Upregulation of stromal cell-derived factor 1α expression is associated with the resistance to neoadjuvant chemoradiotherapy of locally advanced rectal cancer: Angiogenic markers of neoadjuvant chemoradiation

HAN JO KIM[1], SANG BYUNG BAE[1], DONGJUN JEONG[2], EUN SEOG KIM[3], CHANG-NAM KIM[4], DONG-GUK PARK[5], TAE SUNG AHN[6], SUNG WOO CHO[6], EUNG JIN SHIN[6], MOON SOO LEE[6] and MOO JUN BAEK[6]

Departments of [1]Internal Medicine, [2]Pathology and [3]Radiation Oncology, Soonchunhayng University College of Medicine, Cheonan; [4]Department of Surgery, Eulji University College of Medicine, Daejeon; [5]Department of Surgery, Dankook University College of Medicine, Cheonan; [6]Department of Surgery, Soonchunhayng University College of Medicine, Cheonan, Republic of Korea

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Abstract. The ability to achieve pathologic downstaging after neoadjuvant chemoradiotherapy (NCRT) is correlated with improved survival in locally advanced rectal cancer (LARC). However, there is no effective predictive markers. In this study, the expression of angiogenic markers was evaluated in pre-treatment biopsies and corresponding post-treatment resection specimens, and were correlated to histopathological tumour characteristics and response. Fifty-five patients with stage II/III rectal cancer treated with 5-fluorouracil (5-FU)-based NCRT were studied. All patients were administered NCRT followed by surgical resection. Immunohistochemical staining for angiogenic markers [hypoxia-inducible factor 1α (HIF-1α), vascular endothelial growth factor (VEGF), stromal cell-derived factor 1α (SDF-1α) and placental growth factor (PlGF)] was performed on specimens obtained before NCRT and after surgery. Expression of VEGF, PlGF and HIF-1α protein was downregulated after NCRT in the rectal cancer tissues (P<0.001, P=0.001 and P=0.044, respectively). However, SDF-1α was upregulated after NCRT (P<0.001). Moreover, upregulated expression of SDF-1α (P=0.016) and positive PlGF staining (P=0.001) after NCRT were significantly associated with resistance to NCRT. On multivariate analysis, positive PlGF staining after NCRT was found to be independently associated with resistance to NCRT (P=0.013). Our data suggest that SDF-1α and PlGF should be evaluated as new targets for NCRT in LARC.

Correspondence to: Professor Moo Jun Baek, Department of Surgery, Soonchunhayng University College of Medicine, 23-20 Byeongmyeong-Dong, Dongnam-Gu, Cheonan-Si, Chungcheongnam-Do 330-721, Republic of Korea
E-mail: ssurge@sch.ac.kr

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Introduction

Rectal cancer (RC) is a major health issue and is one of the leading causes of cancer-related death worldwide (1). Neoadjuvant chemoradiotherapy (NCRT) followed by surgical resection is the current standard treatment for locally advanced rectal cancer (LARC). It offers improved local control, reduced toxicity and higher rates of sphincter preservation without compromising oncological outcome compared with post-operative treatment (2,3). A pathologic complete response (pCR) is one of the best predictive markers of a favourable prognosis. However, approximately 15-30% of patients experience a pCR, whereas the majority of patients have some degree of residual disease after NCRT (4). Thus, if patients with tumours that are responsive to NCRT could be identified at the time of diagnosis, then NCRT could be administered in a more individualised manner.

Recent studies have attempted to identify predictive biomarkers such as Ki-67, p53, p21, p27, bax, BCL-2, vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), survivin and thymidylate synthase (5,6). However, clinical use of these biomarkers requires further evaluation in prospective clinical trials (7).

Angiogenesis is necessary for tumour growth and malignant progression, with VEGF being a key angiogenic factor. High VEGF expression was found to be associated with poor survival in colorectal cancers (8). In particular, bevacizumab, a humanised monoclonal antibody inhibiting VEGF-A, in combination with standard chemotherapy regimens was beneficial both in terms of response rate and survival as first- and second-line treatment of patients affected by metastatic colorectal cancer. In patients affected by LARC who underwent radical surgery and adjuvant chemoradiation, tumour

VEGF overexpression was found to be associated with a statistically higher risk of local recurrence and metastasis (9). Not only VEGF, but placental growth factor (PlGF) are potentially useful predictive factors in rectal cancer (10).

Hypoxia is one of the key stimuli for the release of angiogenic markers (AMs) necessary for angiogenesis and tumour growth. Hypoxic tumours not only have a more aggressive nature (11,12), but studies in cervical and head and neck cancer demonstrated that tumour hypoxia decreases the response to radiation treatment (13). The effect of tumour hypoxia on the response to radiation therapy is relevant to the management of rectal cancer.

Hypoxia-inducible factor 1α (HIF-1α) is a protein involved in the cellular response to hypoxia centrally. It activates a variety of downstream genes involved in anaerobic metabolism and angiogenesis (14-16). These downstream gene protein products, which include VEGF, stromal cell-derived factor 1α (SDF-1α) and PlGF, promote cell survival under hypoxic conditions. Among patients with colorectal cancer, expression of these 4 AMs has been shown to correlate with rates of lymph node or liver metastasis, disease-free survival, and overall survival (17-20). However, the effects of pre-operative treatment on expression of AMs in rectal cancer remain unclear. The 4 AMs chosen for this study include HIF-1α, VEGF, SDF-1α and PlGF.

The aims of this exploratory study were to a) characterise expression of AMs in LARC before NCRT, b) investigate the change in AM expression after NCRT and c) evaluate the relationship between AM expression and tumour response.

Patients and methods

Patients and neoadjuvant chemoradiotherapy regimen. Between March 2005 and August 2009 at Soonchunhyang University Hospital, a total of 55 patients with non-metastatic, locally advanced (radiological T3-T4 or N^+ and/or clinically bulky) and biopsy-proven primary rectal cancer received NCRT. The whole pelvic field received 25 fractions of 180 cGy/day over 5 weeks, for a total of 4,500-5,040 cGy, using a four-field box technique. Chemotherapy was administered intravenously and consisted of 5-fluorouracil (5-FU; 425 mg/m^2/day) and leucovorin (20 mg/m^2/day) during the first and fifth weeks of radiotherapy. Surgical resection at 6-8 weeks was performed following the completion of NCRT. All data were collected and recorded prospectively, and the clinical and pathological features were reviewed retrospectively. The patients were classified according to the 6th edition of the American Joint Committee on Cancer staging system (21). Surgical specimens were evaluated for histopathologic staging as well as for pathologic response to NCRT. The detailed characteristics of the patients are listed in Table I. Our study was approved by the Clinical Ethics Review Committee of the Soonchunhyang University Hospital, Cheonan, Republic of Korea. Clinical consent was obtained from all patients.

Tissue microarray (TMA) construction. Areas representative of cancer were marked on haematoxylin and eosin-stained slides and TMAs were constructed. TMAs were created from formalin-fixed by 10% neutral buffered formalin, paraffin-embedded tissues using a 2-mm-diameter punch (Unitma; Unitech Science, Seoul, Korea). TMA blocks were assembled by obtaining duplicate cores from one patient block and re-embedding the two cores in an arrayed recipient block (Unitma; Unitech Science). A TMA block contains 60 cores from 30 samples.

Tumour response. Clinical stage was performed by an independent review conducted by a radiologist, and pathologic stage was reviewed by two independent pathologists. Downstaging was defined as staging reduction from pre-treatment stage (cStage) to pathologic stage (ypStage) (i.e. cIII to ypII, ypI or yp0; cII to ypI or yp0). Pathologic response (tumour regression) to NCRT was semiquantitatively determined by the amount of viable tumour versus the amount of fibrosis, ranging from no evidence of any NCRT effect to a complete response with no viable tumour identified, as described by Dworak *et al* (22). The following were characteristics of each grade: grade 0, no regression; grade 1, minor regression (dominant tumour mass with obvious fibrosis in 25% or less of the tumour mass); grade 2, moderate regression (dominant tumour mass with obvious fibrosis in 26-50% of the tumour mass); grade 3, good regression (dominant fibrosis outgrowing the tumour mass; i.e. >50% tumour regression); and grade 4, total regression (no viable tumour cells, only fibrotic mass). Patients with tumour regression grade (TRG) of 3 or 4 were considered as the responder group in our study.

Immunohistochemical (IHC) staining. The TMAs were sectioned at 4-μm intervals, deparaffinised three times in xylene for 30 min and rehydrated with graded alcohols (100% ethyl alcohol for 5 min, 95% ethyl alcohol for 3 min and 75% ethyl alcohol for 3 min) and then heated in antigen retrieval solution (sodium citrate, pH 6.0) in a microwave for 20 min. Sections were incubated in H_2O_2 for 10 min at room temperature. Furthermore, the sections were incubated with 150 ml of the primary antibodies, VEGF (1:200; Millipore, USA), PlGF (1:200; R&D system, USA), HIF-1α (1:50; Proteintech, USA) and SDF-1α (1:100; Novus Biologicals, USA) at 4˚C overnight. Subsequently, the sections were washed in PBS buffer three times for 3 min, treated with 150 ml secondary antibody for 1 h at room temperature and stained with DAB solution (Dako, USA). The sections were then washed in PBS buffer for 10 min. Finally, the sections were counterstained with hematoxylin for 3 min at room temperature, washed in distilled water 3 times for 3 min and mounted with coverslips.

IHC analysis. The VEGF, PlGF, HIF-1α and SDF-1α stained tissue cores were examined by 2 independent pathologists and a consensus score was determined for each specimen. A positive reaction for both antibodies was scored into 4 grades, according to the intensity of the staining: 0, 1+, 2+ and 3+. The percentages of positive cells were also scored into 4 categories: 0 (0%), 1 (1-33%), 2 (34-66%), and 3 (67-100%). The final score, calculated as the product of the intensity score multiplied by the percentage score, was classified as follows: 0 for negative; 1-3 for weak; 4-6 for moderate; and 7-9 for strong.

Statistical analysis. The correlations between expression levels of hypoxia-related proteins and pathologic response to NCRT were evaluated by the χ^2 or Fisher's exact test. The univariate

Table I. Association between AMs and clinicopathological parameters.

Clinicopathological parameters	HIF-1α expression			VEGF expression			PlGF expression			SDF-1α expression		
	Negative	Positive	P-value	Negative	Positive	P-value	Negative	Positive	P-value	Negative	Positive	P-value
Total patients, n (%)	29 (52.7)	26 (47.3)		24 (43.6)	31 (56.4)		19 (34.5)	36 (65.5)		16 (29.1)	39 (70.9)	
Age (years)			0.153			0.877			0.957			0.466
<65	21	14		15	20		12	23		9	26	
≥65	8	12		9	11		7	13		7	13	
Gender			0.385			0.876			0.733			1.000
Male	24	19		19	24		14	29		13	30	
Female	5	7		5	7		5	7		3	9	
Pre-treatment tumour stage			0.418			0.505			1.000			0.712
3	22	22		18	26		15	29		12	32	
4	7	4		6	5		4	7		4	7	
Pre-treatment nodal stage			0.220			0.078			0.570			0.388
0	8	3		8	3		5	6		5	6	
1	9	13		7	15		6	16		5	17	
2	12	10		9	13		8	14		6	16	
Post-treatment tumour stage			0.938			0.926			0.262			0.648
0	4	2		3	3		3	3		2	4	
1	1	0		0	1		0	1		0	1	
2	2	6		3	5		5	3		1	7	
3	20	18		17	21		10	28		12	26	
4	2	0		1	1		1	1		1	1	
Post-treatment nodal stage			0.138			0.281			0.489			0.277
0	23	15		18	20		15	23		14	24	
1	4	8		5	7		2	10		0	12	
2	2	3		1	4		2	3		2	3	

AMs, angiogenic markers; HIF-1α, hypoxia-inducible factor 1α; VEGF, vascular endothelial growth factor; PlGF, placenta growth factor; SDF-1α, stromal cell-derived factor 1α.

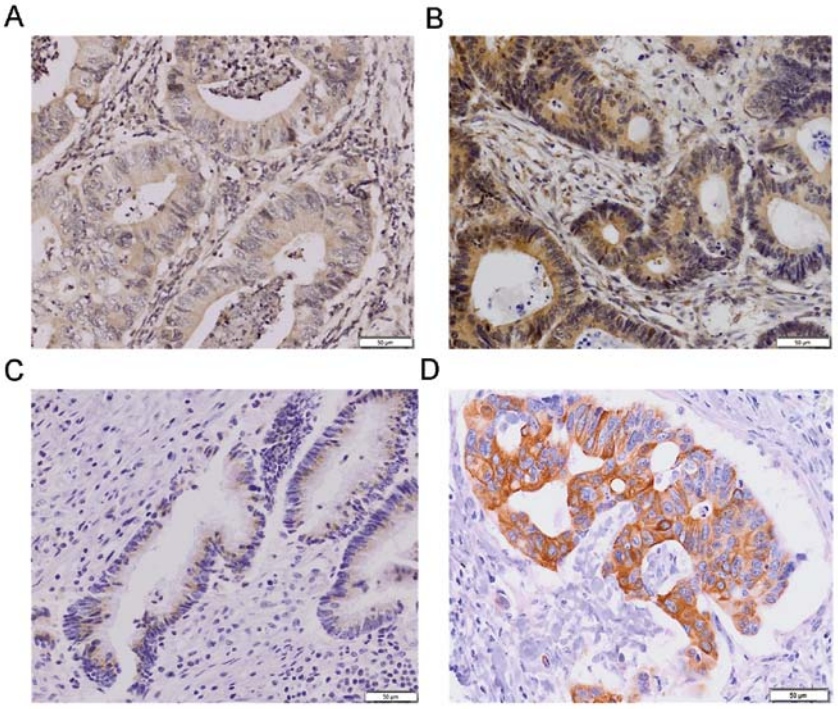

Figure 1. Immunoreactivity in the immunostained rectal carcinoma samples. (A) VEGF was weakly expressed in the cytoplasm. (B) HIF-1α was moderately expressed in the cytoplasm and nuclei. (C) PlGF was weakly expressed in the cytoplasm. (D) SDF-1α was strongly expressed in the cytoplasm and cytoplasmic membrane (magnification, x400). Scale bar, 50 μm.

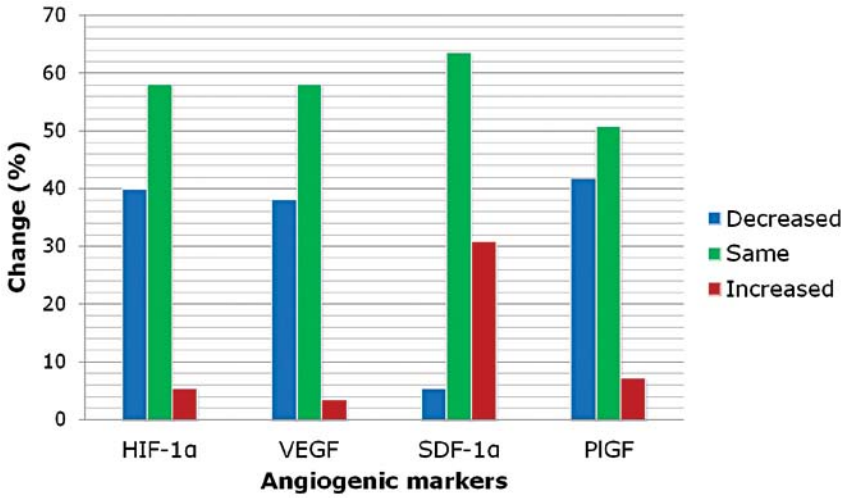

Figure 2. Change in staining status after neoadjuvant chemoradiation (NCRT). Expression of VEGF, PlGF and HIF-1α protein was downregulated after NCRT (P<0.001, P=0.001 and P=0.044, respectively). However, SDF-1α was upregulated after NCRT (P<0.001).

and multivariate analyses between response to NCRT and clinical or histopathologic parameters were performed by binary logistic regression model. All P-values quoted were two-sided, and P<0.05 was considered to indicate a statistically significant difference. All the analyses were performed using SPSS v. 17.0 (SPSS, Inc., Chicago, IL, USA).

Results

Association between AM expression and clinicopathological variables. The mean age of the 55 patients with LARC was 56 years (range, 18-82 years). In regards to gender, 43 (78.2%) were male, and 12 (21.8%) were female. Regarding the stage of disease, 11 (20.0%) were at stage II, and 44 (80.0%) were at stage III. Concerning the T stage, 44 (80.0%) were T3 and 11 (20.0%) were T4. The number of negative lymph node metastases was 11 (20.0%); N1 was 22 (40.0%), and N2 was 22 (40.0%). A pCR was obtained in 9.1% cases (5 patients). Patient characteristics are summarised in Table I. As shown in Table I, expression levels of AMs were not statistically correlated to the clincopathological variables.

Change in AM expression in LARC before NCRT and after surgery. The positive expression rate of HIF-1α, VEGF, PlGF

Table II. Results of AM immunoreactivity before NCRT and after surgery.

	HIF-1α		VEGF		SDF-1α		PlGF	
Staining score	Before NCRT	After surgery	Before NCRT	After surgery	Before NCRT	After surgery	Before NCRT	After surgery
Negative	29	48	24	39	16	10	19	30
Weak	17	7	16	12	17	12	19	19
Moderate	8	0	11	2	15	19	8	4
Strong	1	0	4	2	7	14	9	2

NCRT, neoadjuvant chemoradiotherapy; AMs, angiogenic markers.

Table III. Association between tumour response and clinicopathological parameters.

Clinicopathological parameters	Tumour response		P-value
	R	NR	
Age (years)			0.877
<65	20	15	
≥65	11	9	
Gender			0.416
Male	23	20	
Female	8	4	
Pre-treatment tumour stage			0.180
3	27	17	
4	4	7	
Pre-treatment nodal stage			0.517
0	4	7	
1	15	7	
2	12	10	
Pre-treatment VEGF staining			0.773
Negative	13	11	
Positive	18	13	
Pre-treatment PlGF staining			0.190
Negative	13	6	
Positive	18	18	
Pre-treatment SDF-1α staining			0.235
Negative	11	5	
Positive	20	19	
Pre-treatment HIF-1α staining			0.368
Negative	18	11	
Positive	13	13	
Post-treatment VEGF staining			0.074
Negative	19	20	
Positive	12	4	
Post-treatment PlGF staining			0.001
Negative	23	7	
Positive	8	17	
Post-treatment SDF-1α staining			0.159
Negative	8	2	
Positive	23	22	
Post-treatment HIF-1α staining			0.686
Negative	28	20	
Positive	3	4	
Change of staining status			
VEGF			0.400
Decreased	10	11	
Same	20	12	
Increased	1	1	
PlGF			0.568
Decreased	12	11	
Same	19	9	
Increased	0	4	

Table III. Continued.

Clinicopathological parameters	Tumour response		P-value
	R	NR	
SDF-1α			0.016
Decreased	3	0	
Same	22	13	
Increased	6	11	
HIF-1α			0.343
Decreased	11	11	
Same	19	13	
Increased	1	0	

R, responder; NR, non-responder.

and SDF-1α was 47.3% (26/55), 56.4% (31/55), 65.5% (36/55) and 70.9% (39/55) before NCRT, respectively. Weak, moderate and strong staining intensity of AMs is illustrated in Fig. 1. The expression rate of HIF-1α, VEGF, SDF-1α and PlGF was increased by 1.8% (1/55), 3.6% (2/55), 30.9% (17/55) and 7.3% (4/55) after NCRT, respectively. Expression of VEGF, PlGF and HIF-1α protein was downregulated after NCRT in the rectal cancer tissues (P<0.001, P=0.001 and P=0.044, respectively). However, SDF-1α was upregulated after NCRT (P<0.001; Table II, Fig. 2).

Relationship between tumour response to NCRT and clinicopathological variables. Upregulated expression of SDF-1α (P<0.016) and positive PlGF staining (P=0.001) after NCRT were significantly associated with resistance to NCRT. However, other clinicopathologic variables showed no correlation with tumour response (Table III). In multivariate analyses, positive PlGF staining after NCRT was found to be associated with resistance to NCRT [P=0.013; OR=0.197, 95% confidence interval (CI), 0.055-0.705]. Only low pre-treatment tumour lymph node staging was associated with pCR (P=0.002; Table IV).

Relationship with AM expression. Before NCRT, an association was identified between HIF-1α expression and SDF-1α (P=0.034). HIF-1α was not correlated with VEGF and PlGF. However, SDF-1α had an association with PlGF (P=0.005). After surgery, HIF-1α expression was not correlated with SDF-1α (P=0.621), and SDF-1α tended to be associated with PlGF (P=0.052).

Discussion

Recently, studies have attempted to identify predictive biomarkers, yet various studies only compared pre-treatment and post-treatment changes in biomarker expression (5). In this study, we investigated the predictive relevance of AM expression both in pre-treatment biopsies and in corresponding surgical specimens of 55 patients with LARC treated with standadised 5-FU-based NCRT. Comparing pre-treatment biopsies and surgical specimens, we observed a downregulation of

Table IV. Association between pCR and clinicopathological parameters.

Clinicopathological parameters	pCR		P-value
	(-)	(+)	
Age (years)			1.000
<65	32	3	
≥65	18	2	
Gender			0.298
Male	40	3	
Female	10	2	
Pre-treatment tumour stage			0.571
3	39	5	
4	11	0	
Pre-treatment nodal stage			0.002
0	7	4	
1	21	1	
2	22	0	
Pre-treatment VEGF staining			0.643
Negative	21	3	
Positive	29	2	
Pre-treatment PlGF staining			0.327
Negative	16	3	
Positive	34	2	
Pre-treatment SDF-1α staining			0.622
Negative	14	2	
Positive	36	3	
Pre-treatment HIF-1α staining			0.355
Negative	25	4	
Positive	25	1	
Post-treatment VEGF staining			1.000
Negative	35	4	
Positive	15	1	
Post-treatment PlGF staining			0.056
Negative	25	5	
Positive	25	0	
Post-treatment SDF-1α staining			0.220
Negative	8	2	
Positive	42	3	
Post-treatment HIF-1α staining			1.000
Negative	43	5	
Positive	7	0	
Change of staining status			
VEGF			0.817
Decreased	19	2	
Same	29	3	
Increased	2	0	
PlGF			0.835
Decreased	21	2	
Same	25	3	
Increased	4	0	

Table IV. Continued.

Clinicopathological parameters	pCR (-)	pCR (+)	P-value
SDF-1α			0.053
Decreased	2	1	
Same	31	4	
Increased	17	0	
HIF-1α			0.418
Decreased	21	1	
Same	28	4	
Increased	1	0	

pCR, pathologic complete response.

VEGF, PlGF and HIF-1α. However, SDF-1α was upregulated after NCRT. In addition, upregulated SDF-1α after NCRT was significantly associated with resistance to NCRT. Our findings suggest that SDF-1α is one of the important targets for resistance to NCRT and this finding is significant.

SDF-1α, also known as chemokine ligand 12 (CXCL12), and its receptor CXCR4, play important roles in the onset and progression of primary or metastatic cancer from various organs (23-26). In colorectal cancer (CRC), elevated SDF-1α expression is associated with metastasis and poor prognosis (27,28). In our investigation, upregulation of SDF-1α in surgical specimens was related to resistance to NCRT. Thus, SDF-1α appears to be a predictive marker to chemoradiation treatment. In an *in vitro* study using a CRC cell line, the results indicate that CXCR4 antagonistic therapy might prevent tumour cell dissemination and metastasis in CRC patients, consequently improving survival (29). Therefore, the targeting of SDF-1α represents an attractive adjuvant treatment to eradicate cancer cells and induce anti-angiogenic effects in highly hypoxic tumours. Further study evaluating the distinctive value of SDF-1α expression in LARC patients receiving NCRT is warranted. However, we did not observe a relationship between expression of AMs before NCRT and tumour reponse. Therefore, it is not possible to choose the 'right' patients who may require additional therapeutics (such as anti-angiogenesis), except NCRT, by analhysis of the specimen before treatment. These findings are difficult for clinical application.

We also found that positive expression of PlGF after NCRT was correlated with resistance to NCRT in multivariate analyses. PlGF is a cytokine in the VEGF family of growth factors, with 53% homology to VEGF (30). It primarily regulates the angiogenic switch under pathologic states (31). PlGF recruits smooth muscle precursors that envelop developing vessels in tumours and together with VEGF produces more stable and mature vessels. PlGF may also facilitate metastasis by increasing the motility and invasion of malignant cells (32). Tumour overexpression of PlGF and VEGF together is associated with increased tumour angiogenesis and cancer growth (33,34). However, in general, there was no correlation between elevated VEGF expression and survival (35,36). Our results suggest that PlGF, than VEGF, is also an important target for resistance to NCRT. It would be worthwhile to determine whether or not PlGF is a predictive biomarker for patients with LARC receiving NCRT.

As shown in this study, an association was identified between HIF-1α and SDF-1α (P=0.034). HIF-1α was not correlated with VEGF and PlGF. However, SDF-1α had an association with PlGF (P=0.005). Although HIF-1α expression is known to drive expression of downstream proteins, differences in individual protein half-lives may not allow for a direct relationship between HIF-1α and other proteins (37). Downstream proteins may have been influenced by other signaling pathways independent of HIF-1α, making their expression levels somewhat variable in relation to HIF-1α. The limited sample size and the heterogeneity of intratumoural oxygenation may also be responsible for these findings.

In summary, SDF-1α and PlGF are relevant for resistance to NCRT. By comparison of pre-therapeutic and post-therapeutic intratumoural SDF-1α and PlGF, our results suggest that therapeutic strategies to downregulate expression of SDF-1α and PlGF during pre-operative treatment or to inhibit SDF-1α/PlGF mediated signaling pathways may further increase the individual tumour response and, as a consequence, improve patient prognosis. Based on our results, patients with increased expression of SDF-1α or positive expression of PlGF after NCRT might benefit from additional anti-SDF-1α/PlGF therapeutics.

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