were divided into two groups. Tumors were ranked according to the relative number of measurements below or equal to 2.5 mm Hg in their metastatic lymph nodes, and divided into two groups where the largest possible gene expression discrimination was observed. The 15 genes in the classifier were characterized by their mean expression (and estimated variance) in each of the two groups, "more hypoxic" or "less hypoxic". For each patient in the DAHANCA 5 trial, the expression level of each gene was compared to the mean expression levels of the genes in the two pre-defined groups from the independent test study. Each patient was then assigned individually to the group where the sum of the distance to mean expressions (weighed by the estimated variance) was the lowest. As the classifier was developed in an independent data set, no attempts were made to optimize the number of genes or expression levels prior to the classification of patients in the DAHANCA 5 cohort. Not all genes could be measured in all patients, but the 15 genes represent a robust signature and only failed to classify 3 out of 326 patients where FFPE material was available for RNA extraction (Fig. 1A). The investigator performing the classification procedure (KT) was blinded to patient treatment and clinical outcome data until the classification had been performed.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.radonc.2011.09.010.

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