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ORIGINAL ARTICLE

Strong Correlation Between mRNA Expression Levels of HIF-2α, VEGFR1, VEGFR2 and MMP2 in Laryngeal Carcinoma

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Abstract The hypoxia that arises due to the rapid proliferation of tumor cells is a fundamental driving force for the canonical pathway of neovascularization. In the current study we report a very strong correlation between mRNA expression levels of HIF-2α (but not HIF-1α), VEGFR-1, VEGFR-2 and MMP2 in ex vivo samples from laryngeal carcinoma. Sixty-three samples from patients with histopathologically verified carcinoma of the larynx were examined in this study. Total RNA was isolated from both normal and tumor fresh frozen tissues of each patient and real-time quantitative PCR reactions were performed. The mRNA expression levels of HIF-1α, HIF-2α, VEGFR1, VEGFR2 and MMP2 were acquired. We found strong positive correlations between mRNA expression levels of HIF-2α and VEGFR-1, $r_s(98) = .671$, $p < .0005$; HIF-2α and VEGFR-2, $r_s(98) = .742$, $p < .0005$; HIF-2α and MMP2, $r_s(98) = .566$, $p < .0005$; VEGFR-1 and VEGFR-2, $r_s(98) = .791$, $p < .0005$; VEGFR-1 and MMP2, $r_s(98) = .709$, $p < .0005$; VEGFR-2 and MMP2, $r_s(98) = .793$, $p < .0005$. Our results provide evidence for the regulatory connection between HIF-2α and VEGFR-1, VEGFR-2 and MMP2 in the light of ETS1/ HIF-2α regulatory axis on a non-in-vitro level in carcinoma tissue, uncover some of the differences between the homologues HIF-1α and HIF-2α and round up and support the results from different experimental models in this field.

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Introduction

The proliferation of vascular endothelial cells is a key process of tumor neoangiogenesis which is one of the six classical hallmarks of cancer [1]. The hypoxia that arises due to the rapid proliferation of tumor cells is a fundamental driving force for the canonical pathway of neovascularization. This oxygen deprivation leads to accumulating hypoxia inducible factors (HIF-1α, HIF-2α and HIF-3α), which are heterodimeric transcription factors, and this activation of HIFs upregulates the transcription of a large number of HIF target genes, such as phosphoglycerate kinase (PGK), erythropoietin (Epo), and vascular endothelial growth factor (VEGF-A) [2]. HIF1α and HIF2α were previously suspected of promoting tumor progression through largely overlapping functions. However, this relatively simple model has now been challenged in light of recent data uncovering the differences in function and interaction with other molecules [3]. Endothelial cell proliferation is mediated primarily by vascular endothelial growth factor, which has two major tyrosine kinase receptors involved in neoangiogenesis—VEGF receptor 1 (Flt-1) and VEGF receptor 2 (KDR in humans/Flk-1 in mouse) [4, 5]. Tyrosine phosphorylation of VEGFR-1 in response to VEGF stimulation is hard to detect, and, in endothelial cells, no direct proliferative, migratory or cytoskeletal effects mediated by this receptor are apparent [6] and despite its vital role in embryogenesis [7], it is considered as more of a decoy receptor [8]. VEGFR-2 on the contrary is much more efficiently upon ligand binding and in endothelial cells leads to mitogenesis, chemotaxis and changes in cell morphology [9]. The intricate regulatory mechanisms of those

molecules and their interrelationship, their connection with extracellular matrix alteration via matrix metalloproteinases and the specific differences between HIF-1α and HIF-2α are still an area of research with the ultimate goal of finding new therapeutic opportunities against cancer.

In the current study we report a very strong correlation between expression levels of HIF-2α (but not HIF-1α), VEGFR-1, VEGFR-2 and MMP2 in ex vivo samples from laryngeal carcinoma. Our results provide evidence for the regulatory connection between HIF-2α and VEGFR-1, VEGFR-2 and MMP2 on a non-in-vitro level in carcinoma tissue. To our knowledge this is the first study that reports such correlation pattern in ex vivo carcinoma tissue. It is a continuation of a previous one focused on pattern of mRNA expression levels of HIF-1α, HIF-2α, HIF-3α and VEGF-A in laryngeal carcinoma, where we found a specific phenotype of HIFs expression in laryngeal carcinoma, where the HIF switch is absent in contrast to other malignant lesions [10].

Materials and Methods

Patient Recruitment and Assessment

The study was carried out in the ENT department of University Hospital "Queen Jovanna", Sofia, Bulgaria in co-operation with the Molecular Medicine Center at Medical University of Sofia over the period 2012–2014. Sixty-three patients with histopathologically verified carcinoma of the larynx were enrolled in the study. Informed consent was solicited from every patient and the protocol of the study was approved by the Ethics committee of Medical University of Sofia. A standardized history was obtained for each patient. Detailed description of the endoscopic/microscopic direct laryngoscopy findings were recorded along with the computer tomography examination results. All of the patients underwent operative intervention—either total laryngectomy or organ saving surgery depending on the extend of the disease. Tumor and normal laryngeal tissue samples were obtained from each patient during the surgery and immediately frozen in liquid nitrogen. The tissue samples were stored at −80 °C until use. The study was approved by the Ethical Committee of Medical University—Sofia, Bulgaria and written informed consent was obtained from every patient.

Genetic Testing

Total RNA Extraction and cDNA Synthesis

Total RNA was isolated from both normal and tumor fresh frozen tissues of each patient using RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The quality of RNA was checked by denaturing electrophoresis on a formaldehyde gel. The amount of RNA was determined spectrophotometrically on NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE).

One μg RNA of each sample underwent reverse transcription using High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA) according to manufacturer's recommendations. In brief, 2x RT Master mix prepared according to the supplied protocol was added to RNA in a total volume of 20 μl. Reverse transcription was performed in 3 steps: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min.

Quantitative Real-Time PCR

In the present study we analyzed the expression of 4 genes—HIF-1α, HIF-2α, VEGF-A, Flt-1 (VEGFR1), KDR (VEGFR2) and MMP2. Real-time quantitative PCR reactions were performed in 25 μl volume and the mixture included:1x RotorGene SYBR Green PCR Mix (Qiagen), 1x QuantiTect Primer Assay (Qiagen) for the respective gene and 100 ng cDNA. The conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 15 s, primer annealing at 55 °C for 30 s and synthesis with data acquisition at 72 °C for 30 s. Each sample was examined in triplicates and mean values of Ct from the three repeats were used for the data analysis. Negative and no template controls were evaluated as well. Beta-actin (Hs_ACTB_1_SG QuantiTect Primer Assay) was used as a reference gene for normalization. To determine the relative expression of each gene in the tumor, the 2-ddCt method was applied [11]. Briefly, mean threshold cycles (Ct) for a certain gene of interest (GOI) and a reference gene in tumor ($Ct_{T,GOI}$ and $Ct_{T,Ref}$, respectively) and normal ($Ct_{N,GOI}$ and $Ct_{N,Ref,}$ respectively) tissues were used to calculate dCt ($dCt = Ct_{GOI} - Ct_{Ref}$) for each tissue and then to derive the relative quantity (RQ) of the gene in the tumor compared to the normal tissue ($RQ = 2^{-ddCt}$ where $ddCt = dCt_T - dCt_N$). RQ over 2 was defined as overexpression and RQ less than 0.5—as underexpression of the gene, in agreement with previous publications [12, 13]. Two-sided t-test was used to calculate the statistical significance of the results. P values below 0.05 were accepted as statistically significant. Three outliers were removed from all statistical analysis due to strong deviation from the mean visual inspection of a scatterplot.

Results

The mean age of the study group was 60.5 with a standard deviation of 7.8. The youngest patient was 41 and the oldest one was 84. From the whole group there are two female patients. All of the patients had a histologically verified

squamous cell carcinoma of the larynx. Distribution according to TNM classification was as follows: two patients were staged T1 (3.2 %), seven—T2 (11.1 %), twenty-three—T3 (36.5 %) and thirty-one—T4 (49.2 %). Fourteen patients (13,6 %) had histologically verified lymph node metastases at the time of operation.

HIF-1α was up-regulated (RQ > 2) in the majority of patients—68.33 % and normally expressed (0.5 < RQ < 2) in 23,33 % of the patients, only 8.33 % were down-regulated. In contrast, only 11.11 % patients from the whole group had HIF-2α overexpression (RQ > 2). From the other 88.89 % patients, 41.27 % of the patients showed almost silenced mRNA HIF-2α expression (RQ < 0.5) and the other 47.62 % had an expression similar to the one in the matched normal laryngeal samples (0.5 < RQ < 2). VEGFR-1 mRNA levels were up-regulated in 38.10 % of the patients, down-regulated in 17.46 % and in 44.44 % normal expression levels were found. VEGFR-2 levels were distributed as follows: 17.46 % up-regulated, 52.38 % normal levels of expression and 30.16 % down-regulated; respectively for MMP2 distribution was: 58.73 % up-regulated, 26.98 % normal levels of expression and 14.29 % down-regulated (Fig. 1).

Quantitative analysis of the whole study group display mean values of mRNA expression for HIF-1α 2,71 times higher than the corresponding normal laryngeal epithelium (RQ), respectively HIF-2α—0,92 RQ, VEGFR-1—1.80 RQ, VEGFR-2—1.04 RQ and MMP2—4.62 RQ.

We explored for statistically significant associations between the expression levels of the studied genes. We found no correlation between HIF-1α and the other molecules. Between the rest of them we identified strong correlation pattern. A Spearman's rank-order correlation was used, Shapiro-Wilk test of normality was $p < .05$. Preliminary analysis showed the relationships to be monotonic, as assessed by visual inspection of a scatterplot. We found strong positive correlations between mRNA expression levels of HIF-2α and VEGFR-1, $r_s(98) = .671$, $p < .0005$ (Fig. 2a); HIF-2α and VEGFR-2, $r_s(98) = .742$, $p < .0005$ (Fig. 2b); HIF-2α and MMP2, $r_s(98) = .566$, $p < .0005$ (Fig. 2c); VEGFR-1 and VEGFR-2, $r_s(98) = .791$, $p < .0005$ (Fig. 2d); VEGFR-1 and MMP2, $r_s(98) = .709$, $p < .0005$ (Fig. 2e); VEGFR-2 and MMP2, $r_s(98) = .793$, $p < .0005$ (Fig. y2f).

Discussion

In this study we present a strongly correlated expression pattern of HIF-2α, VEGFR-1, VEGFR-2 and MMP2 in

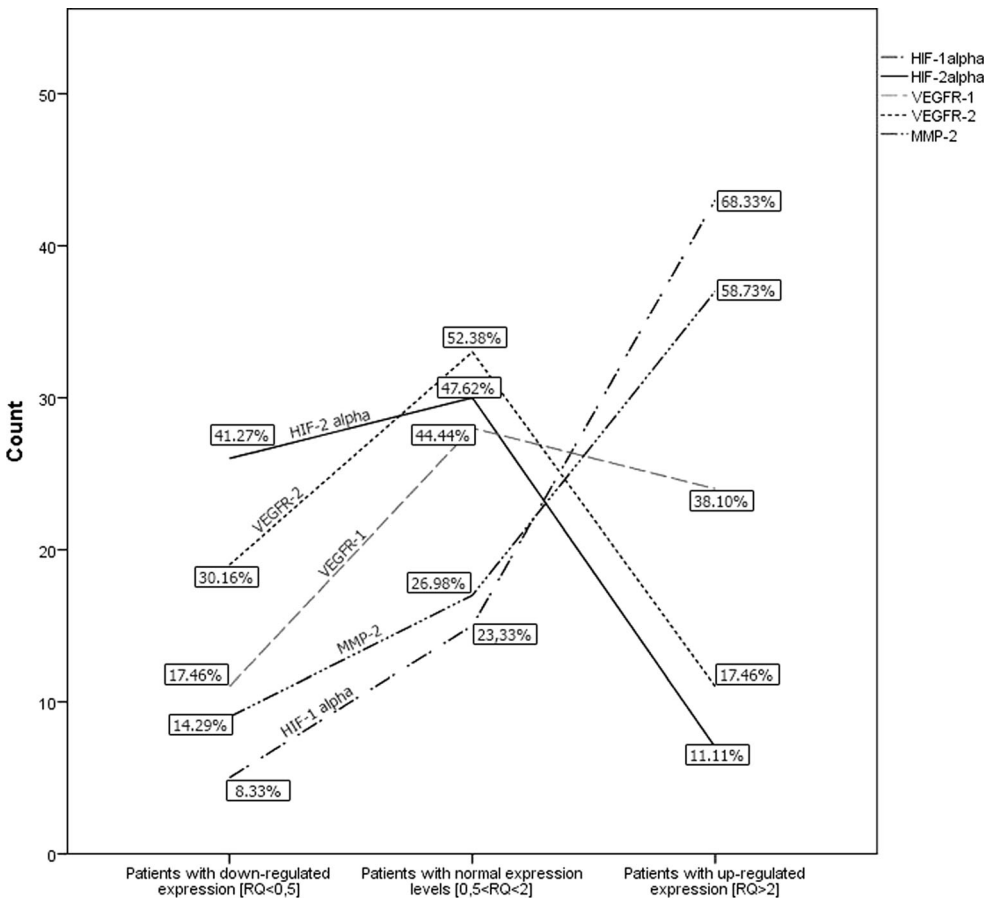

Fig. 1 Expression of HIF-1α, HIF-2α, VEGFR-1, VEGFR-2 and MMP2 distributed in groups according to RQ

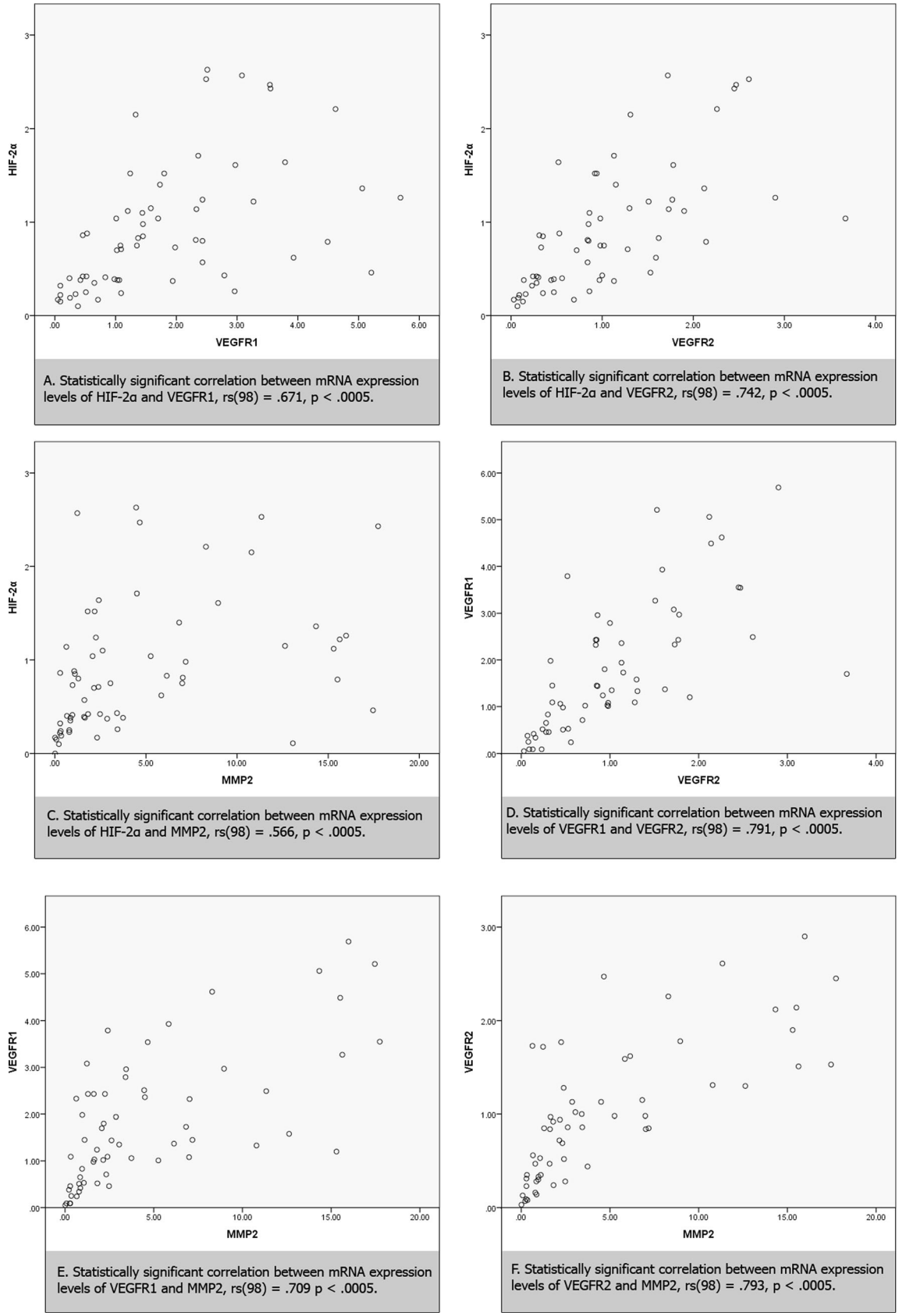

Fig. 2 Scatterplots with correlation data between HIF-2α, VEGFR-1, VEGFR-2 and MMP2

ex vivo samples from laryngeal carcinoma. When we analyzed the published data we found experimental studies connecting all those molecules with the ETS1/ HIF-2α regulatory axis [14]. ETS-1 is implicated in tumor vascularization and angiogenesis, as well as in contributing to tumor proliferation and invasion by acting within both neoplastic cells and fibroblasts of the tumor stroma [15].

Elvert et al observed in their experiments that HIF-2α but not HIF-1α is synergistic with Ets-1 in stimulating the Flk-1 (VEGFR2 in mice) promoter [16]. HIF-2α and Ets-1 (but not HIF-1α) physically interact via their carboxyl termini and exon-VII domains, respectively. HIF-2 binds to two HRE-related sequences, each in close proximity to functional Ets binding sites in the Flk-1 promoter. These two pairs of transcription factor binding sites constitute enhancer elements that confer strong inducibility by HIF-2α and Ets-1 when fused to heterologous promoters. They are indispensable, positively acting elements for the Flk-1 5′-flanking region and are essential for endothelial cell-specific reporter gene expression in transgenic mice [16].

Dutta et al reported that EGF/FGF2 signaling induces VEGFR1 in endothelial cells by recruiting ETS1 and HIF-2α transcription factors to the VEGFR1 chromatin domain. ETS1 and HIF-2α occupancy is associated with Pol II recruitment and transcriptionally favorable histone modifications at the VEGFR1 locus [14]. Additionally we found supporting evidence for strong association between Ets-1 and MMP2 [17, 18], such as Taki M et al who report that Ets-1 induced the promoter-activation and expression of MMP-2 [17]. Despite the lack of papers connecting HIF-2α and MMP2 we could speculate on the basis of our results that MMP2 regulation could be also intricately linked to the ETS1/ HIF-2α regulatory axis. Interestingly, we did not find such correlation pattern with MMP9 mRNA levels in the same group of samples (unpublished data).

Eubank T et al concludes that HIF-1α "appears to regulate VEGF production" [19], which corresponds with previous results of ours [10], and "VEGFR-1 levels are HIF-2α dependant", whereas we could also add VEGFR-2 and MMP2 to this group in the light of our results and the published data. Additional studies of mRNA expression levels of Ets1 and its role in regulation would be interesting for future investigations.

Conclusion

This study reports a strong quantitative correlation pattern between HIF-2α, VEGFR-1, VEGFR-2 and MMP2 (but not HIF-1α) mRNA expression levels in ex vivo samples from laryngeal carcinoma. Our results provide evidence for the regulatory connection between HIF-2α and VEGFR-1, VEGFR-2 and MMP2 on a non-in-vitro level in carcinoma tissue, uncover some of the differences between the homologues HIF-1α and HIF-2α and round up and support the results from different experimental models in this field. To our knowledge this is the first study that reports such correlation pattern in ex vivo carcinoma tissue.

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Compliance with Ethical Standards

Conflict of Interest Authors report no conflict of interest in the publication of the article.

References

1. Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70
2. Fong GH (2008) Mechanisms of adaptive angiogenesis to tissue hypoxia. Angiogenesis 11:121–140
3. Koh MY, Powis G (2012) Passing the baton: the HIF switch. Trends Biochem Sci 37:364–372
4. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309
5. Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M (1990) Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 5:519–524
6. Park JE, Chen HH, Winer J, Houck KA, Ferrara N (1994) Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem 269:25646–25654
7. Fong GH, Rossant J, Gertsenstein M, Breitman ML (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 376:66–70
8. Robinson CJ, Stringer SE (2001) The splice variants of vascular endothelial growth factor (VEGF) and their receptors. J Cell Sci 114:853–865
9. Quinn TP, Peters KG, de Vries C, Ferrara N, Williams LT (1993) Fetal liver kinase-1 is a receptor for vascular endothelial growth factor and is selectively expressed in vascular endothelium. Proc Natl Acad Sci U S A 90:7533–7537
10. Popov TM, Goranova TE, Stancheva G et al (2015) Relative quantitative expression of HIF-1α, HIF-2α, HIF-3α and VEGF-A in laryngeal carcinoma. Oncol Lett 9:2879–2885
11. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods 25:402–408
12. Scrideli CA, Carlotti CG Jr, Okamoto OK et al (2008) Gene expression profile analysis of primary glioblastomas and non-neoplastic brain tissue: identification of potential target genes by oligonucleotide microarray and real-time quantitative PCR. J Neurooncol 88: 281–291
13. Borel F, Han R, Visser A et al (2012) Adenosine triphosphate-binding cassette transporter genes up-regulation in untreated hepatocellular carcinoma is mediated by cellular microRNAs. Hepatology 55:821–832

14. Dutta D, Ray S, Vivian JL, Paul S (2008) Activation of the VEGFR1 chromatin domain: an angiogenic signal-ETS1/HIF-2alpha regulatory axis. J Biol Chem 283:25404–25413
15. Hahne JC, Kummer S, Heukamp LC et al (2009) Regulation of protein tyrosine kinases in tumour cells by the transcription factor Ets-1. Int J Oncol 35:989–996
16. Elvert G, Kappel A, Heidenreich R et al (2013) Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2alpha) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). J Biol Chem 278:7520–7530
17. Taki M, Verschueren K, Yokoyama K, Nagayama M, Kamata N (2006) Involvement of Ets-1 transcription factor in inducing matrix metalloproteinase-2 expression by epithelial-mesenchymal transition in human squamous carcinoma cells. Int J Oncol 28:487–496
18. Liu DX, Liu XM, Su Y, Zhang XJ (2011) Renal expression of proto-oncogene Ets-1 on matrix remodeling in experimental diabetic nephropathy. Acta Histochem 113:527–533
19. Eubank TD, Roda JM, Liu H, O'Neil T, Marsh CB (2011) Opposing roles for HIF-1α and HIF-2α in the regulation of angiogenesis by mononuclear phagocytes. Blood 117:323–332