Original paper

HIF-1α expression is inversely associated with tumor stage, grade and microvessel density in urothelial bladder carcinoma

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Urothelial bladder carcinoma (UBC) is the most common urinary tract malignancy. The most important histopathological factors affecting prognosis are cancer stage and grade. Studies show that microvessel density (MVD) reflecting angiogenesis is also associated with clinicopathological features and affects the outcome in UBC. One of the most important regulators of angiogenesis is hypoxia inducible factor 1 (HIF-1). Previous reports describing expression of the HIF-1α subunit in UBC showed unclear and inconsistent results. Our study attempted do evaluate the association between HIF-1α expression and tumor stage, grade, lymph nodes status and MVD in UBC. We performed immunohistochemical staining in 99 UBC cases, including 38 non-muscle invasive (NMIBC) and 61 muscle invasive tumors (MIBC). We observed inverse relationships between HIF-1α immunoreactivity score (IRS) and tumor stage, grade and MVD. Significantly lower HIF-1α IRS values were observed in MIBC and high grade cancers. We found a significant negative correlation between HIF-1α IRS and MVD. These results suggest that HIF-1α pathway is not involved in UBC growth and progression, and that angiogenesis in high grade MIBC is not regulated by HIF-1. Our findings contradict previous reports regarding HIF-1α, MVD and UBC which shows the necessity of additional molecular studies in this field.

Key words: angiogenesis, hypoxia-inducible factor 1, microvessel density, urothelial bladder cancer.

Introduction

Bladder cancer is the most common urinary tract malignancy and fourth most common cancer in men in developed countries. It is estimated that in 2012 more than 430.000 new cases were diagnosed worldwide, and approx. 165.000 deaths caused by bladder cancer were reported. In North America and Europe more than 90% of bladder cancers are urothelial bladder carcinomas (UBC) [1]. The most important histopathological factors affecting prognosis are cancer stage, grade, presence of vascular invasion and particular histologic variants [2]. The depth of tumor invasion (pT stage), especially the presence of the muscularis propria invasion, is of fundamental significance since it directly influences patient management [3]. An additional parameter that can be assessed in histopathological evaluation of UBC specimens is microvessel density (MVD). MVD reflects the process of angiogenesis – the formation of new capillaries from pre-existing blood vessels and epithelial progenitor cells. In several malignancies MVD value correlates with cancer stage and affects patient prognosis [4, 5, 6, 7, 8, 9, 10]. Most commonly MVD is measured as a mean number of small blood vessels counted in randomly chosen areas or in hot-spots (selected areas with the richest vascularization). The lack of unified criteria regarding the choice

of endothelial marker (CD31, CD34 or CD105) and selection of area for analysis resulted in limited use of MVD in routine histopathological evaluation, despite a large number of studies confirming the usefulness of MVD in cancer diagnosis [11, 12].

Angiogenesis, or neovascularization, is an essential part of many physiological processes, but it is also of crucial importance for cancer growth and survival of neoplastic cells [13]. The factor that is believed to have the biggest impact on angiogenesis is hypoxia, a common feature of many solid tumors where neoplastic cells proliferate rapidly [14]. Hypoxia leads to activation of hypoxia inducible factors (mainly HIF-1 and HIF-2) which in turn cause the up-regulation of numerous proangiogenic factors as well as suppression of antiangiogenic factors [15].

HIFs are a family of three transcription factors (HIF-1, -2 and -3) among which HIF-1 is best studied and described. HIF-1 is a heterodimeric transcription factor composed of two subunits: HIF-1α and HIF-1β which binds with its corresponding DNA sequences named HRE (hypoxia response element) [16]. HIF-1β expression is continuous and remains at steady levels, while in normoxic conditions the HIF-1α subunit undergoes hydroxylation, ubiquitination and subsequent degradation in proteasomes [17]. However, when oxygen concentration drops HIF-1α cannot be hydroxylated, thus preventing its degradation and resulting in HIF-1α accumulation. Additionally HIF-1α expression is affected by activation of Hsp90, loss of function of p53 or VHL proteins and activation of growth factor signaling pathways such as PI3K/Akt/mTOR and RAS/RAD/MEK/ERK [18]. In hypoxic cells HIF-1 regulates the expression of several proangiogenic factors, including vascular endothelial growth factor (VEGF), transforming growth factors (TGFs), angiopoietin 1 and 2 (Ang-1 and -2), platelet-derived growth factor (PDGF) and placental growth factor (PGF), as well as expression of enzymes such as matrix metalloproteinases (MMPs) [15, 18].

Overexpression of HIF-1 was described in several types of cancers including lung, breast, ovary, prostate, kidney and colon cancer [19]. It was demonstrated that high expression of HIF-1α was associated with poor prognosis in breast and cervical cancer, and with worse response to treatment in esophageal and head and neck cancers [20, 21]. However, many studies evaluating the relationship between HIF-1α expression and histopathological parameters such as tumor stage and grade showed contradicting results [19].

In studies evaluating the expression of HIF-1α in human UBC tissues, HIF-1a overexpression was seen in urothelial neoplastic cells and was strongly associated with high tumor MVD and VEGF expression in cancer cells [22, 23, 24]. On the other hand, the reports describing relationships between HIF-1α expression, tumor stage and grade in UBC are often conflicting. Reports published by Chai *et al.* and Deniz *et al.* showed that high HIF-1α expression correlates with tumor size, depth of invasion, high tumor grade and high risk of recurrence [24, 25]. Ioachim *et al*. and Theodoropoulos *et al*. in their papers reported that high HIF-1α expression in UBC was associated with poor prognosis [22, 23, 26]. However, at the same time they did not show any relationship between HIF-1α overexpression and tumor stage and the association with cancer grade had only borderline statistical significance [22, 23, 26].

These findings indicate that the precise role of HIF-1 in angiogenesis and UBC pathogenesis is still not fully understood. Taking into consideration the discrepancies between different reports on HIF-1α expression in UBC, a question arises: what is the impact of HIF-1 on the process of neovascularization, acquisition of malignant histological features, growth and progression of UBC? The goal of this study is to answer that query by (1) investigating the relationship between tumor microvessel density (MVD) tumor pT stage, grade and status of regional lymph nodes (pN), (2) evaluating the presence and distribution of HIF1-α protein in UBC cells in both non-muscle invasive cancer (NMIBC) and muscle invasive cancer (MIBC), (3) investigating the association between the HIF-1α expression and tumor pT stage, grade, lymph node status and MVD.

Material and methods

Patients

Our study was conducted on histopathological samples obtained from 99 patients diagnosed with UBC between 2005 and 2010. Samples were stored in the archives of our Department. The study design was reviewed and approved by the Local Ethical Committee. The specimens represented 19 cases of pTa tumors, 19 cases of pT1 UBC, 22 cases of pT2 UBC, 20 cases of pT3 and 19 cases of pT4 UBC. Samples of cancers staged pTa and pT1 were obtained from transurethral resection of the tumor (TURT) and were classified as NMIBC tumors (38 cases total, 38.4%). If the patient underwent more than one TURT procedure, we evaluated the specimen obtained during the first biopsy. Samples of more advanced cancers (in stage pT2-pT4) were taken from radical cystectomy specimens and were classified as MIBC (61 cases total, 61.6%). All tumors included in the study presented conventional UBC histology. In cases where tumors had divergent differentiation (squamous or glandular) only the areas of conventional urothelial

morphology were analyzed. UBCs with uncommon morphological variants, tumors with extensive necrosis and TURT samples with widespread coagulation artifact or lacking the muscularis propria were excluded from the study.

24 tumors (24.2%) were diagnosed as low grade as they fulfilled the criteria of low-grade non-invasive papillary urothelial carcinoma according to the 2016 WHO classification. 75 tumors were high grade (75.8%) as they fulfilled the criteria of either high-grade non-invasive papillary urothelial carcinoma or high-grade infiltrating urothelial carcinoma. Among 38 cases of NMIBC 24 tumors were described as low grade (63.2%) and 14 cases as high grade (36.8%). In all cases of MIBCs the tumors were high grade. The status of regional lymph nodes was evaluated only in cases staged pT2-pT4 in which radical cystectomy with lymphadenectomy was performed (48 cases). Among these cases assessment of regional lymph nodes in 23 patients demonstrated a presence of metastases (pN+, 47.9%). The mean patient's age was 68.7 SD ±8.8 years and ranged from 48 to 86 years. 74 patients were male (74.7%) and 25 were females (25.3%).

Immunohistochemistry

All histopathological specimens were fixed in 10% buffered formalin and embedded in paraffin blocks. Immunohistochemical assessment of HIF-1α expression was carried out on 3-μm tissue sections using anti-HIF-1α mouse monoclonal antibody (H1α67, Thermo Fisher Scientific, Rockford, IL USA). Sections were deparrafinized in xylene and rehydrated in graded ethanol solutions. Heat-induced antigen retrieval was performed with 0.01 M citrate buffer (pH 6.0) in a 700W microwave (2 × 5 min, with 5-minute pause). Endogenous peroxidase activity was blocked by incubating slides in 3% hydrogen peroxide for 5 minutes. After washing with PBS, slides with sections were incubated with 2.5% horse serum for 20 minutes to block the non-specific antigen binding sites. Then sections were incubated for 45 minutes at room temperature with primary HIF-1α antibody (1 : 1000, 200 μl/slide). After washing the slides with PBS, ImmPRESS HRP Anti-Mouse Polymer Detection Kit (Vector Labs, Burlingame, Ca USA) was applied for 30 minutes. The peroxidase reaction was visualized using ImmPACT DAB Peroxidase Substrate (Vector Labs) using 1 droplet of DAB concentrate per 1ml of diluent. Sections were then washed with water, counterstained with Mayer's hematoxylin, dehydrated in graded ethanol solutions, cleared in xylen and coverslipped using mounting solution. As a positive control for HIF-1α antibody we used samples of normal human kidney as suggested by the manufacturer and we observed expression of HIF-1α in nuclei of a number of renal tubular epithelial cells. A negative control with UBC section with known HIF-1α expression processed without the primary antibody was set up for every batch of slides to exclude nonspecific binding of the secondary antibody. (Fig. 1).

In our study HIF-1α expression presented a nuclear pattern of distribution in both positive control (human kidney) and UBC cells. Nuclear immunoreactivity was also observed in a number of normal urothelium cells and stromal cells seen in UBC specimens.

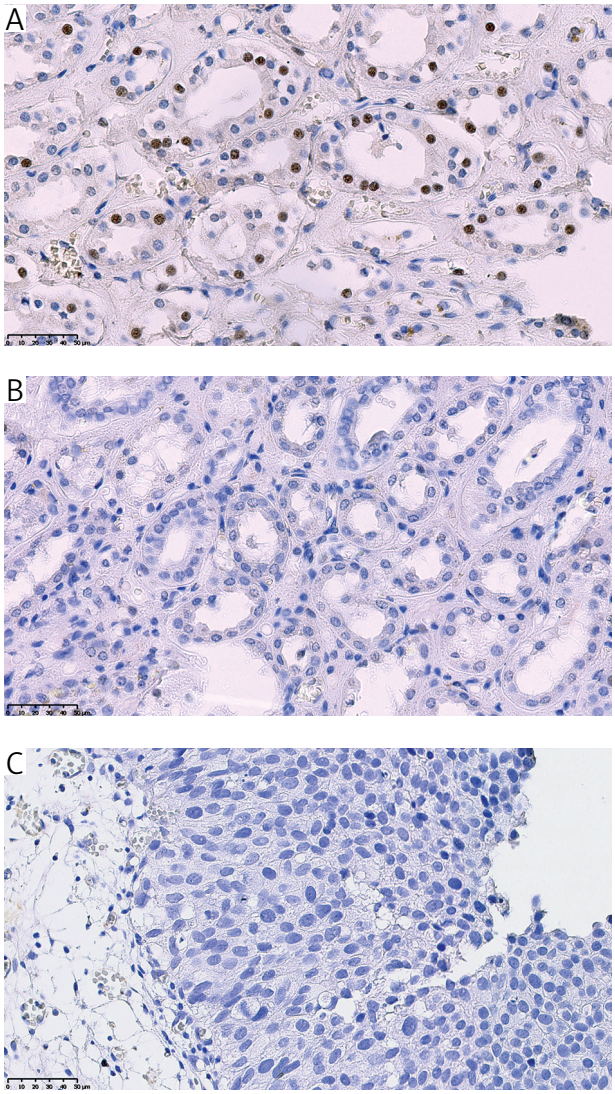

Fig. 1A-C. Examples of positive and negative controls of HIF-1α immunoreactivity. A) Normal human kidney with visible nuclear HIF-1α immunoreactivity in a number of tubular epithelial cells (positive control). B) Normal human kidney with no HIF-1α immunoreactivity in tubular epithelial cells (negative control, section processed without the primary antibody). C) NMIBC with no HIF-1α immunoreactivity in cancer cells (negative control, section processed without the primary antibody). Magnification in photomicrographs: 400×, scale bars – 50 μm

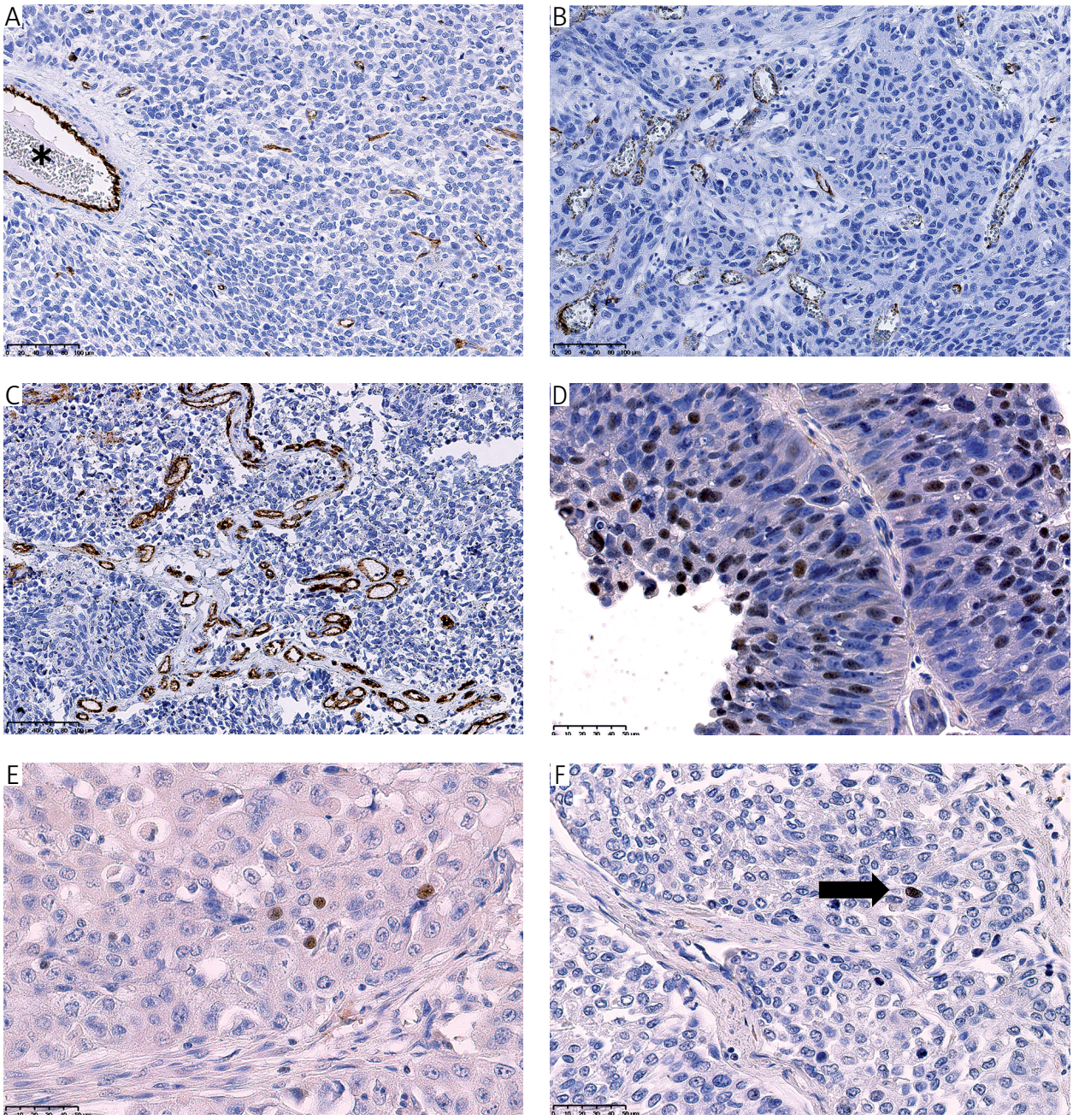

Fig. 2A-F. Examples of CD31 and HIF-1α immunoreactivity in UBC. A) UBC with low MVD, only a few microvessels stained with CD31 are visible; large vessel with a muscular wall is visible on the left (marked with asterix) and serves as internal positive control. B) UBC with moderate MVD. C) UBC with high MVD and large number of microvessels stained with CD31. D) High grade NMIBC with HIF-1α immunoreactivity visible in 11-50% of cancer cells (HIF-1α IRS = 3). E) MIBC with HIF-1α immunoreactivity visible in 1-10% of cancer cells (HIF-1α IRS = 2). F) MIBC with HIF-1α immunoreactivity visible in only one cell (marked with arrow) (HIF-1α IRS = 1). Magnification in photomicrographs A-C: 200×, scale bars – 100 μm; magnification in photomicrographs D-F: 400×, scale bars – 50 μm

Immunoreactivity scoring

Immunostained slides were digitized using Hamamatsu NanoZoomer 2.0-HT scanner and evaluated using NDP.view2 software (Hamamatsu). The expression of HuR in cancer cells was assessed with semiquantitative immunoreactivity scoring system (IRS) as described previously by Wan-Tzu Chen *et al*. and Chee-Yin Chai *et al*. [25, 27]. Slides were initially evaluated at low magnification (40-100×) in search of areas with the strongest immunoreactivity (hot-spots). According to the mean percentage of positive cells found in three high-power hot-spots (200×) the nuclear expression of HIF-1α was scored: IRS = 0 (no positive cells), IRS = 1 (< 1% positive cells), IRS = 2 (1-10% positive cells), IRS = 3

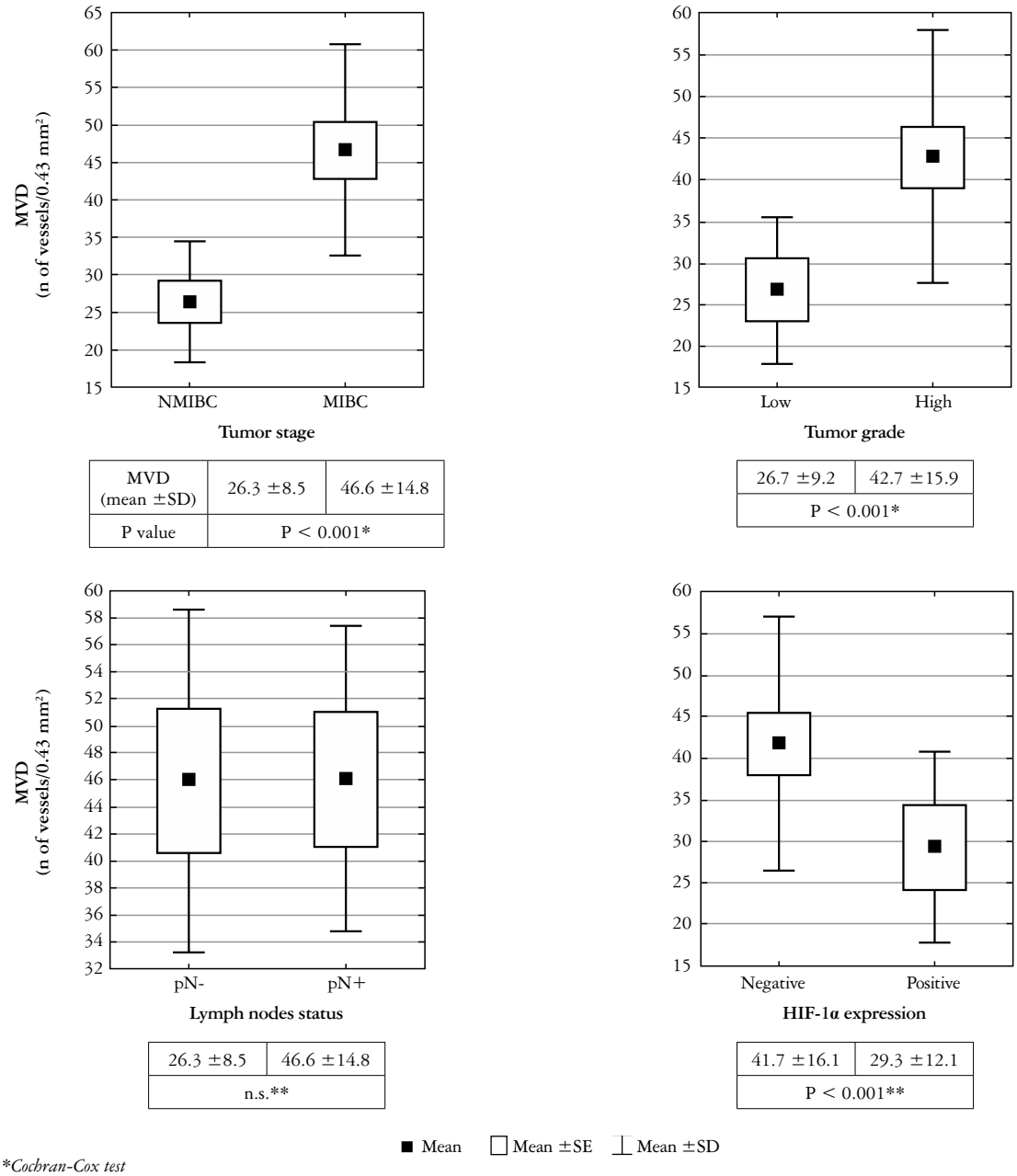

Fig. 3. Comparison of mean MVD values in groups classified according to clinicopathological data (tumor stage, grade, lymph nodes status) and HIF-1α expression. MVD is significantly higher in groups of MIBC and high grade UBCs as well as in tumors with negative HIF-1α expression. Regional lymph nodes status was not associated with MVD.

(11-50% positive cells) and IRS = 4 (> 50% positive cells). For statistical analysis, we categorized the final immunoreactivity score (HIF-1α IRS) as negative (IRS 0-2) or positive (IRS 3-4).

Microvessel density evaluation

To evaluate tumor microvessel density CD31 (platelet/endothelial cell adhesion molecule-1) immunostaining was performed. CD31 is a pan-endothelial marker and is considered to be a marker of choice for paraffin sections [12]. Staining was done using automatic stainer (BOND-MAX, Leica Biosystems, Nussloch, Germany) and a set of dedicated IHC reagents as specified in the manufacturer's instructions and protocols. In brief: after deparaffinization, antigen-retrieval procedure and blocking of endogenous peroxidase activity, 3-μm sections were incubated for 15 minutes with Ready-to-Use Primary CD31 antibody (Leica Biosystems). Subsequently, tissues were incubated with HRP-polymer secondary antibody for 8 minutes, DAB

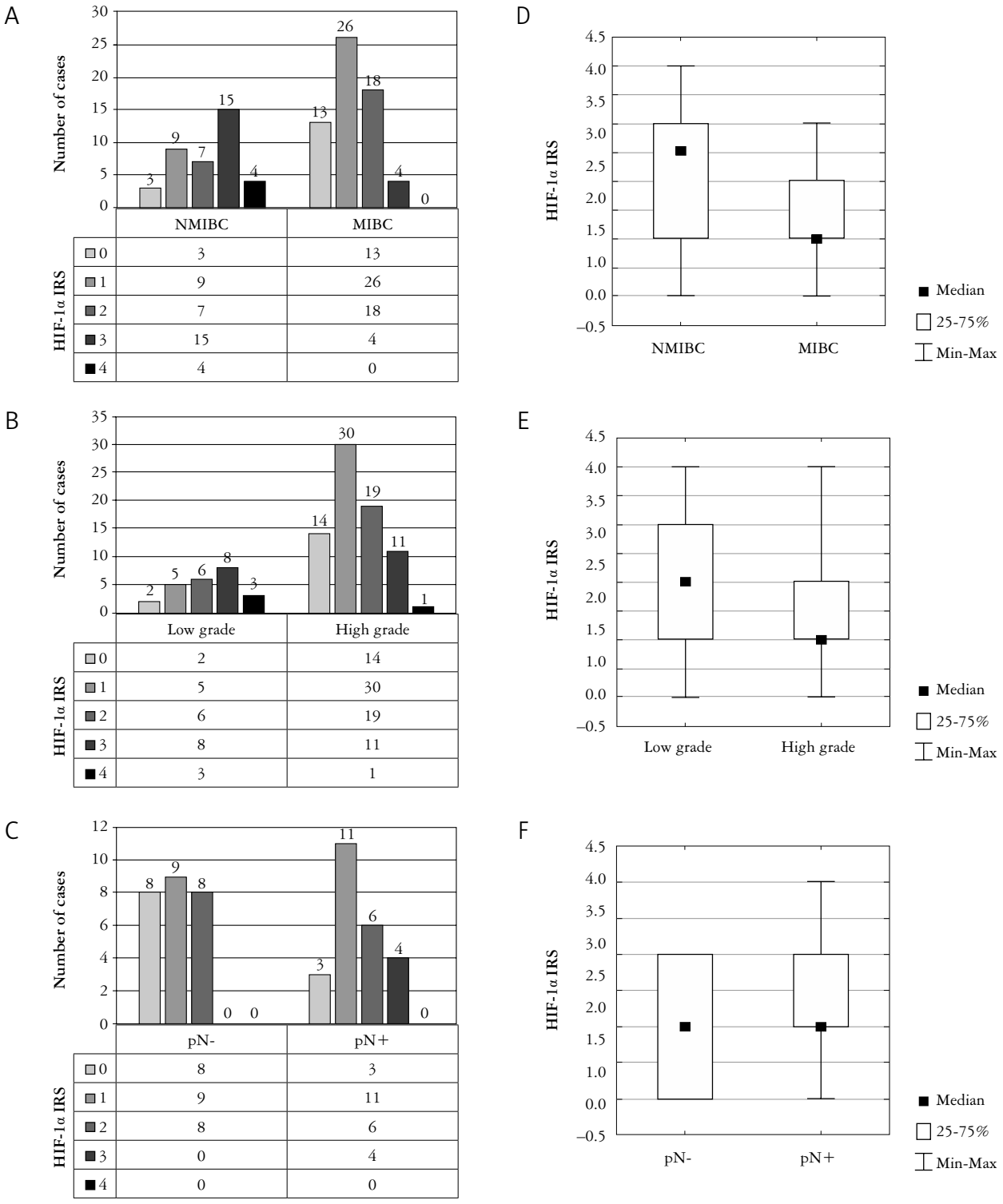

Fig. 4A-F. Distribution of HIF-1α IRS values and their corresponding medians in groups classified according to clinicopathological data (tumor stage, grade, lymph nodes status). A) Comparison of distribution of HIF-1α IRS values in NMIBC and MIBC cases. MIBC cases showed predominantly low HIF-1α IRS values (0-2, classified as negative reaction) in contrast to NMIBC cases in which the most frequent score was 3 (positive reaction). B) Comparison of distribution of HIF-1α IRS values in low and high grade UBCs. In high grade tumors HIF-1α IRS values were predominantly low (0-2) in contrast to high grade tumors in which the most frequent score was 3 (positive reaction). C) Comparison of distribution of HIF-1α IRS values in cases without regional lymph nodes involvement (pN–) and with lymph nodes metastases (pN+). In both groups HIF-1α IRS values were low. D) Differences in median HIF-1α IRS values in NMIBC and MIBC cases. Cancers without invasion of the muscularis propria showed significantly higher HIF-1α IRS median than MIBCs (2.5 vs. 1, $p < 0.001$). E) Differences in median HIF-1α IRS values in low and high grade tumors. Low grade UBCs presented significantly higher HIF-1α IRS median than high grade tumors (2 vs. 1, $p < 0.001$). F) We observed no differences in median HIF-1α IRS values in pN– and pN+ cases (1 vs. 1, $p > 0.05$)

chromogen for 10 minutes and counterstained with Mayer's hematoxylin. To measure tumor MVD we used the vascular hot-spot technique described by Weidner *et al*. and modified by other authors [6, 11, 12, 28]. At first we identified three areas of highest vascular density within the tumor at low magnification (40×), and then evaluated each hot-spot at high magnification (200×). Each evaluated visual field covered 0.43 mm^2. Any brown stained endothelial cell that was clearly separate from adjacent blood vessels, tumor cells and connective tissue elements was considered to be a single, countable vessel (Fig. 2). Vessels with a muscular wall and areas with extensive necrosis, inflammatory infiltrate or fibrosis were not included in the vessel count. The final MVD score was the average of the microvessels counts obtained from all three hot-spots and given as continuous variable (mean number of vessels/0.43 mm^2).

Statistical analysis

Mann-Whitney U Test was performed to compare the median levels of ordinal values (HIF-1α IRS) in groups selected according to their histopathological parameters (NMIBC vs MIBC, LG vs HG, pN- vs. pN+). Student's t-test or Cochran-Cox test were performed for comparison of the mean values of continuous variables (MVD). Spearmann's rank correlation coefficient was used to analyze the correlation between HIF-1α IRS and MVD. For all tests the significance threshold was set at $p \leq 0.05$. All analyses were performed using STATISTICA 13 (Dell Software).

Results

Mean MVD value in the analyzed cases was 38.8 SD ±16.2 vessels/0.43 mm^2 and ranged from 14.3 to 92. Microvessel density was shown to be significantly higher in MIBC and high grade tumors in comparison to NMIBC and low grade tumors (46.6 ±14.8 SD vs 26.3 ±8.5 SD Cochran-Cox $t = 8.63$, $p = 0.0000$ and 42.7 ±15.9 SD vs. 26.7 ±9.2 SD Cochran-Cox $t = 6.07$, $p = 0.0000$ respectively). We observed no differences in the MVD in patients with (pN+) and without lymph nodes metastases (pN–) (46.1 ±11.9 SD vs. 45.9 ±13.9 SD, Student's $t = -0.04$, $p = 0.9661$) (Fig. 3).

As mentioned earlier UBC cells showed nuclear HIF-1α immunoreactivity only (Fig. 2). The staining pattern was diffuse and we observed no association between immunoreactivity pattern and the presence of tumor necrosis, inflammation or fibrosis. Positive staining (HIF-1α IRS 3 or 4) was observed in 76 cases (76.8%) and weak or negative staining (HIF-1α IRS 0 to 2) in 23 cases (23.2%). There were significant differences between HIF-1α IRS values in NMIBC vs MIBC cases and in low grade vs high grade cases. Median HIF-1α IRS in NMIBC group was significantly higher than in MIBC group (2.5 vs. 1, Mann-Whitney $U = 599.5$, $p = 0.0001$) and was significantly higher in low grade tumors when compared to high grade tumors (2 vs. 1, Mann-Whitney $U = 548.5$, $p = 0.0042$). No association between HIF-1α expression and regional lymph nodes status was found (1 vs. 1, Mann-Whitney $U = 203.5$, $p = 0.084$; Fig. 4).

A moderate negative correlation between HIF-1α IRS and MVD was demonstrated ($R = -0.39$, $p = 0.0001$). HIF-1α-positive tumors had significantly lower MVD values than tumors showing no HIF-1α immunoreactivity (29.3 SD ±12.1 vs. 41.7 SD ±16.1, Student's $t = 3.41$, $p = 0.0009$).

Discussion

The formation of new blood vessels is essential in the process of cancer growth and progression. The relationship between tumor microvessel density and tumor stage and grade was described in several types of cancer [4, 5, 6, 7, 8]. In UBC high MVD correlates with tumor stage, grade, recurrence rate, risk of progression, risk of distant metastases and poor overall survival [29, 30, 31, 32].

Our study demonstrates that in UBC angiogenesis is associated with tumor growth and invasion of muscularis propria. We observed that high microvessel density is a characteristic feature of advanced, muscle invasive and high-grade cancers in comparison to NMIBC and low grade tumors. This is in concordance with previous studies evaluating MVD in UBC [33]. We did not observe any association between tumor vascularization and the presence of metastases in regional lymph nodes. One of the reasons for that may be the lack of data regarding lymph nodes involvement in a number of patients that underwent radical cystectomy, which resulted in relatively smaller group of patients in whom MVD and pN status could be evaluated.

The exact mechanisms behind neovascularization in neoplasms are still being investigated. One of the best-known events in the initial phase of angiogenesis is the activation of HIF-1 by hypoxic conditions in the growing tumor. The extensive IHC studies in multiple human neoplasms have shown that the HIF-1 pathway is a major contributor to tumor growth and progression in several malignancies, however its exact role in cancer may be different in different stages of tumor development and among tumor subtypes [34]. HIF-1α expression was demonstrated to correlate with high tumor stage, grade and rich vascularization in breast cancer, colon cancer, pancreatic cancer and hepatocellular

carcinoma [34-39]. At the same time studies evaluating HIF-1α immunoreactivity in lung cancer, renal cell carcinoma, gastric cancer and oral squamous cell carcinoma showed no association between HIF-1α expression and clinicopathological factors or the results were contradictory [34, 40, 41, 42, 43, 44, 45, 46]. The reasons behind those differences are still unknown. It is proposed that some tumors do not require HIF-1 associated signal transduction to acquire malignant phenotype and that other hypoxic-independent pathways are involved in their pathogenesis and neovascularization.

As mentioned previously, reports on association between HIF-1α expression in UBC and clinicopathological factors such as tumor stage, grade and course of the disease are equivocal [22, 23, 24, 25, 26, 27]. A study conducted by Chai *et al*. showed that HIF-1α overexpression correlated with tumor size, depth of invasion, high tumor grade and high risk of recurrence [25]. Similar results were published by Deniz *et al*. who demonstrated a significant association between HIF-1α expression and high cancer stage, grade, high MVD and high mitotic activity of cancer cells [24]. In studies performed by Ioachim *et al.* and Theodoropoulos *et al*. HIF-1α overexpression was showed to be associated with high risk of recurrence, disease progression and poor overall survival [22, 23, 26]. However, at the same time Theodoropoulos *et al*. did not show any relationship between HIF-1α expression and tumor stage, and the association between HIF-1α and cancer grade reported in their study and in the study by Ioachim *et al*. had only borderline statistical significance. It is worth mentioning that many of the previous attempts to evaluate the role of HIF-1α in UBC focused mainly on tumors in stage pTa, pT1, with a moderate number of pT2 cases and small number of pT3 and pT4 tumors.

In our study HIF-1α expression showed an inverse relationship with tumor stage and grade, with significantly lower HIF-1α IRS values in MIBCs and high grade tumors. We also observed higher mean MVD values in cases demonstrating negative HIF-1α expression. These observations are in contrast to studies by Chai *et al*. and Deniz *et al.* who reported that HIF-1α overexpression in UBC was associated with high MVD, high tumor stage and high grade [24, 25]. In two published papers Theodoropoulos *et al*. did not show significant association between tumor pT stage and HIF-1α expression, and the correlation between HIF-1α and MVD was weak [22, 23]. Results similar to ours were demonstrated by Nakanishi *et al.* in the context of urothelial carcinoma of the upper urinary tract. They showed that HIF-1α expression was found to be associated with grade but not with tumor stage, they also showed that in invasive tumors there was no relationship between HIF-1α immunoreactivity and microvessel density [46].

When comparing and discussing the results reported by different authors it is important to take into consideration the differences in the design of those studies. Nearly all studies evaluating HIF-1α expression in UBC concentrated on cancers in pTa, pT1 and pT2 stage. In our assessment we attempted to include relatively equal number of tumors in every pT stage and as a result the expression of HIF-1α protein was evaluated in a comparable number of both NMIBC and MIBC. It is important to realize that HIF-1α is a labile protein that is rapidly degraded in cells. In our study the UBC tissue samples were obtained through standard surgical procedures without any special attention to assure appropriate preservation of unstable proteins. We are aware that might have influenced the levels of HIF-1α expression in tissues and may have affected our results.

The inverse association between the levels of HIF-1α expression and tumor depth of invasion or tumor grade may indicate that the activation of HIF-1α pathway is not necessary for the UBC cells to acquire more aggressive phenotype and that other, hypoxia-independent mechanisms are involved in high grade, muscle invasive bladder cancers. The loss of HIF-1α expression in UBC may also be caused by the acquisition of additional genetic alterations associated with progression of the disease.

Angiogenesis may be controlled by hypoxia-induced HIF-1 expression in the initial stages of UBC development when the tumor is well differentiated as we found that low grade NMIBC tumors have a low number of blood vessels and show higher HIF-1α expression. However, considering the inverse relationship between MVD and HIF-1α expression as well as diffuse pattern of HIF-1α immunostaining with no connotation to the areas of tumor necrosis, we conclude that neovascularization in UBC – especially in high grade, muscle invasive tumors – is not regulated by HIF-1. We assume that other, hypoxia-independent pathways are involved in the angiogenesis in UBC.

Considering the complex role of HIF-1 in carcinogenesis, additional studies on larger number of cases must be conducted to explain in detail the influence of HIF-1α protein expression on UBC pathogenesis. The expression of other factors stimulating angiogenesis in UBC should be evaluated in different stages of the disease to investigate what is the main driving force behind neovascularization in advanced, muscle invasive UBC. Finally, molecular studies should be performed to differentiate if the loss of HIF-1α expression in high grade, muscle invasive tumors is caused by abundant blood vessels in tumor or rather the accumulation of genetic alterations in cancer cells leads to diminished HIF-1α protein production.

The authors declare no conflict of interest.

References

1. Ferlay J, Soerjomataram I, Ervik M, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11. [Online] 2013 (cited 15 November 2017). Available from: http://globocan.iarc.fr.
2. Moch H, Humphrey PA, Ulbright TM, et al. WHO Classification of Tumours of the Urinary System and Male Genital Organs. International Agency for Research on Cancer, Lyon 2016; 81-107.
3. Amin MB, Edge SB, Greene FL, et al. AJCC Cancer Staging Manual 8th ed. Springer, 2017.
4. Cheng SH, Liu JM, Liu QY, et al. Prognostic role of microvessel density in patients with renal cell carcinoma: a meta-analysis. Int J Clin Exp Pathol 2014; 7: 5855-5863.
5. Wang Y, Yao X, Ge J, et al. Can vascular endothelial growth factor and microvessel density be used as prognostic biomarkers for colorectal cancer? A systematic review and meta-analysis. Scientific World Journal 2014; 2014: 102736.
6. Uzzan B, Nicolas P, Cucherat M, et al. Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. Cancer Res 2004; 64: 2941-2955.
7. Saponaro C, Malfettone A, Ranieri G, et al. VEGF, HIF-1α expression and MVD as an angiogenic network in familial breast cancer. PLoS One 2013; 8: e53070.
8. Nadkarni NJ, Geest KD, Neff T, et al. Microvessel density and p53 mutations in advanced-stage epithelial ovarian cancer. Cancer Lett 2013; 331: 99-104.
9. Benckert C, Thelen A, Cramer T, et al. Impact of microvessel density on lymph node metastasis and survival after curative resection of pancreatic cancer. Surg Today 2012; 42: 169-176.
10. Kadota K, Huang CL, Liu D, et al. The clinical significance of lymphangiogenesis and angiogenesis in non-small cell lung cancer patients. Eur J Cancer 2008; 44: 1057-1067.
11. Fox SB, Harris AL. Histological quantitation of tumour angiogenesis. APMIS 2004; 112: 413-430.
12. Hasan J, Byers R, Jayson GC. Intra-tumoural microvessel density in human solid tumours. Br J Cancer 2002; 86: 1566-1577.
13. Radomska-Leśniewska DM, Bałan BJ, Skopiński P. Angiogenesis modulation by exogenous antioxidants. Centr Eur J Immunol 2017; 42: 370-376.
14. Kerbel RS. Tumor angiogenesis. N Engl J Med 2008; 358: 2039-2049.
15. Rankin EB, Giaccia AJ. The role of hypoxia-inducible factors in tumorigenesis. Cell Death Differ 2008; 15: 678-685.
16. Lee JWM, Bae SH, Jeong JW, et al. Hypoxia-inducible factor (HIF-1) alpha: its protein stability and biological functions. Exp Mol Med 2004; 36: 1-12.
17. Semenza GL. Hypoxia-Inducible Factor 1 and Cancer Pathogenesis. IUBMB Life 2008; 60: 591-597.
18. Masoud GN, Li W. HIF-1α pathway: role, regulation and intervention for cancer therapy. Acta Pharm Sin B 2015; 5: 378-389.
19. Zhong H, De Marzo AM, Laughner E, et al. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 1999; 59: 5830-5835.
20. Koukourakis MI, Giatromanolaki A, Skarlatos J, et al. Hypoxia inducible factor (HIF-1a and HIF-2a) expression in early esophageal cancer and response to photodynamic therapy and radiotherapy. Cancer Res 2001; 61: 1830-1832.
21. Koukourakis MI, Giatromanolaki A, Sivridis E, et al. Hypoxia-inducible factor (HIF1A and HIF2A), angiogenesis, and chemoradiotherapy outcome of squamous cell head-and-neck cancer. Int J Radiat Oncol Biol Phys 2002; 53: 1192-1202.
22. Theodoropoulos VE, Lazaris ACh, Sofras F, et al. Hypoxia-inducible factor 1 alpha expression correlates with angiogenesis and unfavorable prognosis in bladder cancer. Eur Urol 2004; 46: 200-208.
23. Theodoropoulos VE, Lazaris AC, Kastriotis I, et al. Evaluation of hypoxia-inducible factor 1alpha overexpression as a predictor of tumour recurrence and progression in superficial urothelial bladder carcinoma. BJU Int 2005; 95: 425-431.
24. Deniz H, Karakök M, Yagci F, et al. Evaluation of relationship between HIF-1alpha immunoreactivity and stage, grade, angiogenic profile and proliferative index in bladder urothelial carcinomas. Int Urol Nephrol 2010; 42: 103-107.
25. Chai CY, Chen WT, Hung WC, et al. Hypoxia-inducible factor-1alpha expression correlates with focal macrophage infiltration, angiogenesis and unfavourable prognosis in urothelial carcinoma. J Clin Pathol 2008; 61: 658-664.
26. Ioachim E, Michael M, Salmas M, et al. Hypoxia-inducible factors HIF-1alpha and HIF-2alpha expression in bladder cancer and their associations with other angiogenesis-related proteins. Urol Int 2006; 77: 255-263.
27. Chen WT, Hung WC, Kang WY, et al. Overexpression of cyclooxygenase-2 in urothelial carcinoma in conjunction with tumor-associated-macrophage infiltration, hypoxia-inducible factor-1α expression, and tumor angiogenesis. APMIS 2009; 117: 176-184
28. Weidner N, Semple JP, Welch WR, et al. Tumor angiogenesis and metastasis. Correlation in invasive breast carcinoma. N Engl J Med 1991; 324: 1-8.
29. Shirotake S, Miyajima A, Kosaka T, et al. Angiotensin II type 1 receptor expression and microvessel density in human bladder cancer. Urology 2011; 77: e19-25.
30. Ajili F, Kacem M, Tounsi H, et al. Prognostic impact of angiogenesis in nonmuscle invasive bladder cancer as defined by microvessel density after immunohistochemical staining for CD34. Ultrastruct Pathol 2012; 36: 336-342.
31. Inoue K, Slaton JW, Karashima T, et al. The prognostic value of angiogenesis factor expression for predicting recurrence and metastasis of bladder cancer after neoadjuvant chemotherapy and radical cystectomy. Clin Cancer Res 2000; 6: 4866-4873.
32. Canoğlu A, Göğüş C, Bedük Y, et al. Microvessel density as a prognostic marker in bladder carcinoma: correlation with tumor grade, stage and prognosis. Int Urol Nephrol 2004; 36: 401-405.
33. Huang J, Ma X, Chen X, et al. Microvessel density as a prognostic factor in bladder cancer: a systematic review of literature and meta-analysis. Cancer Biomark 2004; 14: 505-514.
34. Mabjeesh NJ, Amir S. Hypoxia-inducible factor (HIF) in human tumorigenesis. Histol Histopathol 2007; 22: 559-572.
35. Kronblad A, Jirström K, Rydén L, et al. Hypoxia inducible factor-1alpha is a prognostic marker in premenopausal patients with intermediate to highly differentiated breast cancer but not a predictive marker for tamoxifen response. Int J Cancer 2006; 118: 2609-2616.
36. Kitada T, Seki S, Sakaguchi H, et al. Clinicopathological significance of hypoxia-inducible factor-1alpha expression in human pancreatic carcinoma. Histopathology 2003; 43: 550-555.
37. Couvelard A, O'Toole D, Leek R, et al. Expression of hypoxia-inducible factors is correlated with the presence of a fibrotic focus and angiogenesis in pancreatic ductal adenocarcinomas. Histopathology 2005; 46: 668-678.
38. Kuwai T, Kitadai Y, Tanaka S, et al. Expression of hypoxia-inducible factor-1alpha is associated with tumor vascularization in human colorectal carcinoma. Int J Cancer 2003; 105: 176-181.
39. Yoshimura H, Dhar DK, Kohno H, et al. Prognostic impact of hypoxia-inducible factors 1alpha and 2alpha in colorectal cancer patients: correlation with tumor angiogenesis and cyclooxygenase-2 expression. Clin Cancer Res 2004; 10: 8554-8560.
40. Kim SJ, Rabbani ZN, Dewhirst MW, et al. Expression of HIF-1alpha, CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. Lung Cancer 2005; 49: 325-335.
41. Swinson DE, Jones JL, Cox G, et al. Hypoxia-inducible factor-1 alpha in non-small cell lung cancer: relation to growth

factor, protease and apoptosis pathways. Int J Cancer 2004; 111: 43-50.
42. Giatromanolaki A, Koukourakis MI, Sivridis E, al. Relation of hypoxia inducible factor 1 alpha and 2 alpha in operable non-small cell lung cancer to angiogenic/molecular profile of tumours and survival. Br J Cancer 2001; 85: 881-890.
43. Lidgren A, Hedberg Y, Grankvist K, et al. Hypoxia-inducible factor 1alpha expression in renal cell carcinoma analyzed by tissue microarray. Eur Urol 2006; 50: 1272-1277.
44. Urano N, Fujiwara Y, Doki Y, et al. Overexpression of hypoxia-inducible factor-1 alpha in gastric adenocarcinoma. Gastric Cancer 2006; 9: 44-49.
45. Fillies T, Werkmeister R, van Diest PJ, et al. HIF1-alpha overexpression indicates a good prognosis in early stage squamous cell carcinomas of the oral floor. BMC Cancer 2005; 84.
45. Nakanishi K, Hiroi S, Tominaga S, et al. Expression of hypoxia-inducible factor-1alpha protein predicts survival in patients with transitional cell carcinoma of the upper urinary tract. Clin Cancer Res 2005; 11: 2583-2590.

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