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Strong Prolyl Hydroxylase Domain 1 Expression Predicts Poor Outcome in Radiotherapy-treated Patients with Classical Hodgkin's Lymphoma

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Abstract. *Background: Hypoxia-inducible factors (HIFs) and prolyl hydroxylase domain (PHD) proteins control cellular oxygen homeostasis and a wide range of other processes. Materials and Methods: We immunohistochemically assessed the expression of HIF1α, HIF2α, PHD1, PHD2 and PHD3 in 115 cases of classical Hodgkin's lymphoma, all treated in the first line with doxorubicin, bleomycin, vinblastine and darcabazine (ABVD) chemotherapy. Results: In advanced-stage patients treated with involved-field radiotherapy (IFRT), nuclear HIF1α expression in reactive cellular infiltrate predicted prolonged relapse-free survival (RFS) (p=0.026). Strong cytoplasmic PHD1 expression in Reed–Sternberg cells was associated with poor RFS among patients treated with IFRT and advanced-stage patients treated with ABVD and IFRT (p=0.0028 and p=0.0058, respectively). In Cox regression analysis, PHD1 was a more significant predictor of relapse (risk ratio=18.383; 95% confidence interval(CI)=1.521-222.246; p=0.022) than the International Prognostic Score. Conclusion: HIF and PHD expression appear to be novel prognostic biomarkers in classical Hodgkin's lymphoma.*

Hodgkin's lymphoma (HL) is classified as either classical Hodgkin's lymphoma (cHL) and nodular lymphocyte-predominant Hodgkin's lymphoma (1). Most (95%) patients in Western countries present with cHL. Worldwide, age at diagnosis shows a bimodal distribution, the first peak occurring at 15-30 years of age and the second after 60 years (2).

With modern treatments, the prognosis of HL is among the most favourable in comparison to other malignancies (cure rate at least 80%). However, in particular, the overall survival of young patients is reduced because of the increased incidence of cardiovascular diseases and secondary malignancies resulting from treatment-related long-term toxicity (3, 4). After relapse, survival is significantly worse, even though there are new targeted agents for the treatment of relapsed cHL (5).

Hypoxia, a low level of oxygen, is the result of imbalance between availability and consumption of oxygen. It occurs in both non-pathological and pathological conditions. The most important hypoxia regulators are hypoxia-inducible factors (HIFs). These transcription factors regulate both the consumption and delivery of oxygen (6). They have two subunits, -α and -β. Hypoxia leads to HIFα stabilization and its attachment to constitutively expressed HIFβ. HIF1α and HIF2α combine with HIFβ and consequently activate HIF (6). Activated HIF triggers target gene expression, with the potential to accelerate cancer progression, affecting angiogenesis, metabolism, proliferation, apoptosis and reactive oxygen species (ROS) homeostasis (7-12). Increased expression of HIF1α and HIF2α has been linked to many types of cancer, including ovarian, brain and breast cancer (13). HIF1α and HIF2α have also been suggested to be prognostic factors in various cancer types (8).

Prolyl hydroxylase domain proteins (PHD1, PHD2 and PHD3) are also involved in oxygen homeostasis, triggering

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the proteosomal degradation of HIF1α and HIF2α (14). PHD2 in particular is known to act as an oxygen sensor for HIF stabilization (15). In addition to controlling the oxygen level, PHD proteins are part of the DNA damage response and regulate metabolism under oxidative stress in an HIF-independent fashion (16, 17). PHD proteins have been proposed to be tumour suppressors, but they are also associated with chemoresistance and tumour growth (18,19).

In the present study, we retrospectively explored the roles of HIF1α, HIF2α, PHD1, PHD2 and PHD3 expression in patients with previously untreated cHL. Special emphasis was placed on the localization of expression, association with previously recognized prognostic factors of cHL and possible usability of immunostaining in terms of prognostic or predictive value.

Materials and Methods

Patient collection. The study material consisted of lymph node samples from 115 patients with histologically confirmed cHL before the initiation of any treatment. All patients were treated with doxorubicin-bleomycin-vinblastine-dacarbazine (ABVD) chemotherapy in the first-line setting. Sixty-five patients also underwent involved-field radiotherapy (IFRT) after their chemotherapy (Table I). All lymphomas were diagnosed and treated in Finland in 1997-2015. Sixty-nine patients were diagnosed and treated at Oulu University Hospital and 42 patients at Kuopio University Hospital. Four patients were diagnosed and treated at the Central Hospitals of Kajaani, Kemi or Rovaniemi. Diagnoses were reviewed by a specialist haematopathologist. Accurate and updated patient information was gathered in each case from the hospital records.

Limited-stage risk factors included bulky mediastinal mass, elevated sedimentation rate, four or more involved nodal regions and age of 50 years or more. The International Prognostic Score (IPS) was calculated, based on the following factors: serum albumin ≤40 g/l, haemoglobin level ≤105 g/l, male sex, age ≥45 years, stage IV, leucocytosis $\geq 15\times10^9$/l and lymphocytopenia $\leq 0.6\times10^9$/l. Complete response (CR) was defined as no detectable tumour after first-line ABVD treatment. The Ethics Committee of the Northern Ostrobothnia Hospital District approved the study design (reference number 42/2010).

Immunohistochemistry. HL samples collected from the patients at the time of diagnosis were fixed in formalin and embedded in paraffin. Representative tumour areas from the paraffin blocks were cut in 3-μm sections and placed on SuperFrostPlus glass slides (Menzel-Gläser, Braunschweig, Germany). The slides were deparaffinised in Histo-Clear (National Diagnostics, Atlanta, GA, USA) and rehydrated through a graded series of alcohol solutions and rinsed in distilled water. Next, the slides were microwaved for 10 minutes in Tris-EDTA solution at pH 9 (PHD1, PHD2, PHD3 and HIF1α) or for 20 minutes in citrate buffer solution at pH 6 (HIF1α) to retrieve the epitopes and after 20 minutes' cooling at room temperature, endogenous peroxidase activity was neutralized in 3% H_2O_2 solution for 5 minutes. The next step was incubation with primary antibodies (Table II) in a humidity chamber at room temperature for 1 hour (PHD1 and PHD2), overnight at room temperature (HIF1α), or overnight at 4˚C (PHD3 and HIF2α). Immunostaining was continued using a Dako REAL™ EnVision™ Detection System (Dako Denmark, A/S, Glostrup, Denmark) according to the instructions of the manufacturer. Diaminobenzidine was used to detect the immunoreaction. Between all stages of the immunostaining procedure, the slides were washed with PBS-Tween. Finally, the slides were counterstained with Mayer´s haematoxylin, dehydrated and mounted.

Evaluation of immunohistochemical staining. Evaluation of immunostaining was performed by an experienced haematopathologist (KMH) together with another investigator (HB) blinded to the clinical data. Immunostaining was graded (i) separately in Reed–Sternberg (RS) cells and in the surrounding reactive cellular infiltrate; (ii) separately in nuclei and cytoplasm; and (iii) separately according to the extent (0-100%) and the intensity of immunostaining (1: weak, 2: moderate, 3: strong, and 4: very strong immunostaining intensity).

Statistical analysis. For statistical analyses, the staining intensity (0-4) was multiplied by the extent of immunostaining (0-100%), resulting in a continuous variable of 0-400. This continuous variable was used in all statistical analyses involving the Mann–Whitney *U*-test. Associations between protein expression and patient survival were analysed using the Kaplan–Meier method, and the statistical significance of differences was evaluated using the log-rank test. In survival analyses, continuous variables were divided into two classes (low or high expression) based on the median expression of each variable. Relapse-free survival (RFS) was calculated from the date of diagnosis to the date of the first confirmed relapse of cHL. Cox regression analysis was applied in multivariate analysis. Statistical analyses were carried out using IBM SPSS Statistics 24.0.0.0 software (IBM Corp., Armonk, NY, USA) and the results were considered significant when the two-sided *p*-value was less than 0.05.

Results

Clinical and histological data are presented in Table I. The median follow-up time was 64 months (range=4-153 months). After chemotherapy, 81 (70.4%), 30 (26.1%) and four (3.5%) of the patients had CR, partial response and progressive disease, respectively. After IFRT, CR was achieved by 66 (91.7%) of the patients, while six (8.3%) had only a partial response. There were seven (6.1%) lymphoma-specific deaths and four (3.5%) deaths due to other causes (*e.g.* infection). Nineteen (16.5%) patients suffered a relapse during follow-up.

The extent of HIF1α, HIF2α, PHD1, PHD2 and PHD3 expression in RS cells and in reactive cellular infiltrate are presented in Table III and representative examples of the immunostaining patterns are shown in Figure 1.

Strong cytoplasmic HIF2α staining in RS cells was associated with fewer complete responses after IFRT (p=0.014) in the whole cohort and in the subgroup with limited-stage disease (p=0.036), but not among the patients with advanced-stage cHL. In those with limited-stage cHL, strong nuclear HIF1α staining in reactive cellular infiltrate correlated inversely with the achievement of CR after first-line ABVD chemotherapy (p=0.021). HIF1α and HIF2α did

Table I. *Demographics of the patients*.

Characteristic	Limited stage		Advanced stage		Total	
Median age (range) at diagnosis, years	26	(11-85)	28	(16-70)	28	(11-85)
Gender, n (%)						
Male	24	52.1%	30	50.8%	61	53.0%
Female	32	57.1%	29	29.2%	54	47.0%
Histology (ICD-10 code), n (%)						
C81.1	47	83.9%	41	69.5%	88	76.5%
C81.2	4	7.1%	14	23.7%	18	15.7%
C81.7	5	8.9%	1	1.7%	6	5.2%
C81.9	0	0%	3	5.1%	3	2.6%
B-Symptoms, n (%)						
Absent	54	96.4%	22	37.3%	76	66.1%
Present	2	3.6%	37	62.7%	39	33.9%
Stage, n (%)						
Limited					56	48.7%
Advanced					59	51.3%
Limited-stage risk factors, n (%)						
None	24	42.9%				
≥1	32	57.1%				
International Prognostic Score, n (%)						
0-2			50	89.2%		
3-5			9	20.0%		
WHO performance status ≥1, n (%)	4	7.1%	24	40.7%	28	24.3%
Number of ABVD cycles received, n (%)						
2-3	5	8.9%	1	1.7%	6	5.2%
4-5	34	60.7%	2	3.4%	36	31.3%
6-7	15	26.8%	33	55.9%	48	41.7%
8	2	3.6%	23	39.0%	25	17.6%
Complete response with first-line ABVD, n (%)						
None	14	25.0%	20	33.9%	34	29.6%
Yes	42	75.0%	39	66.1%	81	70.4%
Radiotherapy, n (%)						
No	11	19.6%	32	54.2%	43	37.4%
Yes	45	80.4%	27	45.8%	72	62.6%
Complete response after radiotherapy, n (%)						
No	3	6.7%	3	11.1%	6	8.3%
Yes	41	93.3%	24	88.9%	66	91.6%
Relapse, n (%)						
No	50	89.3%	46	78.0%	96	83.5%
Yes	6	10.7%	11	22.0%	19	16.5%
Deaths, n (%)						
Lymphoma-specific	1	1.8%	6	10.2%	7	6.1%
Other causes	3	5.4%	1	1.6%	4	3.5%

ABVD: Doxorubicin, bleomycin, vinblastine and darcabazine. C81.1: Nodular sclerosis classical Hodgkin lymphoma; C81.2: Mixed cellularity classical Hodgkin lymphoma; C81.7: Other (classical) Hodgkin lymphoma; C81.9: Hodgkin lymphoma, unspecified.

not show associations with limited-stage risk factors, IPS, stage or B-symptoms. Low-level cytoplasmic PHD1 staining in RS cells was associated with limited-stage risk factors (p=0.042) and low-level cytoplasmic PHD1 and PHD3 immunostaining in reactive cellular infiltrate was also associated with a low rate of CR to chemotherapy in advanced-stage patients (p=0.0020 and p=0.019, respectively). Low-level nuclear PHD3 staining in RS cells was associated with a reduced degree of CR after the IFRT in patients with advanced-stage cHL (p=0.030). PHD1, PHD2 and PHD3 showed no association with advanced IPS, stage or B-symptoms.

Survival analysis. Strong nuclear HIF1α expression in reactive cellular infiltrate was associated with prolonged RFS in patients with advanced-stage cHL who had received IFRT (p=0.026, Figure 2A). When combined with IPS in multivariate analysis, HIF1α expression in this subgroup

Table II. *Antibodies used in immunohistochemical analyses using the Dako REAL™ EnVision™ Detection System (Dako Denmark A/S, Glostrup, Denmark).*

Primary antibody	Source of primary antibody	Dilution
PHD1, NB100-310	Novus Biologicals, Oxford, UK	1:300
PHD2, NB100-138	Novus Biologicals, Oxford, UK	1:300
PHD3, NBP1-30440	Novus Biologicals, Oxford, UK	1:500
HIF1α, 610958	BD Transduction Laboratories, Franklin Lakes, NJ, USA	1:40
HIF2α, ab8365	Abcam, Cambridge, UK	1:100

appeared to have more prognostic power, although neither variable remained significant in this model [for HIF1α: risk ratio (RR)=0.223, 95% confidence interval (CI)=0.043-1.157, p=0.074; and for IPS: RR=1.301, 95% CI=0.251-6.730, p=0.754]. Strong nuclear HIF1α expression in RS cells was not associated statistically significantly with prolonged RFS in patients with advanced-stage cHL who had received IFRT (p=0.11, Figure 2B).

Strong cytoplasmic PHD1 expression in RS cells was associated with poor RFS among patients treated with IFRT and among those with advanced-stage cHL who had received IFRT (p=0.0028 and p=0.0058, respectively, Figure 2C and D). In Cox regression analysis, the statistically significant effect greatly exceeded that of IPS in the patients treated with IFRT (for PHD1: RR=10.073, 95% CI=1.549-65.520, p=0.016; and for IPS: RR=0.340, 95% CI=0.387-1.388, p=0.34) and also in the patients with both advanced-stage and treated with IFRT (for PHD1: RR 18.383, 95% CI=1.521–222.246, p=0.022; and for IPS: RR=0.263, 95% CI=0.021–3.229, p=0.297).

Table III. *Percentages of cases showing any expression of hypoxia-inducible factor 1α (HIF1α), HIF2α, prolyl hydroxylase domain 1 (PHD1), PHD2 and PHD3.*

	Reed–Sternberg cells		Reactive cellular infiltrate	
	Nuclei	Cytoplasm	Nuclei	Cytoplasm
HIF1α	73.9%	0%	66.6%	0%
HIF2α	0%	85.8%	0%	0%
PHD1	0%	9.6%	0%	100%
PHD2	97.6%	0%	0%	99.1%
PHD3	86.5%	0%	0%	86.5%

Discussion

To our knowledge, this is the first report in which the expression of HIF1α, HIF2α, PHD1, PHD2 and PHD3 in cHL has been examined. The results show that HIF1α and PHD1 are linked to relapse-free time, but that HIF1α, HIF2α, PHD1 and PHD3 may also have roles in the evolution of resistance to first-line treatment.

Both PHD1 and PHD3 have significant independent roles in inhibiting signalling of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) (20, 21). NF-κB has a significant role in the pathogenesis of RS cells in cHL and is constitutively active, ensuring the survival of RS cells (22). PHD3 also promotes growth inhibition through epidermal growth factor receptor (23) and mediates alpha-ketoglutarate-induced apoptosis and tumour suppression (24). Considering the essential role of NF-κB in cHL pathogenesis, it may be a connecting factor between low nuclear PHD3 expression in RS cells and worsened probability of achieving CR after IFRT in patients with advanced-stage disease. On the other hand, there is significant crosstalk between reactive cellular infiltrate and RS cells. If cHL treatment drives (sensitive) reactive cellular infiltrate to apoptosis, the balance of crosstalk will suffer, which might explain why low levels of cytoplasmic PHD1 and PHD3 expression in reactive cellular infiltrate were associated with a lower rate of CR to chemotherapy in patients with advanced-stage cHL.

Stronger cytoplasmic PHD1 expression in RS cells was linked to poorer RFS in the patients with advanced-stage cHL who were treated with ABVD and IFRT. In more detail, PHD1 was expressed in RS cells in six patients treated with ABVD and IFRT, and three of them experienced relapse during follow-up. In patients with advanced-stage treated with ABVD and IFRT, PHD1 was expressed in four cases; three of these suffered relapse and two died of lymphoma. Based on the current results, cytoplasmic PHD1 expression thus seems to have diverse roles in reactive cellular infiltrate and in RS cells, being associated with sensitivity to ABVD in the former and poorer prognosis in the latter. In patients with pancreatic endocrine tumours, prostate adenocarcinoma and non-small-cell lung carcinoma, PHD1 has also been linked to poorer survival (25-27).

Strong HIF1α and HIF2α expression was associated with a low rate of CR to first-line treatment, especially in patients

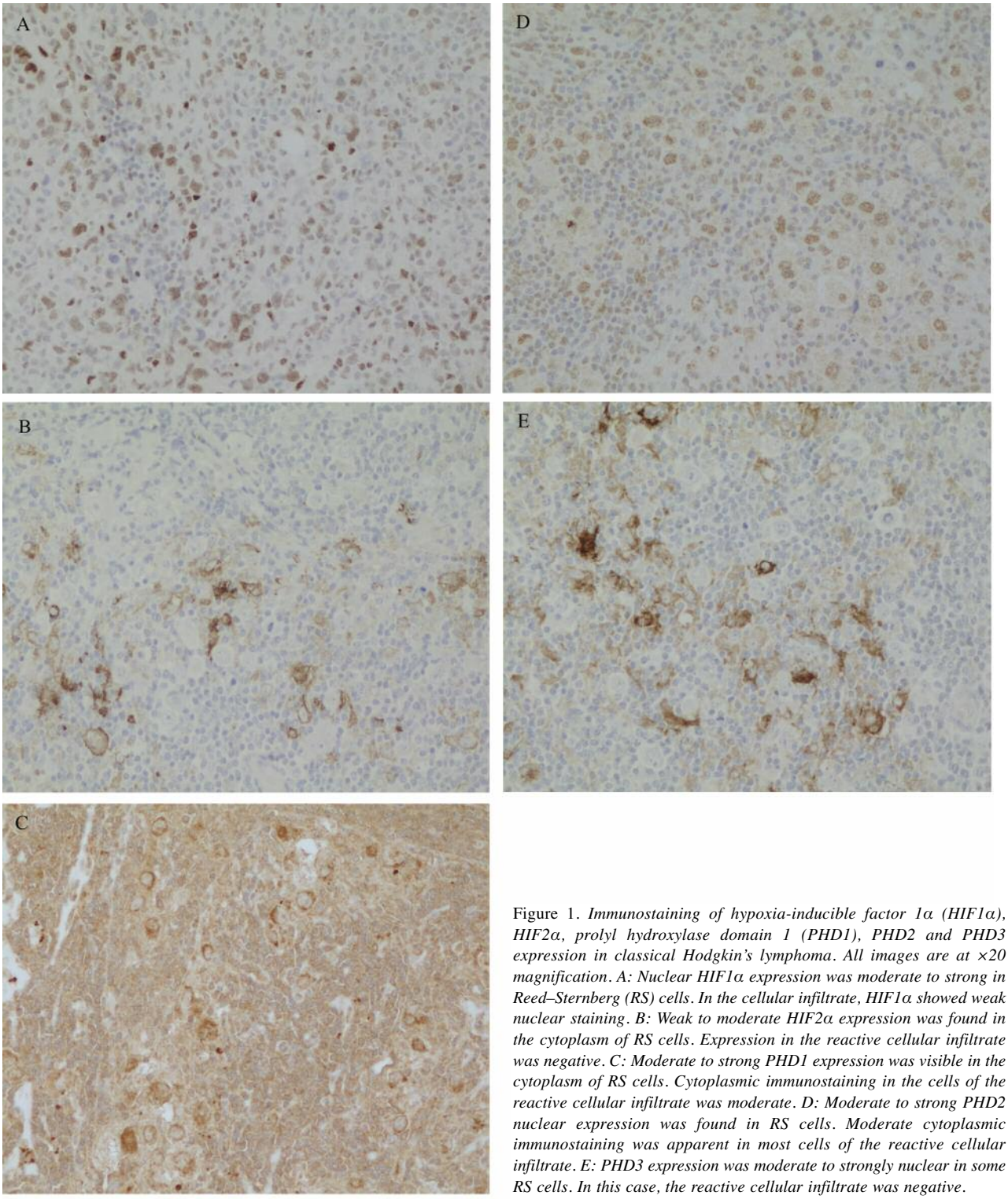

Figure 1. *Immunostaining of hypoxia-inducible factor 1α (HIF1α), HIF2α, prolyl hydroxylase domain 1 (PHD1), PHD2 and PHD3 expression in classical Hodgkin's lymphoma. All images are at ×20 magnification. A: Nuclear HIF1α expression was moderate to strong in Reed–Sternberg (RS) cells. In the cellular infiltrate, HIF1α showed weak nuclear staining. B: Weak to moderate HIF2α expression was found in the cytoplasm of RS cells. Expression in the reactive cellular infiltrate was negative. C: Moderate to strong PHD1 expression was visible in the cytoplasm of RS cells. Cytoplasmic immunostaining in the cells of the reactive cellular infiltrate was moderate. D: Moderate to strong PHD2 nuclear expression was found in RS cells. Moderate cytoplasmic immunostaining was apparent in most cells of the reactive cellular infiltrate. E: PHD3 expression was moderate to strongly nuclear in some RS cells. In this case, the reactive cellular infiltrate was negative.*

with limited-stage disease. HIF1α has been linked to chemoresistance by inhibiting apoptosis and attenuating the rate of intracellular drug accumulation (28). A hypoxic environment causes radioresistance, mainly due to re-oxygenation and ROS formation, which enables stabilization of the DNA damage response (29). Activated HIF brings

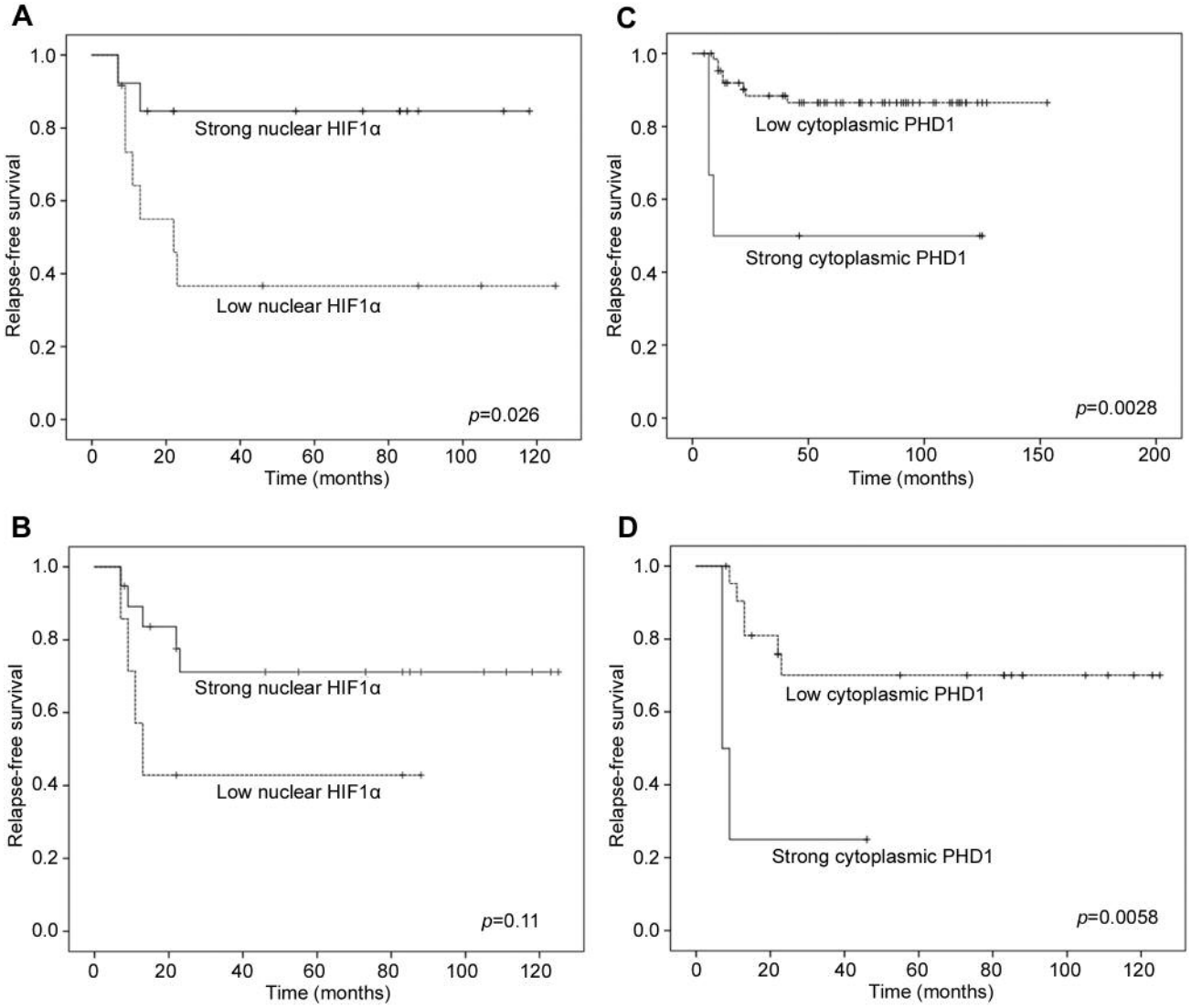

Figure 2. *Kaplan–Meier analysis of relapse-free survival in patients with advanced-stage disease who had received involved-field radiotherapy according to nuclear hypoxia-inducible factor 1α (HIF1α) expression in reactive cellular infiltrate (A), nuclear HIF1α expression in Reed–Sternberg (RS) cells (B), cytoplasmic prolyl hydroxylase domain 1 (PHD1) expression in RS cells (C), and cytoplasmic PHD1 expression in RS cells (D).*

about target gene expression and epigenetic post-translational histone modifications, which partly cause radiotherapy resistance (30). We have also shown that strong expression of the epigenetic regulators lysine (K)-specific demethylase 4B (KDM4B) and KDM4D are linked to radioresistance in cHL (31).

Strong nuclear HIF1α expression in reactive cellular infiltrate was associated with prolonged RFS. There was also a similar tendency among the patients with advanced-stage cHL who received both ABVD and IFRT, although this observation was not statistically significant. HIF1α has been linked to inferior survival in most types of cancer (8). However, there are results concerning renal cell carcinoma, diffuse large B-cell lymphoma and head and neck squamocellular carcinoma where HIF1α has been linked to improved survival (32-34). HIF1α contributes to regulatory T-cell (Treg) differentiation and in cHL, Tregs have an essential role in reactive cellular infiltrate by providing vital survival signals for the RS cells (35, 36). On the other hand, the high proportion of active Tregs in reactive cellular infiltrate has been associated with a favourable prognosis in patients with HL (37, 38). Furthermore, we reported earlier that strong expression of the oxidative stress marker 8-hydroxydeoxyguanosine (8-OHdG) predicts prolonged RFS in advanced stages of cHL

(39). Hypoxia produces oxidative stress *via* ROS generation and a strong positive correlation between 8-OHdG and HIF1α has been reported in healthy humans (40).

In conclusion, expression of HIF1α, HIF2α, PHD1 and PHD3 was associated with treatment resistance in cHL. Expression of PHD1 and HIF1α may predict RFS, but this varies depending on the cellular compartment of expression and the stage of the disease. Hypoxia biomarkers could be promising as predictive factors as regards treatment response, but our findings should be confirmed in a larger independent patient population.

Competing Interests

None.

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