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Primary tumor vascularity in esophagus cancer. CD34 and HIF1-α expression correlate with tumor progression

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Summary: Objective: Hypoxia inducible factor α (HIF1-α) is a key protein regulating the response of a variety of genes and pathways, including angiogenesis, to hypoxic stimuli. High vascularity in various carcinomas correlates with invasion and metastasis. Assessment of primary tumor vascularity and HIF1-α expression in esophageal carcinomas was an objective of this study.

Methods: The vascularity in esophageal carcinomas (n=52) was quantified by Chalkley method on CD34 immunostained sections. HIF1-α expression was examined by immunohistochemistry. The relationships between CD34 Chalkley count, HIF1-α and various clinico-pathological characteristics with clinical outcome were evaluated.

Results: High HIF1-α expression in squamous cell carcinoma (SCC) was significantly associated with the T3-4 group (p=0.02). A higher percentage of SCC with high HIF1-α expression compared to its expression in adenocarcinoma (AC) (p=0.005) was observed. In the SCC group, high CD34 Chalkley count and high HIF1-α expression implied a significantly reduced survival (p=0.003 and p=0.001). No such significant association was found in the AC group.

Conclusions: HIF1-α expression is different in two separate tumor microenvironments: SCCs and ACs of the esophagus. This suggests that different mechanisms may be involved in HIF1-α expression- and activity between the two histological types of esophageal carcinoma.

Key words: Esophageal cancer, Primary tumor vascularity, CD34, HIF1-α, Survival

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Introduction

At present, the overall 5-year survival for patients with esophagus cancer varies between 10-26% (Enzinger and Mayer, 2003; Parkin et al., 2005). The initially asymptomatic development of the disease, combined with central tumor localization causing direct invasion of adjacent organs, does challenge early detection and treatment (Koshy et al., 2004).

Tumor growth and development of metastasis requires angiogenesis. Early angiogenesis is induced by pro-angiogenic factors, but when further advanced, the vascular development is dependent on adequate oxygen and nutrient supply (Folkman, 1986).

Hypoxia is a key driver for angiogenesis, regulated by a series of stimulating and inhibiting angiogenic factors, including HIF1-α (Semenza and Wang, 1992; Hendriksen et al., 2009).

HIF1-α is a transcriptional factor regulating the response of a variety of genes under cellular hypoxic stress. Apoptosis, glycolysis and angiogenesis are all influenced by it (Fukuda et al., 2002, 2003; Moriyama et al., 2008). Over-expression of HIF1-α, associated with angiogenesis and tumor progression, has been reported in different types of human malignancies, including esophageal SCC (Weidner, 1998; Zhong et al., 1999; Koukourakis et al., 2002).

Hypoxia activates cancer cells and stimulates angiogenesis, which in turn strongly correlates with tumor progression (Krock et al., 2011; Wahl et al., 2013). High vascularity in various carcinomas correlates with invasion and metastasis (Fidler and Ellis, 1994; Osinsky et al., 2011; Balac et al., 2012), including breast-, colon- and gastric carcinoma patients (Hansen et al., 2000; Liang et al., 2004; Dhakal et al., 2008).

For quantification of angiogenesis using immunohistochemistry, various markers, i.e. CD31,

CD34, CD105 or VEGF, have been used (El Gehani et al., 2011; Balac et al., 2012). Among different methods used, the Chalkley counting method is considered to be simple, fast and easy for daily clinical use (Dhakal et al., 2009).

In the present study we used a standard immunochemical technique with an anti-CD34 monoclonal antibody to study primary tumor vascularity in esophageal carcinomas measured by the Chalkley method. We examined HIF1-α expression as well, and compared it with tumor vascularity, clinico-pathological features and clinical outcomes of the patients with both adeno- and squamous cell carcinoma of the esophagus.

Materials and methods

Patients and specimens

Fifty-two patients (24 with SCC and 28 with AC) undergoing esophagectomy at the Department of Surgery, The Norwegian Radium Hospital between November 1988 and November 2002 were enrolled in this study. The clinical material has been reported previously (Goscinski et al., 2008a,b, 2009). Esophagectomy was performed through laparotomy and a right-sided thoracotomy. Subsequent reconstitution was carried out mostly by means of esophagogastrostomy using a gastric tube through the retrosternal route. The tissue samples were then fixed in neutral buffered formalin embedded in paraffin and stained with hematoxylin and eosin.

In accordance with the WHO classification, the esophageal carcinomas were histologically subtyped and graded into three groups: well-, moderately- and poorly differentiated (WHO, 1990). TNM classification was done according to the UICC Global Cancer Control (UICC, 2005). The patients' age ranged from 38 to 87 years. The clinico-pathological features of the patients are summarized in Table 1.

The study has been approved by the Regional Ethical Committee.

Immunohistochemistry

Flex plus method

Four-micrometer-thick sections with representative tumor tissue were cut from the formalin-fixed paraffin embedded blocks and immunostained for CD34 and HIF1-α using Dako EnVision™ Flex+ System (K8012, Dako) and Dakoautostainer. After deparaffinization of sections, epitopes were unmasked using PT-Link (Dako) and EnVision™ Flex target retrieval solution, high pH (Dako). Blocking of endogenous peroxidase with 0.03% H_2O_2 treatment for five minutes was carried out. Thereafter, the slides were incubated with primary monoclonal murine antibody (IgG1) against CD34, QBend-10 (Monosan, the Netherlands) in 1:1000 dilution and with mouse monoclonal antibody IgG1 against HIF1-α (BD Biosciences, San Diego, CA, USA) in 1:25 dilution for 30 minutes at room temperature and further with EnVision™ Flex+ mouse or rabbit (linker) and EnVision™ Flex/HRP for 30 minutes. At the end, the slides were stained with DAB for 10 minutes, counterstained with haematoxylin and mounted in Diatex. Appropriate positive controls were used for each series and gave satisfactory results.

Quantification of tumor vascularity

Tumor vascularity was quantified on CD34 stained slides using the Chalkley method following recommendations from a recent international consensus meeting (Vermeulen et al., 2002). A careful scanning of the tumor sections was done with a simple microscope at x40 and then x100 magnification. Thereafter, the most intensely vascularized areas in each tumor section (hotspots) were identified and selected subjectively as

Table 1. Clinico-pathological features of patients with esophageal carcinoma (n=52).

Characteristics		Frequency
Histology	Adenocarcinoma	28
	Squamous cell carcinoma	24
	Total	52
T	T1	7
	T2	5
	T3	37
	T4	3
	Total	52
N	N0	21
	N1	25
	Nx	6
	Total	52
M	M0	22
	M1	29
	Mx	1
	Total	52
Local recurrence	No	30
	Not settled	11
	Yes	11
	Total	52
Sex	Male	42
	Female	10
	Total	52
Histological grading	Well differentiated	2
	Moderately differentiated	22
	Poorly differentiated	27
	Non-classified	1
	Total	52

T, primary tumor; T1, tumor invades lamina propria or submucosa; T2, tumor invades muscularis propria; T3, tumor invades adventitia; T4, tumor invades adjacent structures; N, regional lymph nodes; Nx, regional lymph nodes cannot be assessed; N0, no regional lymph node metastasis; N1, regional lymph node metastasis; M, distant metastasis; Mx, distant metastasis cannot be assessed; M0, no distant metastasis; M1, distant metastasis.

described by Weidner (1995). A 25-point Chalkley eyepiece graticule was applied to each hotspot area at x200 magnification which creates a Chalkley grid area of 0.1886 mm^2 (Eclipse E400, Nikon microscope). Areas with carcinoma invasion, including the tumor periphery, were used for counting purposes, but the sclerotic and necrotic areas were avoided. The graticule was oriented in a way to allow the maximum number of black dots (points) to fall on or within the areas of immunohistochemically stained microvessel profiles. The number of dots hitting the stained vessels and endothelial cells in each hotspot were counted as Chalkley count. For further analyses the highest score among the three hotspots counts was used. All Chalkley counts were done by one experienced pathologist without knowledge of clinical data or patients' prognostic outcome.

Immunoscoring of HIF1-α

The immunoscoring of HIF1-α was performed with a simple microscope in the invasive carcinoma portion of the section. Nuclear expression of the marker was categorized semi-quantitatively based on percentage of positive tumor cells as 0-25% positive tumor cells = no or low expression of HIF1-α, and 25-100% as high expression of the marker for analyses.

Statistical analysis

Associations between CD34 Chalkley count, HIF1-α expression and various clinico-pathological variables were assessed using the Pearson's Chi Square test and the Fischer Exact test as required. Univariate survival analyses were performed using log-rank tests. The Kaplan Meier method was applied to plot survival curves. Binary logistic regression was performed to examine survival and local recurrence status. All p-values were two-sided, and a p-value of <0.05 was considered statistically significant. Statistical analyses were performed using SPSS 18.0 for Windows.

Results

Vascularity and clinico-pathological parameters

The CD34 Chalkley counts for primary tumor vascularity in esophageal carcinomas ranged from 3 to

Table 2. Clinico-pathological characteristics of patients with SCC and their relation with CD34 and HIF1-α.

Squamous cell carcinoma	CD34			HIF1-α		
	Low	High	p-value	Low	High	p-value
All patients (n=24)	10 (41.7%)	14 (58.3%)		9(37.5%)	15 (62.5%)	
Sex						
Female	3 (42.9%)	4 (57.1%)	0.659	2 (33.3%)	4 (66.7%)	0.618
Male	10 (58.8%)	7 (41.2%)		5 (29.4%)	12 (70.6%)	
Histological grading						
Well diff.	1 (100.0%)	-		1 (100.0%)	-	
Moderately diff.	3 (30.0%)	7 (70.0%)	0.089	3 (30.0%)	7 (70.0%)	0.424
Poorly diff.	9 (69.2%)	4 (30.8%)		3 (25.0%)	9 (75.0%)	
T						
T 1-2	4 (100.0%)	-	0.067	3 (100.0%)	-	0.020
T 3-4	9 (45.0%)	11 (55.0%)		4 (20.0%)	16 (80.0%)	
N						
N0	9 (75.0%)	3 (25.0%)	0.221	4 (36.4%)	7 (63.6%)	0.962
N1	4 (50.0%)	4 (50.0%)		3 (37.5%)	5 (62.5%)	
M						
M0	7 (63.6%)	4 (36.4%)	0.329	4 (40.0%)	6 (60.0%)	0.337
M1	6 (46.2%)	7 (53.8%)		3 (23.1%)	10 (76.9%)	
Local recurrence						
Yes	1 (16.7%)	5 (83.3%)	0.070	2 (33.3%)	4 (66.7%)	0.622
No	9 (64.3%)	5 (35.7%)		5 (38.5%)	8 (61.5%)	
CD34						
Low count				5 (41.7%)	7 (58.3%)	0.222
High count				2 (18.2%)	9 (81.8%)	
HIF1-α						
Low count	5 (71.4%)	2 (28.6%)	0.240			
High count	7 (43.7%)	9 (56.3%)				

TNM classification, see Table 1. CD34 Chalkley count from 4 to 13 with cut off =7. Low CD34 vascularity group <7. High CD34 vascularity group ≥7. HIF1-α count from 1 to 6 with cut off =4. Low HIF1-α count group <25% nuclear stained cells. High HIF1-α count group ≥25% nuclear stained cells. P-value computed by log-rank test.

13 (median=7). We used the median value as a cut off for stratifying patients into low and high vascular groups. A tendency for positive association was observed between CD34 expression and T status (p=0.067) with lower Chalkley count in small tumors (T1-2) compared to larger and advanced tumors (T3-4). Fifty percent (5/10) of the patients with high vascularity had local recurrence, whereas only 10% (1/10) of the patients with low vascularity developed local recurrence (p=0.070) (Table 2). No association between CD34 Chalkley count and various parameters in patients with AC was seen (Table 3).

Figure 1 shows CD34 immunostaining.

HIF1-α expression and clinico-pathological parameters

High HIF1-α expression in SCC was significantly associated with the T3-4 group (p=0.02). No significant correlation between HIF1-α expression and sex, nodal status, metastasis, histological grading and local recurrence was detected (Table 2). No association was seen between HIF1-α expression and various parameters in ACs (Table 3).

No correlation was noted between HIF1-α and CD34 Chalkley count in either group (Tables 2,3).

We observed a significantly higher percentage of SCC with high HIF1-α expression compared to its expression in AC (p=0.005), but no association with the Chalkley count.

Immunostaining of HIF1-α is shown in figure 1.

CD34 Chalkley count, HIF1-α and survival

Survival analyses for CD34 Chalkley count and HIF1-α expression were performed separately for patients with SCC and AC.

In the SCC group, high CD34 Chalkley count and high HIF1-α expression implied a significantly reduced survival (p=0.003 and p=0.001, log rank test) (Fig. 2).

No significant association between CD34 and HIF1-α expressions and survival was found in the AC group (Fig. 2).

Discussion

Angiogenesis is a biologically complex process

Table 3. Clinico-pathological characteristics of patients with AC and their relation with CD34 and HIF1-α.

Adenocarcinoma	CD34			HIF1-α		
	Low	High	p-value	Low	High	p-value
All patients (n=28)	7 (25.0%)	21 (75.0%)		18 (64.3%)	10 (35.7%)	
Sex						
Female	2 (66.7%)	1 (33.3%)	0.556	-	-	0.286
Male	13 (52.0%)	12 (48.0%)		14 (73.7%)	5 (26.3%)	
Histological grading						
Well diff.	1 (100.0%)	-		-	-	
Moderately diff.	7 (58.3%)	5 (41.7%)	0.568	6 (60.0%)	4 (40.0%)	0.092
Poorly diff.	6 (42.9%)	8 (57.1%)		8 (100.0%)	-	
T						
T 1-2	5 (62.5%)	3 (37.5%)	0.686	4 (80.0%)	1 (20.0%)	0.603
T 3-4	10 (50.0%)	10 (50.0%)		10 (71.4%)	4 (28.6%)	
N						
N0	5 (55.6%)	4 (44.4%)	0.500	5 (71.4%)	2 (28.6%)	0.686
N1	8 (47.1%)	9 (52.9%)		7 (70.0%)	3 (30.0%)	
M						
M0	5 (45.5%)	6 (54.5%)	0.704	5 (71.4%)	2 (28.6%)	0.956
M1		9 (56.3%)	7(43.8%)		8 (72.7%)	
3(27.3%)						
Local recurrence						
Yes	2 (33.3%)	4 (66.7%)	0.635	5 (100.0%)	-	0.264
No	8 (53.3%)	7 (46.7%)		7 (70.0%)	3 (30.0%)	
CD34						
Low count				7 (70.0%)	3 (30.0%)	0.556
High count				7 (77.8%)	2 (22.2%)	
HIF1-α						
Low count	7 (50.0%)	7 (50.0%)	0.556			
High count	3 (60.0%)	2 (40.0%)				

TNM classification, see Table 1. CD34 Chalkley count from 4 to 13 with cut off =7. Low CD34 vascularity group <7. High CD34 vascularity group ≥7. HIF1-α count from 1 to 6 with cut off =4. Low HIF1-α count group <25% nuclear stained cells. High HIF1-α count group from ≥25% nuclear stained cells. P-value computed by log-rank test.

comprising basal membrane breakdown, endothelium cell proliferation and migration, tube formation and finally the initiation of neo blood flow (Folkman, 1986). Tumor vascularity has been measured as MVD or with the Chalkley method and has been shown to be of prognostic value (Weidner, 1998; Dhakal et al., 2009).

The HIF1-α protein is a key regulator of angiogenesis in many organs, including the gastrointestinal tract, especially under hypoxic stress (Battacharyya et al., 2010; Wang et al., 2011). HIF1-α is highly expressed in epithelial cells in esophageal erosions or ulcers, known to require angiogenesis for healing, and disappears once the lesions are healed (Baatar et al., 2002). In human tumors, over-expression of HIF1-α correlates with the activation of various pathways related to tumor invasion and metastasis (Begg et al., 2001; Semenza, 2011).

In our present study, we found high primary tumor vascularity associated with a shorter survival for the SCC patients. The 5-year survival for SCC patients was significantly reduced in the patient group with high Chalkley count compared to the low Chalkley count group (p=0.003, log-rank). However, no such association was seen in patients with ACs. A similar correlation of vascularity and survival in SCC has been reported previously (Tanigawa et al., 1997; Torres et al., 1999; Shih et al., 2000), strengthening the hypothesis that angiogenesis is essential in tumor progression. We further detected a significantly shorter survival among patients with SCC who had a high HIF1-α expression, but not in patients with AC. Larger and advanced SCCs had a significantly higher expression of HIF1-α. Also, a higher percentage of SCC patients with high vascularity had a high expression of HIF1-α compared to the patients with low vascularity in this group. Our results indicate an important role of the HIF1-α pathway: high

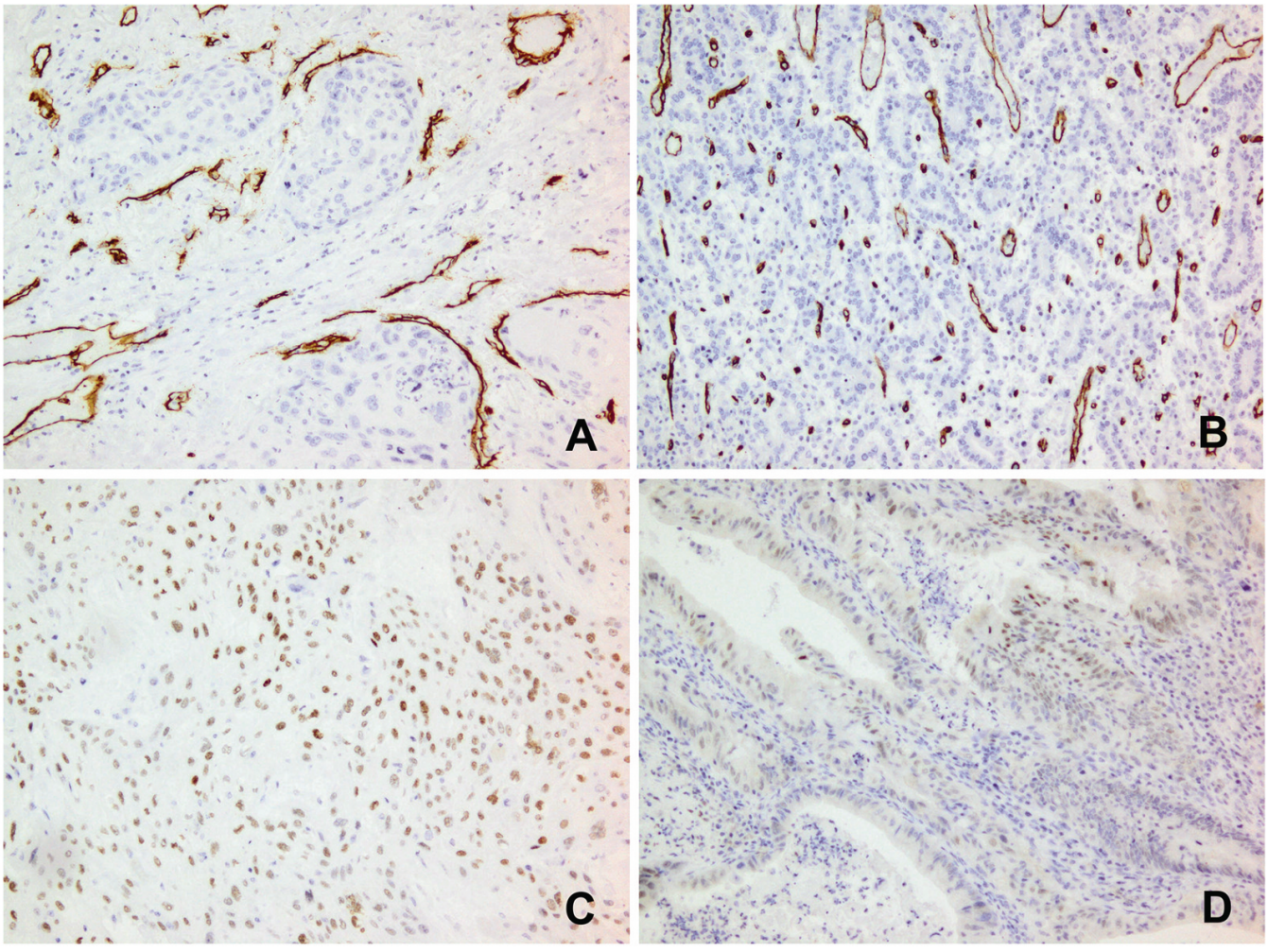

Fig. 1. Immunostaining for CD34 and HIF1-α in esophageal carcinomas. CD34 vascular staining in SCC **(A)** and in AC **(B).** HIF1-α staining in SCC cells **(C)** and in AC cells **(D).** x 100

vascularity of the primary tumor is associated with the HIF1-α pathway due to HIF1-α-dependent up-regulation of angiogenic factors. High metabolic demands during tumor progression with an increasing diffusion distance for oxygen, irregular development pattern of an adequate blood supply or even non-functional collapsed vasculature due to increased interstitial pressure and defective vascular maturation may explain the coexistence of hypoxia and high vascular density (Koukourakis et al., 2004).

We found that high expression of HIF1-α correlated with tumor invasion in SCCs. Locally advanced tumors

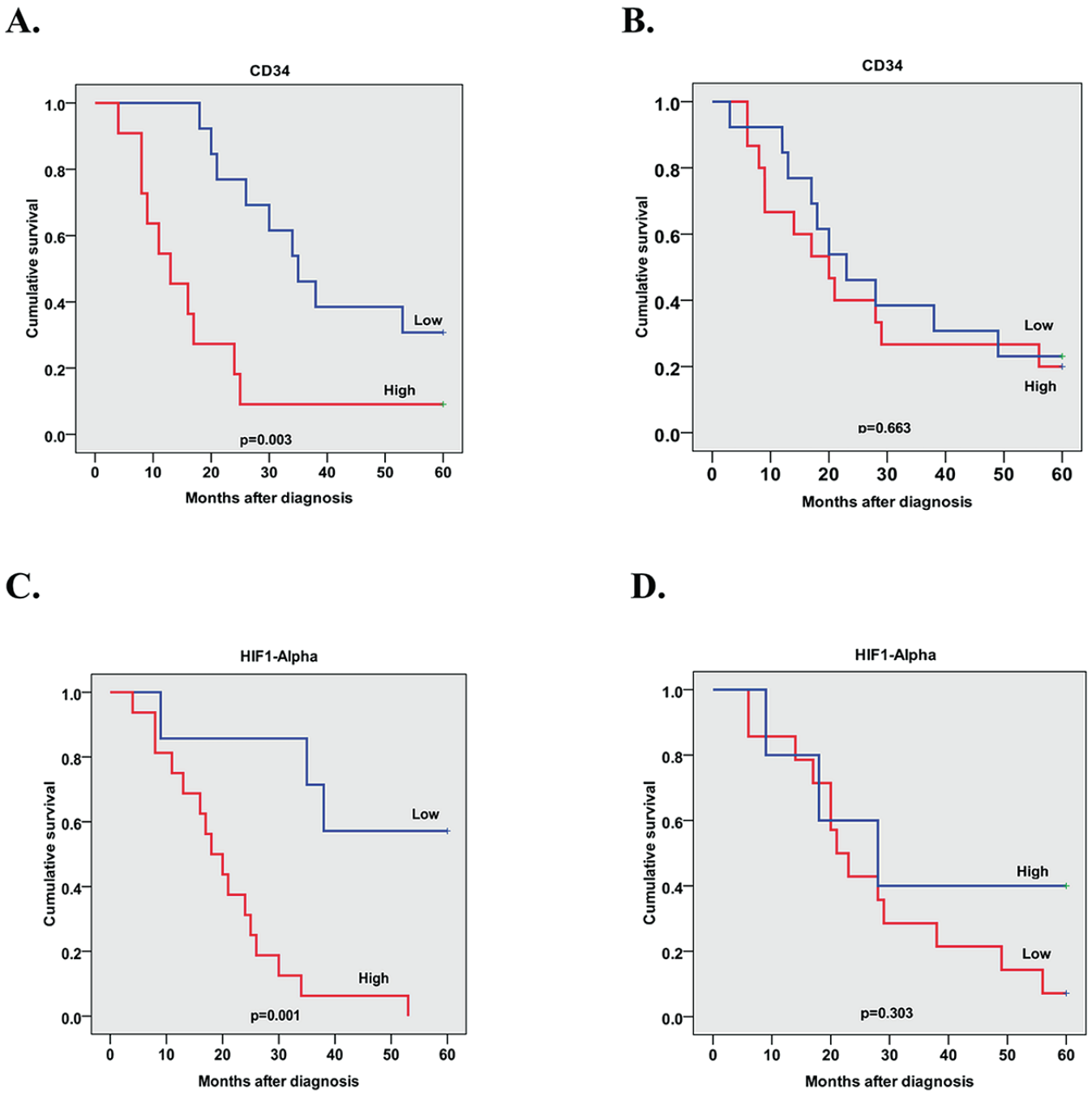

Fig. 2. Kaplan-Meier survival curves for patients with SCC (n=24) and AC (n=28) in low and high CD34 vascular groups (**A and B** respectively) and low and high HIF1-α expression groups (**C and D** respectively).

showed a higher HIF1-α expression. A high Chalkley count corresponding with larger SCCs was also found. We did not find such relations in the AC group. This difference could be explained by the disparate carcinoma histology, the dissimilar pattern of angiogenesis development in these tumors and the fact that high vascular density is not the only factor which can facilitate tumor progression (Chen and Kelly, 2003; Goscinski et al., 2008a,b). Also, the possibility of greater hypoxic stress in SCC compared to AC could explain the differences in outcome for these vascular markers (Lee et al., 2003).

In summary, we conclude that HIF1-α expression is different in two separate hypoxic tumor micro-environments: SCCs and ACs of the esophagus. High HIF1-α expression correlates with shorter survival among patients with SCC, but not in patients with AC. This suggests that different mechanisms may be involved in HIF1-α expression and activity between the two histological types of esophageal carcinoma.

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References

Baatar D., Jones M.K., Tsugawa K., Pai R., Moon W.S., Koh G.Y., Kim I., Kitano S. and Tarnawski A.S. (2002). Esophageal ulceration triggers expression of hypoxia-inducible factor-1 alpha and activates vascular endothelial growth factor gene: implications for angiogenesis and ulcer healing. Am. J. Pathol. 16, 1449-1457.

Balac I., Jurisic V., Laban A., Randelovic T., Knezevic P., Pantic I. and Dzodic R. (2012). The CD34-microvascular density in colorectal cancer patients. J. BUON 17, 97-101.

Begg A.C., Janssen H., Sprong D., Hofland I., Blommestijn G., Raleigh J.A., Varia M., Balm A., Van Velthuyzen L., Delaere P., Sciot R. and Haustermans K.M.G. (2001). Hypoxia and perfusion measurements in human tumors--initial experience with pimonidazole and IUdR. Acta Oncol. 40, 924-928.

Bhattacharyya A., Chattopadhyay R., Hall E.H., Mebrahtu S.T., Ernst P.B. and Crowe S.E. (2010). Mechanism of hypoxia-inducible factor 1 alpha-mediated Mcl1 regulation in Helicobacter pylori-infected human gastric epithelium. Am. J. Physiol. Gastrointest. Liver Physiol. 299, 1177-1186.

Chen W.T. and Kelly T. (2003). Seprase complexes in cellular invasiveness. Cancer Metastasis Rev. 22, 259-269.

Dhakal H.P., Naume B., Synnestvedt M., Borgen E., Kaaresen R., Schlichting E., Wiedswang G., Bassarova A., Giercksky K.E. and Nesland J.M. (2008). Vascularization in primary breast carcinomas: its prognostic significance and relationship with tumor cell dissemination. Clin. Cancer Res. 148, 2341-2350.

Dhakal H.P., Bassarova A., Naume B., Synnestvedt M., Borgen E., Kaaresen R., Schlichting E., Wiedswang G., Giercksky K.E. and Nesland J.M. (2009). Breast carcinoma vascularity: a comparison of manual microvessel count and Chalkley count. Histol. Histopathol. 24, 1049-1059.

El Gehani K., Al-Kikhia L., Mansuri N., Syrjänen K., Al-Fituri O. and Elzagheid A. (2011). Angiogenesis in urinary bladder carcinoma as defined by microvessel density (MVD) after immunohistochemical staining for Factor VIII and CD31. Libyan J. Med. 10, 3402.

Enzinger P.C. and Mayer R.J. (2003). Esophageal cancer. N. Engl. J. Med. 349, 2241-2252.

Fidler I.J. and Ellis L.M. (1994). The implications of angiogenesis for the biology and therapy of cancer metastasis. Cell 79, 185-188.

Folkman J. (1986). How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture. Cancer Res. 46, 467-473.

Fukuda R., Hirota K., Fan F., Jung Y.D., Ellis L.M. and Semenza G.L. (2002). Insulin-like growth factor 1 induces hypoxia-inducible factor 1-mediated vascular endothelial growth factor expression, which is dependent on MAP kinase and phosphatidylinositol 3-kinase signaling in colon cancer cells. J. Biol. Chem. 277, 38205-38211.

Fukuda R., Kelly B. and Semenza G.L. (2003). Vascular endothelial growth factor gene expression in colon cancer cells exposed to prostaglandin E2 is mediated by hypoxia-inducible factor 1. Cancer Res. 63, 2330-2334.

Goscinski M.A., Suo Z.H., Nesland J.M., Chen W.T., Zakrzewska M., Wang J., Zhang S., Flørenes V.A. and Giercksky K.E. (2008a). Seprase, dipeptidyl peptidase IV and urokinase-type plasminogen activator expression in dysplasia and invasive squamous cell carcinoma of the esophagus. A study of 229 cases from Anyang Tumor Hospital, Henan Province, China. Oncology 75, 49-59.

Goscinski M.A., Suo Z.H., Nesland J.M., Flørenes V.A. and Giercksky K.E. (2008b). Dipeptidyl peptidase IV expression in cancer and stromal cells of human esophageal squamous cell carcinomas, adenocarcinomas and squamous cell carcinoma cell lines. APMIS 116, 823-831.

Goscinski M.A., Larsen S.G., Warloe T., Stoldt S., Nesland J.M., Suo Z.H. and Giercksky K.E. (2009). Adenocarcinomas on the rise - does it influence survival from oesophageal cancer? Scand. J. Surg. 98, 214-220.

Hansen S., Grabau D.A., Sørensen F.B., Bak M., Vach W. and Rose C. (2000). The prognostic value of angiogenesis by Chalkley counting in a confirmatory study design on 836 breast cancer patients. Clin. Cancer Res. 6, 139-146.

Hendriksen E.M., Span P.N., Schuurig J., Peters J.P.W., Sweep F.C.G.J., van der Kogel A.J. and Bussink J. (2009). Angiogenesis, hypoxia and VEGF expression during tumour growth in a human xenograft tumour model. Microvasc. Res. 77, 96-103.

Koshy M., Esiashvilli N., Landry J.C., Thomas C.R. Jr and Matthews R.H. (2004). Multiple management modalities in esophageal cancer: combined modality management approaches. Oncologist 9, 147-159.

Koukourakis M.I., Giatromanolaki A., Sivridis E., Simopoulos C., Turley H., Talks K., Gatter K.C. and Harris A.L. (2002). Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis and chemoradiotherapy outcome of squamous cell head-and-neck cancer. Int. J. Radiat. Oncol. Biol. Phys. 53, 1192-1202.

Koukourakis M.I., Giatromanolaki A., Sivridis E., Pastorek J., Karapantzos I., Gatter K.C. and Harris A.L. (2004). Tumour and Angiogenesis Research Group: Hypoxia-activated tumor pathways of angiogenesis and pH regulation independent of anemia in head-and-neck cancer. Int. J. Radiat. Oncol. Biol. Phys. 59, 67-71.

Krock B.L., Skuli N. and Simon M.C. (2011). Hypoxia-induced angiogenesis: good and evil. Genes Cancer 2, 1117-1133.

Lee C.H., Lee M.K., Kang C.D., Kim Y.D., Park D.Y., Kim J.Y., Sol M.Y. and Suh K.S. (2003). Differential expression of hypoxia inducible factor-1 alpha and tumor cell proliferation between squamous cell carcinomas and adenocarcinomas among operable non-small cell lung carcinomas. J. Korean Med. Sci. 18, 196-203.

Liang J.T., Huang K.C., Jeng Y.M., Lee P.H., Lai H.S. and Hsu H.C. (2004) Microvessel density, cyclo-oxygenase 2 expression, K-ras mutation and p53 overexpression in colonic cancer. Br. J. Surg. 91, 355-361.

Moriyama N., Amano Y., Mishima Y., Okita K., Takahashi Y., Yuki T., Ishimura N., Ishihara S. and Kinoshita Y. (2008). What is the clinical significance of stromal angiogenesis in Barrett's esophagus? J. Gastroenterol. Hepatol. 23, 210-215.

Osinsky S., Bubnovskaya L., Ganusevich I., Kovelskaya A., Gumenyuk L., Olijnichenko G. and Merentsev S. (2011). Hypoxia, tumour-associated macrophages, microvessel density, VEGF and matrix metalloproteinases in human gastric cancer: interaction and impact on survival. Clin. Transl. Oncol. 13, 133-138.

Parkin D.M., Bray F., Ferlay J. and Pisani P. (2005). Global cancer statistics, 2002. CA Cancer J. Clin. 55, 74-108.

Semenza G.L. (2011). Hypoxia. Cross talk between oxygen sensing and the cell cycle machinery. Am. J. Physiol. Cell Physiol. 550-552.

Semenza G.L. and Wang G.L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol. Cell Biol. 12, 5447-5454.

Shih C.H., Ozawa S., Ando N., Ueda M. and Kitajima M. (2000). Vascular endothelial growth factor expression predicts outcome and lymph node metastasis in squamous cell carcinoma of the esophagus. Clin. Cancer Res. 1161-1168.

Tanigawa N., Matsumura M., Amaya H., Kitaoka A., Shimomatsuya T., Lu C., Muraoka R. and Tanaka T. (1997). Tumor vascularity correlates with the prognosis of patients with esophageal squamous cell carcinoma. Cancer 79, 220-225.

Torres C., Wang H., Turner J., Shahsafaei A. and Odze R.D. (1999). Prognostic significance and effect of chemoradiotherapy on microvessel density (angiogenesis) in esophageal Barrett's esophagus-associated adenocarcinoma and squamous cell carcinoma. Hum. Pathol. 30, 753-758.

UICC (2005). TNM Atlas: An illustrated guide to the TNM/pTNM classification of malignant tumors. Digestive system tumors, 5th ed. Springer. Heidelberg. pp 74-83.

Vermeulen P.B., Gasparini G., Fox S.B., Colpaert C., Marson L.P., Gion M., Beliën J.A., de Waal R.M., Van Marck E., Magnani E., Weidner N., Harris A.L. and Dirix L.Y. (2002). Second international consensus on the methodology and criteria of evaluation of angiogenesis quantification in solid human tumours. Eur. J. Cancer 38, 1564-1579.

Wahl P., Schmidt A., Demarees M., Achtzehn S., Bloch W. and Mester J. (2013). Responses of angiogenic growth factors to exercise, to hypoxia and to exercise under hypoxic conditions. Int. J. Sports Med. 34, 95-100.

Wang T., Leng Y.F., Zhang Y., Xue X., Kang Y.Q. and Zhang Y. (2011). Oxidative stress and hypoxia-induced factor 1· expression in gastric ischemia. World J. Gastroenterol. 17, 1915-1922.

Weidner N. (1995). Current pathologic methods for measuring intratumoral microvessel density within breast carcinoma and other solid tumors. Breast Cancer Res. Treat. 36, 169-180.

Weidner N. (1998). Tumoral vascularity as a prognostic factor in cancer patients: the evidence continues to grow. J. Pathol. 184, 119-122.

WHO (1990). Histological typing of esophageal and gastric tumors. Geneva. pp 20-26.

Zhong H., De Marzo A.M., Laughner E., Lim M., Hilton D.A., Zagzag D., Buechler P., Isaacs W.B., Semenza G.L. and Simons J.W. (1999). Overexpression of hypoxia-inducible factor 1 alpha in common human cancers and their metastases. Cancer Res. 59, 5830-5835.

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