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ORIGINAL ARTICLE

Hypoxic gene expression is a prognostic factor for disease free survival in a cohort of locally advanced squamous cell cancer of the uterine cervix

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ABSTRACT

Introduction: Tumour hypoxia in locally advanced squamous cervical cancer (LACC) has been shown to be of substantial prognostic importance. The aims of the present study were therefore to investigate if hypoxia could be identified by a newly validated hypoxic gene expression classifier and used as a prognostic factor for disease free survival (DFS).

Material and methods: Paraffin embedded biopsies were obtained from 190 patients with LACC with squamous cell carcinoma treated 2005–2016 with chemo-radiation and image guided adaptive brachytherapy. Analysis of hypoxia was successful in 183 patients (96%). Hypoxic classification of tumours into 'more' or 'less' hypoxic was based on 15 genes using the same method as in a prospective head and neck cancer trial (NCT02661152). HPV was genotyped using INNO-LiPA. Local tumour invasion was evaluated by the T-score. Primary endpoint was DFS analysed by Kaplan-Meier and Cox regression. Events were death of any cause, persistent disease, or recurrence.

Results: The $FIGO_{2009}$ stage distribution was IB–IIA 9%, IIB 64%, and III–IVA 27%, and mean T-score was 7.2. Pathological nodes were present in 53%. Median observation time was 5.2 years. Local control rate at 5 years was 96%, and pelvic (loco-regional) control 91%. Overall, 36% of the tumours were classified as 'more' hypoxic. The frequency of 'more' hypoxic tumours increased with local tumour intrusion (30% for T-score 0–9 vs. 55% for T-score $\geq$10, $p = 0.004$). Hypoxia was associated with decreased DFS in univariate, HR 1.71 (1.04–2.82), and multivariate analysis, HR 1.75 (1.04–2.92), and the effect was particularly observed among tumours with a T-score $\geq$10. HPV 16/18 was not associated with improved DFS in neither in univariate nor in multivariate analysis.

Conclusion: Hypoxic gene expression is a prominent prognostic factor for DFS in LACC with SCC histology and should be considered for treatment stratification in clinical trials.

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KEYWORDS
Locally advanced SCC cervical cancer; hypoxia; gene expression; prognostic factor

Introduction

Locally advanced cervical cancer (LACC) patients with a poor prognosis often present with significant vaginal bleeding and anaemia [1]. Gynaecological examination may then reveal a large, endophytic and infiltrative tumour with central necrosis. Such indirect clinical suggestions of tumour hypoxia have fuelled a long-standing interest in the prognostic role of tumour oxygenation in the treatment of LACC with radiotherapy [2]. Direct transvaginal measurements of tumour oxygenation with polarographic O_2 needle electrodes have clearly demonstrated levels of tumour hypoxia in LACC compatible with the presence of radioresistance [3]. Endogenous markers using immunohistochemical analysis of the hypoxia inducible factor system, exogenous markers by use of bioreductive compounds, gene expression signatures, as well as hypoxia imaging using functional MRI and hypoxia tracers for PET-CT have also shown evidence of tumour hypoxia in LACC [4,5].

Several interventions have been tested to circumvent the negative impact of hypoxia on the radiation response in LACC, including increasing oxygen delivery as well as targeting of hypoxic tumour cells and tumour vasculature [6]. Radiation-based approaches, by selectively raising the dose of radiation to the primary tumour by use of image guided adaptive brachytherapy (IGABT) now a days produce local control rates in excess of 90% even in advanced cases of LACC [7], and this is probably obtained by overcoming the influence of hypoxia. This is especially seen in patients with squamous cell carcinoma (SCC) where the dose response curve for local control approaches 95% and becomes almost flat when the dose to the brachytherapy target (CTV_{HR} D90) surpasses 90 Gy [8–10].

Despite the success of IGABT in LACC, significant problems with the control of distant disease have emerged [11]. Since tumour hypoxia is a well-known and strong driver of metastases [4], identification of hypoxia in LACC therefore remain very important to identify patients in potential need of systemic treatment intensification. In addition, patients with low levels of hypoxia may benefit from treatment de-escalation to improve the therapeutic ratio by diminishing

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morbidity. Unfortunately, translational studies of hypoxia in LACC have so far provided variable results in terms of prognostication of disease control and survival, and have been difficult to implement in routine clinical practice [2,4,6,12,13]. This may be further complicated in multicentre settings where substantial efforts are needed to calibrate and synchronise the methods for measurement of hypoxia.

A 15-hypoxic gene expression classifier has been successful used in SCC of the head and neck for both retrospective and prospective multicentre studies using formalin-fixed paraffin-embedded tumour biopsy material [14–17]. This 15-gene hypoxia classifier is currently being used in a prospective trial (NCT02661152) for specific targeting of hypoxia with nimorazole [18]. Thus, we speculated whether the gene classifier could be applied to tissue samples from patients with SCC of the uterine cervix.

The aims of the present study were to evaluate the prognostic potential of the 15-gene classifier for disease-free survival in multivariate analysis including HPV status in LACC patients with SCC histology, and to compare the prognostic value of the gene classifier with the T-score, a recently described prognostic marker for tumour aggressiveness [19].

Material and methods

Patient cohort and treatment

In the years 2005–2016 a total of 324 patients were treated with definitive chemoradiation (CRT) and image guided adaptive brachytherapy (BT) for primary LACC. Formalin-fixed paraffin-embedded biopsies (FFPE) with histologically verified invasive carcinoma constituting $>$ 5% of the total area of the sample were retrospectively obtainable from 230. Of these, 190 were SCC and analysis of hypoxic gene expression was successful in 183 (96%) and used for further analysis (Supplementary Figure 1). Patient characteristics are described in Table 1. Median age was 55 years, with most patients in $FIGO_{2009}$ stage IIB and beyond (91%). Local tumour invasion was further evaluated by gynaecological examination and MRI using the T-score as previously described [19]. In short, a ranked ordinal scale of 0–3 points was used to assess eight anatomic locations typical for local involvement (cervix, left parametrium, right parametrium, uterine corpus, vagina, bladder, ureters, and rectum). The T-score was calculated by the sum of points obtained from the eight locations with a mean T-score of 7.2 and an even distribution of patients into four prognostic T-score groups (0–4, 5–6, 7–9, $\geq$10).

Pathological nodes on PET-CT were present in the pelvis ($FIGO_{2018}$ stage IIIC1) and in the para-aortic region ($FIGO_{2018}$ stage IIIC2) in 40 and 13% of the patients, respectively. All patients received pelvic external beam radiotherapy of 45–50 Gy/25–30 fractions, including the para-aortic region in 39 (21%) patients. Concomitant weekly cisplatin was given to 142 (78%) patients. All patients were treated with BT with a mean CTV_{HR} volume of 35 cm^3 and a CTV_{HR} D90 of 92 Gy_{EQD2}.

Expression of the 15 hypoxia-induced genes were compared to a representative cohort of 108 patients with head and neck squamous cell carcinoma from the DAHANCA 18 and 24 trials [20,21]. These data were used as part of the validation of the classifier for prospective use in head and neck cancer [14] (see Supplementary Document for details).

Hypoxia classification and HPV

Hypoxic gene classification of tumours into 'more' or 'less' hypoxic was based on expression levels of 15 hypoxia induced genes in whole sections from FFPE (i.e., without preceding macro-dissection of the tumour area) and was done using the same method as currently used to classify head and neck cancer patients in an ongoing prospective clinical trial [14] (ClinicalTrials.gov ID: NCT02661152).

The classifier was developed to correlate with pO_2 levels in tumours from a cohort of head and neck cancer patients (measured by polarographic O_2 needle electrodes [22]) and could separate these patients into two groups, a 'more' hypoxic, and a 'less' hypoxic group [15]. The mean expression values of the 15 genes in these two original groups of patients constitute the basis of the classification. Basically, the expression pattern in a new head and neck cancer sample is compared to these two sets of mean values, one characteristic of the 'more' hypoxic group and one characteristic of the 'less' hypoxic group, and the new head and neck cancer sample is then classified based on which set of values it resembles the most.

The cervical cancer patients in the present study are classified using the same procedures as for head and neck and using the exact same sets of expression values characterising 'more' hypoxic and 'less' hypoxic tumours, respectively. See Supplementary Document for further information on the

Table 1. Patient and treatment characteristics.

	All patients $N = 183$ (100%)		'Less' hypoxic $N = 117$ (64%)		'More' hypoxic $N = 66$ (36%)		
	N	%	N	%	N	%	*p* value
Age							
Median (range)	56	(22–90)	55	(22–90)	57	(27–81)	
<55 years	89	49%	57	49%	32	48%	1.00
≥55 years	94	51%	60	51%	34	52%	
FIGO2009 stage							
IB-IIA	16	9%	8	7%	8	12%	0.012
IIB	117	64%	84	72%	33	50%	
III-IVA	50	27%	25	21%	25	38%	
T-score							
Mean (95% CI)	7.2	(6.6–7.7)	6.7	(6.1–7.3)	8.0	(7.1–9.0)	
0–4	41	22%	28	24%	13	20%	0.011
5–6	57	31%	44	38%	13	20%	
7–9	43	23%	26	22%	17	26%	
≥10	42	23%	19	16%	23	35%	
N stage							
N0	86	47%	55	47%	31	47%	0.20
N1: pelvic	73	40%	43	37%	30	45%	
N2: para-aortic	24	13%	19	16%	5	8%	
HPV-status							
Negative	4	2%	3	3%	1	2%	0.90
Type 16/18	128	70%	80	68%	48	73%	
Other types	51	28%	34	29%	17	26%	
Para-aortic external RT							
No	144	79%	91	78%	53	80%	0.85
Yes	39	21%	26	22%	13	20%	
Chemotherapy							
No	41	22%	24	21%	17	26%	0.46
Yes	142	78%	93	79%	49	74%	

development of the classifier and for a detailed description of the classification procedure.

HPV was genotyped from FFPE sections using INNO-LiPA HPV Genotyping EXTRA II Assay (Fujirebio).

Statistical analysis

Comparisons of patient and treatment characteristics were performed using Fisher's exact test. Endpoints were local and pelvic nodal control (absence of persistent disease or recurrence), loco-regional control (local and pelvic nodal control), and disease-free survival (DFS) calculated from start of CRT. DFS events were persistent disease, recurrence at any site, or death of any cause. Patients alive without evidence of disease were censored at the last day of follow-up for disease-status. Local and pelvic nodal control were analysed by the Aalen-Johansen estimator and competing events were recurrence at other sites and death. DFS was analysed by the Kaplan-Meier estimator and using Cox proportional hazards uni- and multivariate models. Median observation time was estimated using reverse Kaplan–Meier. All analyses were performed using Stata 17.0 (StataCorp LLC). The reporting adheres to the STROBE guidelines for cohort studies.

Results

Hypoxic gene expression

Before classification of the cervical cancer samples, the gene expression levels of the 15 genes were compared to expression levels in a representative head and neck cancer (Figure 1). The SCC data for the uterine cervix are from the 183 patients included here and the data for head and neck are from a cohort of 108 patients from DAHANCA 18/24 [14]. The levels of gene expression between the different sites of squamous cell carcinoma are very similar, and the classification of hypoxia in cervical cancer is therefore based on the same principles and procedures as for head and neck cancer.

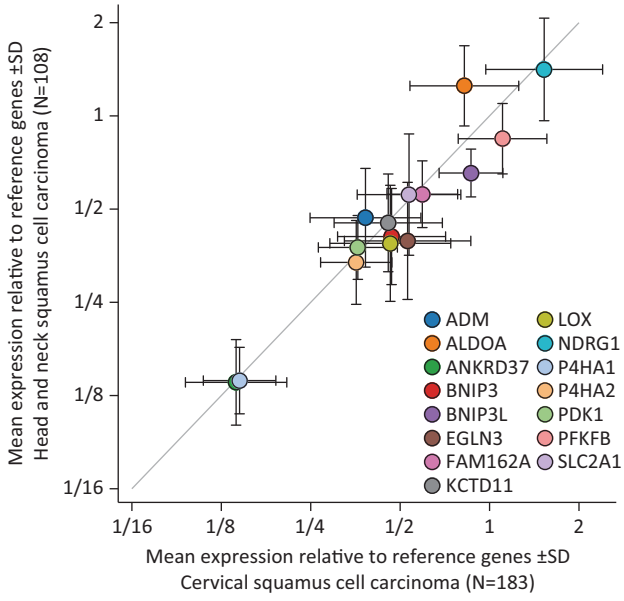

Figure 1. Gene expression levels of hypoxia induced genes (relative to reference genes) in cervical vs head and neck squamous cell carcinoma.

Hypoxia and patient characteristics

Hypoxic gene expression classified 36% (66/183) of the tumours as 'more' hypoxic, and 64% (117/183) as 'less' hypoxic. Table 1 list the characteristics of the patients by hypoxia status. Patients with 'more' hypoxic tumours had significantly higher FIGO stage ($p = 0.012$) and T-score ($p = 0.011$). Thus, the proportion of patients with 'more' hypoxic tumours increased from 41/133 (31%) in stage IB-IIB to 25/50 (50%) in stage III-IVA, and from 43/141 (30%) for T-score 0–9 to 23/42 (55%) in patients with T-score $\geq$10 ($p = 0.004$). Hypoxia was not associated with age and N stage nor with treatment related parameters (para-aortic external RT and concomitant chemotherapy).

Nearly all tumours were HPV positive (98%) with more than one HPV type identified in 11%. HPV 16 and/or HPV18 was found in 70% of the patients (Table 1). HPV16 was most frequently found (58%) followed by HPV18 (14%). HPV33 and HPV52 was identified in 7% and HPV31 and HPV45 in 5%. Other HPV types (11,35,39,53,56,58,59,68,70,73,82) were found in <3%. There was no association between HPV-status and hypoxia.

Hypoxia and clinical outcome

One patient died before first follow-up and was therefore only evaluable for DFS. With a median observation time of 5.2 years, local control was obtained in 176/182 (97%); loco-regional control (local and pelvic nodal) was obtained in 166/182 (91%); and extra-pelvic control (inguinal/para-aortic nodal and systemic) was obtained in 135/182 (74%) patients. In a competing risk model, local control rate at 5 years was estimated at 97% (95% CI: 93–99); pelvic nodal control at 93% (89–96); and extra-pelvic control at 73% (66–80) (Figure 2). Loco-regional persistent disease or recurrence was

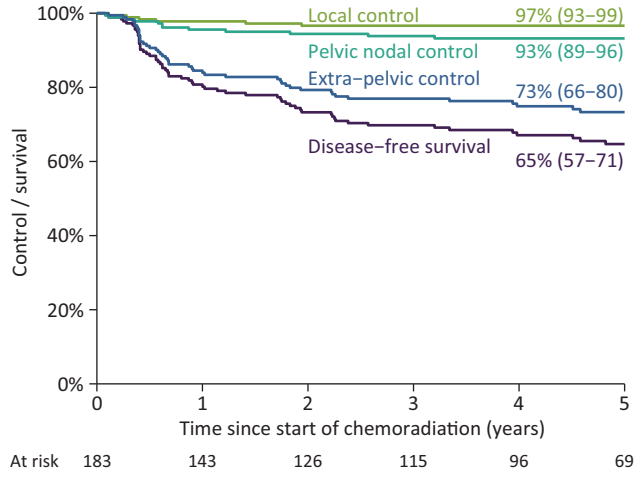

Figure 2. Local control, pelvic nodal control, extra pelvic control, and disease-free survival in 183 consecutive patients with locally advanced cervical cancer with squamous cell histology following state of the art chemoradiation and MRI guided brachytherapy.

observed in 8/117 (7%) patients with 'less' hypoxic and in 8/65 (12%) with 'more' hypoxic tumours ($p = 0.28$). Extra-pelvic persistent disease or recurrence was observed in 26/117 (22%) patients with 'less' hypoxic tumours and in 21/65 (32%) with 'more' hypoxic tumours ($p = 0.16$).

Overall 5 years DFS was 65% (95% CI: 57–71) (Figure 2), and hypoxic gene classification was significantly associated with DFS with a hazard ratio of 1.71 (95% CI: 1.04–2.82) for patients with 'more' hypoxic tumours compared to patients with 'less' hypoxic tumours (Figure 3). Five years DFS was 55% (42–67) in patients with 'more' hypoxic tumours versus 70% (60–78) in patients with 'less' hypoxic tumours ($p = 0.045$). In univariate COX analysis (Table 2), hypoxia, age, T-score, and concomitant chemotherapy were significantly associated with DFS. In multivariate analysis including age, N-stage, HPV, and chemotherapy (excluding T-score which was correlated with hypoxia), hypoxia status remained a significant prognostic factor with a HR of 1.75 (1.04–2.92) (Table 2).

T-score and hypoxia

In the univariate analysis, a T-score ≥10 was associated with a significantly worse DFS compared to patients with a T-score 0–4 (Table 2). Figure 4(A) shows the DFS estimates for the four groups of T-scores (0–4, 5–6, 7–9, ≥10), and illustrates how the group with T-scores ≥10 had a worse prognosis compared to the T-scores 0–9. If the scores 0–9 were combined, the estimated DFS was 39% (23–55) for scores ≥10 versus 71% (63–79) for scores 0–9 ($p < 0.001$) with a HR of 2.56 (1.53–4.31). In a multivariate Cox analysis including age, N-stage, HPV, and chemotherapy, the adjusted HR was 1.92 (1.10–3.36) (Figure 4(A)).

In patients with T-scores 0–9, 43/141 (30%) were 'more' hypoxic and 5 years DFS was 66% (50–79) in patients with 'more' hypoxic tumours versus 74% (64–82) in patients with 'less' hypoxic tumours ($p = 0.38$), HR 1.42 (0.74–2.71) (Figure 4(B)). In patients with T-scores ≥10, 23/42 (55%) were 'more' hypoxic and 5 years DFS was 30% (11–51) in patients with 'more' hypoxic tumours versus 48% (23–70) in patients with 'less' hypoxic tumours ($p = 0.20$), HR 1.73 (0.74–4.03) (Figure 4(C)). As the DFS estimates were comparable for T-scores 0–9 (independent of hypoxia status) and T-scores ≥10 that were 'less' hypoxic, these groups were combined and compared with T-scores ≥10 that were 'more' hypoxic (Figure 4(D)). In patients with T-scores ≥10 and 'more' hypoxic, 5 years DFS was 30% (11–51) versus 69% (61–76) in patients with T-scores 0–9 (independent of hypoxia status) or T-scores ≥10 that were 'less' hypoxic ($p < 0.001$), HR 2.90 (1.59–5.30). In multivariate Cox analysis including age, N-stage, HPV, and chemotherapy, the adjusted HR was 2.30 (1.20–4.40) (Figure 4(D)).

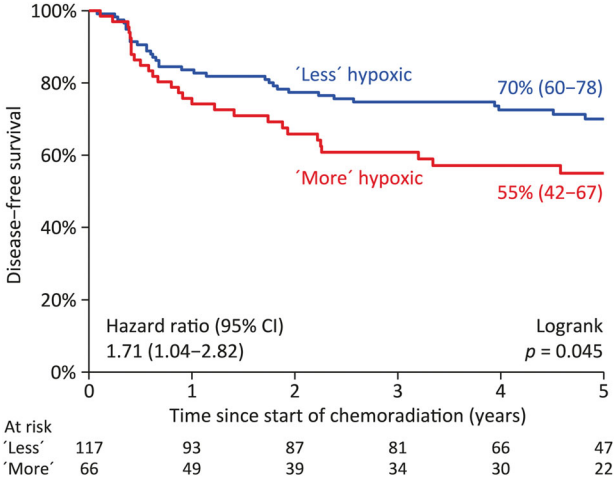

Figure 3. Disease-free survival in patients with locally advanced cervical cancer with squamous cell histology stratified by hypoxia status using a validated 15-hypoxic gene expression classifier.

Discussion

The present results demonstrates that the 15-hypoxic gene expression classifier developed for SCC of the head and neck cancer [14] is also predictive of DFS in SCC of the uterine cervix. We found that DFS was significantly reduced with increasing T-score and that hypoxic tumours were more locally invasive than less hypoxic tumours. By combining T-score and hypoxia status we were able to discriminate between the majority of patients (87%) with good and a minority (13%) with a very poor DFS. However, with the limited sample size we were unable to find any relationship between tumour hypoxia and regional nodal spread (e.g., N1 and N2) at diagnosis.

The present data also clearly underlines that systemic disease now is the main limitation for improving survival in LACC, despite of the loco-regional success of state-of-the-art CRT and BT [7,11]. So far prognostication of in LACC has

Table 2. Uni- and multivariate Cox analysis on disease-free survival.

		Univariate		Multivariate	
	N	HR (95% CI)	*p* value	HR (95% CI)	*p* value
Hypoxia					
'Less' hypoxic	117	1.0 (reference)		1.0 (reference)	
'More' hypoxic	66	1.71 (1.04–2.82)	0.035	1.75 (1.04–2.92)	0.033
Age					
<55 years	89	1.0 (reference)		1.0 (reference)	
≥55 years	94	1.76 (1.06–2.94)	0.030	1.87 (1.06–3.32)	0.031
FIGO2009 stage					
IB-IIA	16	1.0 (reference)		Not included	
IIB	117	0.76 (0.30–1.96)	0.57		
III-IVA	50	1.90 (0.73–4.95)	0.19		
T-score					
0–4	41	1.0 (reference)		Not included	
5–6	57	1.25 (0.55–2.87)	0.59		
7–9	43	1.77 (0.77–4.04)	0.18		
≥10	42	3.40 (1.57–7.37)	0.0019		
N stage					
N0	86	1.0 (reference)		1.0 (reference)	
N1: pelvic	73	1.54 (0.89–2.68)	0.12	2.03 (1.14–3.61)	0.017
N2: para-aortic	24	1.95 (0.95–3.99)	0.069	3.22 (1.48–7.03)	0.0032
HPV-16/18					
No	55	1.0 (reference)		1.0 (reference)	
Yes	128	0.79 (0.47–1.34)	0.39	0.97 (0.55–1.71)	0.92
Para-aortic external RT					
No	144	1.0 (reference)		Not included	
Yes	39	1.48 (0.85–2.59)	0.17		
Chemotherapy					
No	41	1.0 (reference)		1.0 (reference)	
Yes	142	0.49 (0.28-0.84)	0.0098	0.54 (0.29-0.98)	0.042

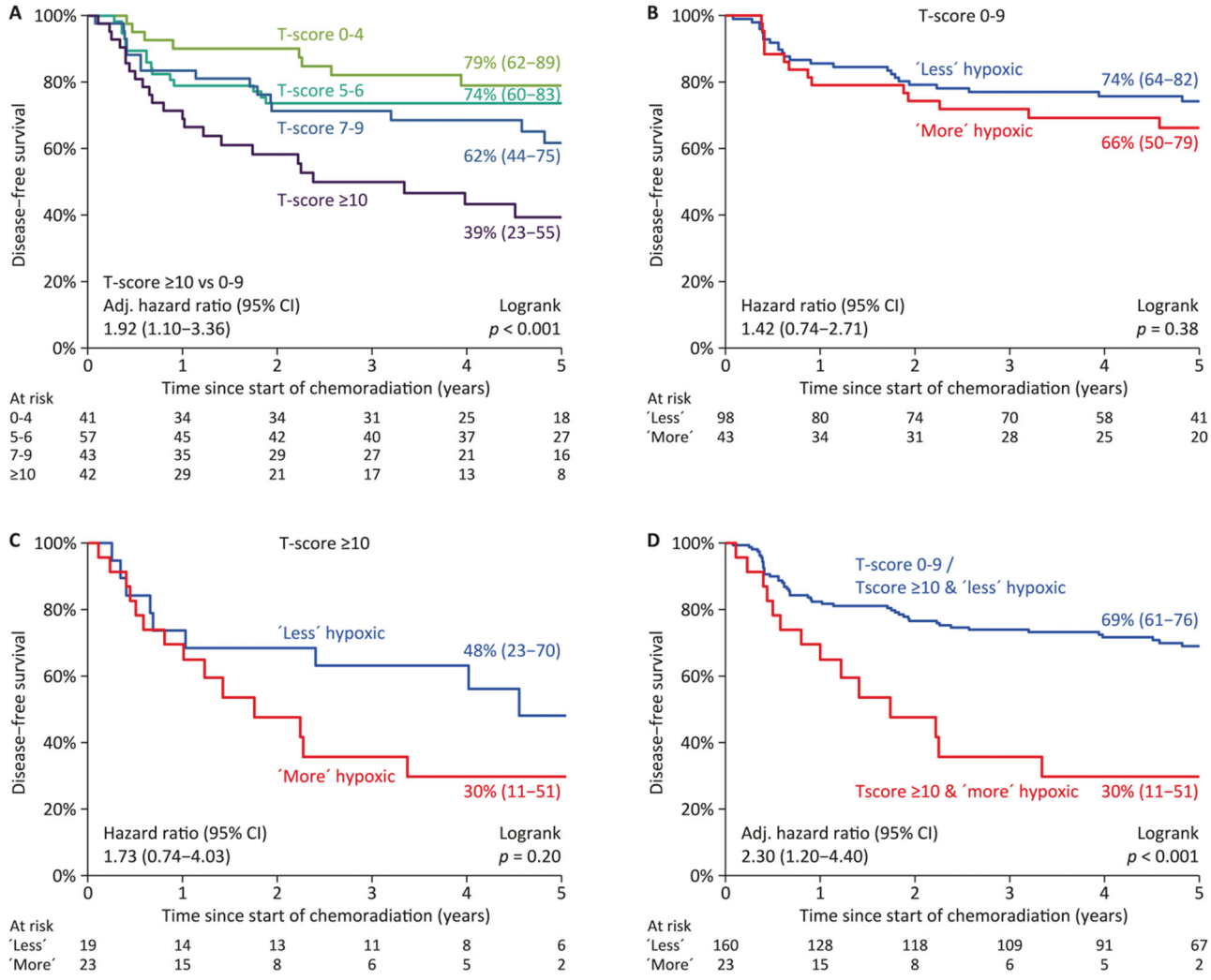

Figure 4. Disease-free survival in patients with locally advanced cervical cancer with squamous cell histology stratified by T-score and hypoxia status using a validated 15-hypoxic gene expression classifier.

mainly been based on disease and treatment related factors with limited impact from biomarker studies [12,19,23]. The strength of the 15-hypoxic gene expression classifier in this context is its ability to provide independent and strong prognostic information on top of the classical factors [4].

A further advantage of the 15-hypoxic gene expression classifier is its high success rate (96%) in classifying hypoxia using whole sections from FFPE, which enables the test to be used not only with archival material but also in prospective multicentre trials, as already demonstrated in head and neck cancer [14]. The use of diagnostic FFPE without the need for macro-dissection of tumour material, renders the technique easy to implement in the daily clinical workflow as opposed to more time consuming and expensive methodologies requiring e.g., handling of fresh biopsies. FFPE further enables verification of carcinoma in the tissue used for the analysis. Since, the FFPE used are obtained as part of the routine diagnostic process, the method is as such non-invasive compared to a procedure like the polarographic O_2 needle electrodes. In principle, the 15-hypoxic gene expression classifier involves only methods which are available in most pathology departments [14]. Thus, it could therefore be implemented more widespread allowing for on-site determination of hypoxia status in multicentre studies.

A similar 6-gene hypoxia classifier have been developed by Fjeldbo at al. using DCE-MRI hypoxic imaging as baseline [5]. In a cohort of patients comparable to the present they identified hypoxia in about one third of the patients and found that it significantly influenced locoregional control and disease-specific survival. This add to the overall impression, that cancer of the uterine cervix, like other squamous cell carcinomas, are likely to have the treatment outcome after radiotherapy to be influenced by the hypoxic status of the tumour.

We attempted to analyse the 6-gene hypoxia classifier [5] in our cohort, but failed due to low expression of the genes in the FFPE samples. See Supplementary Document for details.

The number of local and pelvic failures were almost doubled in the more hypoxic group compared to the less hypoxic group. However, due to a limited number of events, we were unable to detect a significant impact of hypoxia on

local and pelvic control. Other studies of LACC have shown that hypoxia is indeed important for local control [2,5,6,24,25], but these were performed before the advent of MRI guided BT with its ability for tailoring of very high radiation dose to the primary tumour to counteract hypoxia [26,27]. A predictive indication of hypoxia may give an option to stratify local treatment accordingly. This could be explored in a trial design addressing if the very high dose to the primary tumour delivered by MRI guided BT (>90 Gy) could be safely decreased in less hypoxic tumours, in order to reduce morbidity in the near-by organs at risk such as bladder, vagina and rectum [28]. Thus, in patients with low risk of local failure a stepwise lowering of the planning aim for BT could be warranted in a prospective non-inferiority designed observational study. For patients with hypoxic tumours further dose escalation with BT is perhaps questionable, but (re)introduction of hypoxic modification of the radiation response remain an attractive option, and the principal benefit of such treatment has been established in a meta-analysis [6]. With the specific knowledge on hypoxia as provided by the hypoxic gene classifier futures studies could be tailored to the appropriate patients enhancing the chance of a conclusive trial also for survival.

The relationship between hypoxia status and tumour regression during CRT, and the prognostic importance of hypoxia status assessed before/during treatment, are further aspects that needs clarification to optimise the local treatment [5,24,29–31]. The possible relationship between hypoxia and disseminated disease points to several potential clinical applications with regard to extend of elective external beam radiotherapy (small pelvis, ± common iliac, ± para-aortic region) and selection for adjuvant treatment following radical surgery or definitive CRT/BT [32,33]. The strong prognostic potential of the combination of the simple clinical/imaging based T-score and FPPE based hypoxia status as presented is an obvious candidate for identification of high risk patients in future trial addressing these issues.

As expected almost all patients in our study were HPV positive with type 16 and 18 being most frequent. HPV 16/18 have been characterised as 'high risk' in terms of a higher probability for developing intra-epithelial neoplasia [34]. Some data suggest that HPV 16/18 infection is associated with a worse prognosis for cervical cancer especially in early stage disease [35,36], while others [37,38] have found that HVP type 16/18 in fact confer a better prognosis especially in locally advanced disease. In our study where 91% of the patients were in stage IIB-IVA, there was a small trend that HPV type 16/18 might be associated with improved DFS in univariate analysis, but the trend was not seen in multivariate analysis.

In conclusion, hypoxia as estimated by the 15-hypoxic gene expression classifier is a prominent prognostic factor for DFS for locally advanced SCC of the uterine cervix. Dissemination of the 15-hypoxic gene expression technique, which is relying on analysis of routinely processed, FFPE, has a strong potential for improved patient selection to de-intensified or intensified treatment according to hypoxia status of the primary tumour at diagnosis.

Disclosure statement

Jan Alsner and Jens Overgaard are registered as co-inventors on a patent on a method (gene expression profile) for determining clinically relevant hypoxia in cancer (WO2012146259 A1) that is owned by Aarhus University, Aarhus, Denmark.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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