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VEGF AND HIF-1α EXPRESSION IN NEPHROBLASTOMA CORRELATES WITH ANAPLASIA

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HIF-1α (hypoxia inducible factor 1α) is a transcription factor that has a role in maintaining oxygen homeostasis. Increased expression of HIF-1α leads to a rise in amount of VEGF (vascular endothelial growth factor), thus inducing neovascularization. The aim of this study was to determine the expression of VEGF and HIF-1α in nephroblastoma and to compare it with the clinical and pathohistological characteristics of this tumour (dominant histological component, presence of anaplasia and necrosis, size of the tumour, age and sex of the patient). Expression of VEGF and HIF-1α was determined by immunohistochemistry of the tumour tissue. Monoclonal mouse anti-VEGF and polyclonal rabbit anti-HIF-1α antibodies were used. For immunoscoring of both markers the degree of positive staining was evaluated semiquantitatively. The χ^2-test, which includes Spearman's rank correlation and the ANOVA test were used for statistical analysis of data.

Most of the tumours were positive for VEGF (38/40) and HIF-1α (37/40). Significant statistical correlation was found between HIF-1α and the presence of anaplasia (p=0.02986). A clear correlation between VEGF and tumour size was established (Spearman's rank correlation: p=0.0027), showing strong positivity in larger tumours.

Based on this study we can conclude that VEGF and HIF-1α are present in Wilms' tumours, playing an important role in angiogenesis and tumour progression.

Descriptors: VEGF-A protein, human; HYPOXIA-INDUCIBLE FACTOR 1, ALPHA SUBUNIT; WILMS TUMOUR; ANAPLASIA

INTRODUCTION

Wilms' tumour (nephroblastoma) is the most common primary renal tumour of childhood (1).

Age at diagnosis is usually between 3 and 4 years. They occur in hereditary (2%) or nonhereditary (98%) form. Bilateral tumours are present in 5-7% of cases (2), more commonly in those with genetic predisposition than in sporadic ones.

Microscopically, they usually consist of a triphasic combination (blastemal, stromal and epithelial cell types) in variable proportions, with or without the presence of anaplasia.

The most unfavourable prognostic factors are advanced tumour stage and presence of anaplasia.

Angiogenesis is a critical process in the growth and spread of tumours as they cannot enlarge beyond 1-2 mm in diameter unless they are vascularized (4). Otherwise, hypoxia-induced cell death occurs. Vascular endothelial growth factor (VEGF) and hypoxia inducible factor 1 (HIF-1) are among the most significant angiogenic growth factors. VEGF is a potent inducer of blood vessel formation. HIF-1 is a transcription factor that has a role in maintaining oxygen homeostasis by induction of glycolysis, erythropoiesis and angiogenesis in hypoxic states. It can also induce apoptosis (5, 6).

Increased expression of HIF-1α due to lack of oxygen in tumour cells leads to a rise in the amount of VEGF, thus inducing the neovascularization necessary for further progression of the tumour (5).

The aim of this study was to determine the expression of VEGF and HIF-1α in nephroblastoma and to compare it with the clinical and pathohistological characteristics of this tumour.

MATERIALS AND METHODS

We retrospectively analyzed the pathohistologic features and biopsy materials of 40 patients in which nephrectomy was performed for nephroblastoma between 1998 and 2005 at the Children's Hospital, Zagreb.

All patients received the same preoperative chemotherapy treatment according to SIOP-9 protocol.

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We analyzed age, gender, size of the tumour, anaplasia, necrosis and dominant histological component.

Routine histopathologic analysis of tumour tissues was performed at the Department of Pathology, School of Medicine, University of Zagreb.

Specimens were fixed in 10% buffered formalin, embedded in paraffin, cut at 5 μm thickness, deparaffinized and routinely stained with haematoxylin and eosin.

Table 1. *Clinicopathologic features of 40 patients with nephroblastoma*
Tablica 1. *Kliničkopatološke značajke 40-ero bolesnika s nefroblastomom*

Clinicopathologic features Kliničkopatološke značajke	Number of patients Broj bolesnika
Age/Dob	
1-3 years/godina	21
4-6 years/godina	14
7-9 years/godina	5
Sex/Spol	
Male/Muški	26
Female/Ženski	14
Tumour size/Veličina tumora	
2-6 cm	29
>6-10 cm	11
Dominant histological component Dominantna histološka komponenta	
Blastemal	20
Epithelial	11
Stromal	9
Anaplasia/Anaplazija	
Present/Prisutna	8
Absent/Odsutna	32
Necrosis/Nekroza	
Present/Prisutna	27
Absent/Odsutna	13

Expression of VEGF and HIF-1α was determined by immunohistological analysis of the tumour tissue, using antibodies to VEGF (monoclonal mouse; clon VG1; code M 7273; dilution 1:50; Dako Epos, Dennmark) and HIF (polyclonal rabbit, HIF-1α, Abcam, Great Britain).

The peroxidase/antiperoxidase method (PAP) was used on sections with 3.3'-diamino-benzidine-tetrahydrochloride as a chromogene. Briefly, tissue sections were incubated in 10 μmol/L sodium citrate in a pressure cooler. The pressure cooler was placed in a microwave oven and heated for 30 minutes. The slides were then cooled at room temperature for 30 minutes. The endogenous peroxidase activity of the tissue samples was quenched with an aqueous solution of 3% hydrogen peroxidase for 3 minutes, followed by 3 rinses with Tris-buffered saline. As a positive control we used a known positive section of mammary carcinoma. Omission of the primary antibody served as negative control for all antibodies. Microwave pretreatment was performed to improve immunostaining.

For immunoscoring of both markers the degree of positive staining was evaluated semiquantitatively. The percentage of immunoreactive cells was determined under high magnification in 10 whole microscopic fields (400x). The results were reported as: 0 for no reaction; 1 for weak reaction (up to 33% positive cells); 2 for moderate reaction (>33-66% positive cells) and 3 for strong reaction (more than 66% positive cells).

The χ^2-test, which includes Spearman's rank correlation, and the ANOVA test were used for statistical analysis of data. Values of $p<0.05$ were considered statistically significant.

RESULTS

40 cases of primary Wilms' tumour were analyzed in our study. There were 26 male and 14 female patients. The patients' age range was from 1 to 9 years. The average age was 3.7 years. Tumours measured from 2 to 10 cm at the widest diameter. The mean tumour size was 5.3 cm. Anaplasia was present in 8 patients. Microscopically, the blastemal component was the dominant one in 20, epithelial in 11 and stromal in 9 patients. Necrosis was present in 27 tumours. The clinical and pathological features of the patients are shown in Table 1.

Expression of HIF-1α was found in 37 cases. There was no reaction in 3 cases. Weakly positive reaction was observed in 10 cases, moderate in 12 cases and strong in 15 cases (Figure 1a).

Expression of VEGF was found in 38 cases and there was no expression in 2 cases. Sixteen cases showed weak, 10 cases moderate and 12 cases strong reaction (Figure 1b).

Both antigens were expressed in cytoplasm. The surrounding renal tissue showed weak expression of both VEGF and HIF-1α.

There was no difference in localisation of antigens depending on the size of the

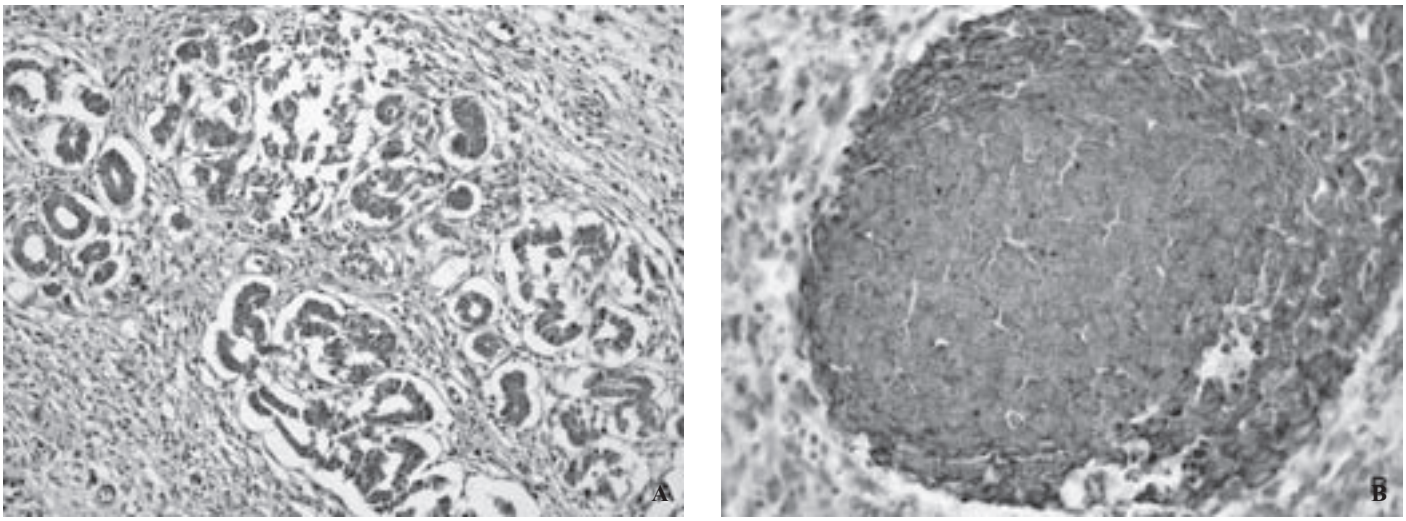

Figure 1. *Moderate immunohistochemical staining for HIF-1α (A) and strong for VEGF (B) in cytoplasm of nephroblastoma cells (A: anti-HIF-1α; B: anti-VEGF, 400x)*
Slika 1. *Umjerena imunohistokemijska reakcija na HIF-1α (A) i jaka reakcija na VEGF (B) u citoplazmi stanica nefroblastoma (A: anti-HIF-1α; B: anti-VEGF, 400x)*

Table 2. *Results of immunohistochemical analysis for VEGF and HIF-1α of 40 patients with nephroblastoma*
Tablica 2. *Rezultati imunohistokemijske analize za VEGF i HIF-1α kod 40-ero bolesnika s nefroblastomom*

Reaction* Reakcija	VEGF	HIF-1α
0	2	3
1	16	10
2	10	12
3	12	15
total	40	40

* 0 = no reaction/nema reakcije
1 = weak reaction, up to 33% of cells positive/slaba reakcija, do 33% stanica pozitivno
2 = moderate reaction, >33-66% of cells positive/umjerena reakcija, >33-66% stanica pozitivno
3 = strong reaction, more than 66% of cells positive/jaka reakcija, više od 66% stanica pozitivno

tumour or other clinicopathologic features studied.

The results of the immunohistochemical analysis for VEGF and HIF-1α expression are presented in Table 2.

χ^2-test was used to compare VEGF and HIF-1α expression in relation to clinical and histological features of the tumour (sex, histological component, anaplasia, necrosis). Values of $p<0.05$ were considered statistically significant.

There was no statistically significant correlation between HIF-1α and patients' sex (p=0.8326), histological components of tumour (p=0.5850) and HIF-1α and necrosis (p=0.1753). There was a statistically significant correlation between HIF-1α expression and anaplasia (p=0.0299).

There was no statistically significant correlation between VEGF expression and patients' sex (p=0.5896), histological components (p=0.6892), VEGF and anaplasia (p=0.0722) and VEGF and necrosis (p=0.4459).

Kruskal –Wallis ANOVA test was used to compare HIF and VEGF expression in relation to patients age and tumour size.

There was no statistically significant correlation between HIF expression and age (p=0.9938), HIF and tumour size (p=0.4853), VEGF and age (p=0.6866). There was a statistically significant correlation between VEGF expression and tumour size (p=0.0357), which was confirmed by Spearman's rank correlation (p=0.0027). This confirmed that neovascularisation is needed for further progression of tumour and VEGF has an important role in it.

Spearman's rank correlation showed no statistically significant correlation between VEGF and HIF (p=0.1802), but the rise of one variable is followed by the rise of the other one.

DISCUSSION

Angiogenesis is a key event in growth and progression of tumour (7). VEGF and HIF-1 play an important role in this process. VEGF mRNA levels are dramatically increased within a few hours of exposing different cell cultures to hypoxia and return to normal when oxygen supply is resumed (8).

Tumour hypoxia is one of the leading factors in neovascularisation development (9). Overexpression of HIF-1 due to tumour hypoxia leads to increase in VEGF, neovascularisation and further tumour progression (5). The importance of HIF-1α transcription regulation of VEGF expression has been confirmed on animal "HIF- knockout" models. Remarkable reduction of VEGF expression and cessation in further tumour growth was observed (9).

There are few studies dealing with clinicopathological features of nephroblastoma and VEGF expression. In one study authors found VEGF expression in 20 of 63 patients (32%) (10). There were 65% male patients and the average age was 3 years. Anaplasia was found in 14% of patients. Average tumour size was 12 cm. They didn't study HIF-1 expression.

Authors of VEGF studies presume that nephroblastoma produces VEGF which than binds to remnants of heparan sulphate in extracellular matrix thus activating host endothelial cells (11).

There are only few angiogenic studies that include Wilms' tumour and they have mostly been done on experimental models (12-15). In English literature there are only 4 studies in which VEGF expression was found in nephroblastoma cells by standard immunohistochemical methods.

Authors found VEGF in all studied cases of nephroblastoma and confirmed correlation between angiogenesis and survival. Patients with worse prognosis had more capillaries in hot spots, increased expression of VEGF in tumour cells and three times higher VEGF concentration in serum (16). Only one study dealt with both VEGF and HIF expression in nephroblastoma (15). They have found VEGF and HIF in all 18 cases. In 78% of cases there were >50% HIF positive cells and 31% had >50% VEGF positive cells. There was no comparison to clinicopathological features of the tumour.

We haven't found any statistically significant difference between VEGF and HIF expression in relation to sex, histological components and necrosis. VEGF and HIF expression was present in most cases (VEGF in 38 and HIF in 37 cases). Both VEGF and HIF expression was found in 37 cases.

In most studies there was a higher proportion of male patients. Percentage varied from 58% to 68% (7, 16, 17). In our study there were 65% of male patients.

We found a statistically significant correlation between HIF expression and anaplasia. Furthermore, correlation between VEGF expression and anaplasia was observed, although not statistically significant, probably due to a small group of patients studied.

Correlation between VEGF expression and tumour size is obvious and statistically significant, demonstrating the importance of VEGF in angiogenesis, a critical process in tumour progression. Increase in VEGF expression is followed by increase in HIF expression.

Based on this study we can conclude that the studied markers, VEGF and HIF-1α, are present in Wilms' tumour, playing an important role in angiogenesis and tumour progression.

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S a ž e t a k

EKSPRESIJA VEGF-A I HIF-1α U NEFROBLASTOMU KORELIRA S ANAPLAZIJOM

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HIF-1α (čimbenik 1α induciran hipoksijom) transkripcijski je faktor koji ima ulogu u homeostazi kisika. Povećana ekspresija HIF-1α dovodi do porasta razine VEGF-a (vaskularni endotelni čimbenik rasta) i na taj način potiče neovaskularizaciju. Cilj ove studije bio je odrediti ekspresiju VEGF-a i HIF-1α u nefroblastomu te je usporediti s kliničkim i patohistološkim značajkama ovog tumora: dominantnom histološkom komponentom, prisutnošću anaplazije i nekroze, veličinom tumora te dobi i spolom pacijenta. Ekspresija VEGF-a i HIF-1α određena je imunohistokemijom tumorskog tkiva. Upotrijebljena su monoklonalna mišja anti-VEGF protutijela te poliklonalna zečja anti-HIF-1α protutijela. Stupanj ekspresije je za oba markera izražen semikvantitativno. Za statističku obradu podataka primijenjeni su χ^2-test, koji uključuje Spearmanov koeficijent korelacije, i ANOVA test. Većina tumora bila je pozitivna na VEGF (38/40) i HIF-1α (37/40). Opažena je statistički značajna povezanost između HIF-1α i anaplazije (p=0.02986) te jasna, statistički značajna povezanost između VEGF-a i veličine tumora (Spearmanov koeficijent korelacije: p=0.0027), uz jaku reakciju u većim tumourima. Temeljem ove studije možemo zaključiti da su VEGF i HIF-1α prisutni u Wilmsovom tumoru te da imaju važnu ulogu u angiogenezi i progresiji tumora.

Deskriptori: VEGF-A protein, humani; ČIMBENIK 1α INDUCIRAN HIPOKSIJOM; WILMSOV TUMOR; ANAPLAZIJA

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