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Significance of Angiogenesis Determination in Pediatric Solid Tumors

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Fifty one patients (27 boys and 24 girls) with solid tumors whose ages ranged between 2 and 168 months were enrolled. Serum Vascular endothelial growth factor by Sandwich Enzyme linked immunosorbent assay was determined for all patients before any treatment and for 17 patients of them while in remission 6-9 months post therapy. Tissue biopsy was obtained for micro vessel count (MVC) determination by staining endothelial cells for factor VIII-related antigen with the use of a standard immunoperoxidase technique. Before therapy, median concentration of serum VEGF was significantly increased in solid tumors' patients when compared to control group ($p<0.001$) with non significant relation to tumor type. Also, patients with different histopathologic diagnoses had comparable median MVC (tissue angiogenesis) with non significant intergroup variability. Serum VEGF increased significantly in patients with neuroblastoma (n = 19) compared to those with ganglioneuroma (n = 6) while MVC values were comparable. When patients with metastatic cancer (n = 15) were compared to those with localized disease (n = 30), median VEGF levels and MVC were slightly higher in the former group, the difference did not reach statistical. In all cancer patients (n = 45), VEGF values correlated with MVC ($r = 0.33$, $p = 0.022$). After treatment, serum concentrations of VEGF dropped significantly ($p<0.001$) but still higher than control values ($p =<0.001$). Estimated OS and EFS of the whole group with malignant solid tumors patients were 86 and 72%, respectively. Serum VEGF did not correlate with outcome for the whole group of patients. Cox regression revealed that MVC ($p<0.001$) and stage of cancer ($p<0.001$) were highly associated with EFS of patients with embryonal tumors. Tumor angiogenesis can be indirectly assessed *in vivo* by serum VEGF measurement which is correlated with tissue MVC. Also, monitoring the levels of VEGF in patients with cancer may prove to be a useful marker for tumor status and response to treatment.

Key words: Tumor angiogenesis, pediatric solid tumors, vascular endothelial growth factor, micro vessel density

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INTRODUCTION

Angiogenesis, neovascularization, is the process leading to formation of new blood vessels (Folkman, 1992). Angiogenesis is a crucial mechanism required for a number of physiological and pathological events. In physiological conditions, angiogenesis is a highly regulated phenomenon. It normally occurs during embryonic development, wound healing and the menstruation cycle. Unregulated angiogenesis is seen in pathological conditions, such as psoriasis, diabetic retinopathy and cancer (Tonini *et al*., 2003). The most dramatic angiogenesis-dependent disease is cancer. During tumor growth, angiogenesis is required for proper nourishment and removal of metabolic wastes from tumor sites (Gasparini and Harris, 1995).

Formation of solid tumors requires coordination of angiogenesis with continued tumor cell proliferation. However, despite such neovascularization, hypoxia is persistent and frequently found in tumors at the time of diagnosis. Hypoxia arises early in the process of tumor development because rapidly proliferating tumor cells outgrow the capacity of the host vasculature. Tumors with low oxygenation have a poor prognosis and strong evidence suggests that this is because of the effects of hypoxia on malignant progression, angiogenesis, metastasis and therapy resistance (Tonini *et al*., 2003). Although hypoxia inhibits cell proliferation and eventually cell death, hypoxia also provides angiogenic and metastatic signals, thus allowing prolonged survival in the absence of oxygen and generation of a persistent angiogenic signal (Dulak and Jozkowicz, 2003; Wouters *et al*., 2003).

Neoplastic cells may produce several angiogenic peptides, but vascular endothelial cells also secrete growth factors and cytokines that stimulate tumor cells proliferate or attract and stimulate inflammatory cells (Hamada *et al*., 2000). The extent of vasculature assessed by immunohistochemistry and micro vessel counting predicts prognosis in many tumor types (Weidner, 1995). Urine and other body fluids of cancer patients have shown to contain endothelial chemokinetic and proliferative activity (Li *et al*., 1994).

Tumor angiogenesis can be indirectly assessed *in vivo* by angiogenic factor measurements in body fluids. There has been much interest in the possible use of angiogenic factors as tumor markers to evaluate tumor status, monitor response to therapy and/or predict outcome (Nguyen, 1997). Micro vessel counting in cancer tissues after immunohistochemical staining remains the reference method of angiogenesis assessment (Weidner, 1995). The present study was conducted to define the role of angiogenesis in pediatric solid tumors, to find out any relation between angiogenesis markers and disease stage, number of metastases and volume of involvement, to evaluate any predictive value of serum angiogenic markers on possibility of response to cytotoxic therapy and to investigate the usefulness of VEGF as a possible prognostic factor in conjunction with the more established parameters.

MATERIALS AND METHODS

Study population: During the period January 2000 till May 2004, 51 patients with solid tumors were enrolled. They were registering to Pediatric Department of NCI (40 patients), Cairo University and Pediatric Hematology/Oncology Clinic (11 patients), Ain Shams University. They were 27 boys and 24 girls whose ages ranged between 2 and 168 months. Twenty healthy children were recruited to determine normal concentration of serum vascular endothelial growth factor (VEGF). A verbal consent was obtained from guardians prior inclusion in study.

Patients' groups: Cancer patients were diagnosed according to standard histopathologic, immunohistochemical, radiomaging and tumor markers relevant to each type of cancer. Six categories were identified;

Malignant solid tumors: Nineteen patients with Neuroblastoma (NB), 13 with Wilms' tumor (WT), 8 Rhabdomyosarcoma (RMS), 3 Retinoblastoma (RB) and 2 with Hepatoblastoma (HB).

Benign solid tumors: Six patients with Ganglioneuroma (GN). Age and sex distribution of patients is shown in Table 1.

Inclusion criteria

Pathologically proven primary solid tumors.
Performance more than 60%.
Life expectancy more than 6 month.
Unified treatment policy and protocols.

Control group: Twenty normal infants and children, 12 boys and 8 girls whose mean age was 38.4 (±30.0) months were included as controls. They were recruited from siblings of patients attending Outpatient Clinic, Children's Hospital, Ain Shams University.

Methodology

All patients were subjected to:

- Complete history taking with particular emphasis on initial presentation, duration of illness and constitutional symptoms.
- Thorough clinical examination laying stress on presence of metastases and disease particulars as hemihypertrophy and aniridia in Wilms' tumor, precocity in hepatoblastoma. Blood pressure was checked being important for patients with Wilms' tumor, neuroblastoma and any abdominal masses with possible compressing manifestations.
- Staging systems: INSS for neuroblastom (Brodeur *et al.*, 1993; Castelbery *et al.*, 1994), NWTS-3 for Wilms'tumor (D;Angio *et al.*, 1989) IRS for rhabdomyosarcoma (Lawrence *et al.*, 1987), St. Jude Children's Research Hospital clinical staging system for retinoblastoma (Howarth *et al.*, 1980; Messmer *et al.*, 1991) and PRETEXT staging for hepatoblastoma (Brown *et al.*, 2000).
- Radioimaging studies; computerized tomography (C/T) scan to assess tumor size and bone scan for presence of metastasis.
- Treatment: Patients in each tumor type received the same protocol. Response to treatment was assessed according to World Health Organization (WHO) criteria for definition of objective of response.

Laboratory investigations:
Routine laboratory requests:

- Complete blood count: Coulter-Microdiff 18.
- Renal function tests: Blood urea nitrogen (BUN) (Patton and Crouch, 1977) and serum creatinine (Höuöt, 1985).
- Liver function tests: Alanine transaminase (ALT) and aspartate transaminase (AST) calorimetrically (Reitman and Frankle, 1957) and total serum bilirubin (Jendrassic and Grof, 1938).
- Lactate Dehydrogenase (LDH) using colorimetric endpoint technique (Henry, 1974) and Neuron Specific Enolase (NSE) using ELISA technique (Sell, 1990) for neuroblastoma patients and alpha fetoprotein (α-FP) for hepatoblastoma group.

Angiogenic marker: Serum Vascular endothelial growth factor by Sandwich Enzyme linked immunosorbent assay (ELISA), (Dosquet *et al.*, 1997), Kit supplied by Oncogeny Research Products (84 Rogers Street, Cambridge, UK). Serum VEGF was determined for all patients before any treatment and for 17 patients (11 NB and 6 WT) while in remission 6-9 months post therapy.

Tissue biopsy: Needle biopsy was obtained for pathological diagnosis and then open biopsy at time due for operative intervention according to protocol of therapy. Tumor specimens from all patients were available for micro vessel density determination. Blood vessels were highlighted by staining endothelial cells for factor VIII-related antigen (DAKO Corporation, Carpinteria, CA, USA) with the use of a standard immunoperoxidase technique (Pinkus *et al.*, 1986).

Statistical analysis: Statistical analysis was performed using SPSS version 10. Statistical significance of differences between variables was analyzed by Mann-Whitney, Kruskal-Wallis and Wilcoxon rank tests. Cox regression models were used to explore association between angiogenic makers as well as other known prognostic factors (age, stage, favorable pathology and tumor markers) and survival rates. Cutoff level of b-FGF was defined as value above the 95th percentile of normal controls. Events were defined as progression of disease under treatment or recurrence after first complete remission. Survival was calculated from first day of diagnosis until last contact, defined as date of death or date of last follow up.

RESULTS

Patients' characteristics: Patients were matched to the control group with respect to mean age and sex distribution (Table 1). Compared to each other, patients with hepatoblastoma were the youngest while those with ganglioneuroma were the eldest.

Disease characteristics: Embryonal tumors constituted 82% (37/45) of cases with malignant solid tumors, whilst RMS patients presented the rest 18% (8/45). Six patients with ganglioneuroma were included as an example of benign solid tumor for angiogenesis comparison. With respect to disease stage, 36 out of 45 (80%) of patients with malignant solid tumors presented at late stages III and IV. With regard to metastasis sites' number, 53.3% (8/15) had only one metastatic site whereas 46.7% had more than one site.

Tumor size: Tumor size as determined by C/T scan (multiplying maximum 2 dimensions) was variable among studied patients; in NB the mean was 101(±70.4) cm^2, in WT was 144.8±65 cm^2 while in RMS 300±169.7 cm^2. In GN, it was 552.5±59.6 cm^2. Individual values in HB were 72 and 108 cm^2.

Tumor pathology: According to Shimada, six out of 19 (31.5%) NB patients were having stroma-rich malignant tissues. Favorable histology was diagnosed in 9 of 13 (69%) of WT group. Histologic subtypes recognized in RMS were embryonal in 4, alveolar in 2 and

Table 1: Age and gender distribution of patients and control group

Group (No.)	Sex: Boys	Sex: Girls	Age (months) Mean ±SD (median, range)
Controls (20)	12	8	38.4±30.0 (34, 2-172)
All solid tumors' patients (45) I-Malignant	24	21	43.7±28.7 (36, 2-168)
Neuroblastoma (19)	8	11	33.4±21.3 (36, 2-72)
Wilms' tumor (13)	9	4	33.6±25.1 (30, 3-96)
Rhabdomyosarcoma (8)	3	5	52.3±47.8(38.5,15-168)
Others (5)	4	1	18.2±12.6 (18, 4-36)
Retinoblastoma (3)	2	1	
Hepatoblastoma (2)	2	0	
II-Benign Ganglioneuroma (6)	3	3	91.2±34.6 (72, 60-144)

Table 2: Serum Vascular Endothelial Growth Factor (VEGF) in patients and controls

Group (No.)	VEGF (pg mL^{-1}) Mean±SD (median, range)	Mann-Whitney test "Z "	p-value
Controls (20)	175.0±84.2 (148.5, 42-305)	4.961	<0.001
All patients (45)	447.6±191.5 (394.8, 62.8-726.6)		
Solid Tumors' Groups		Kruskal-Wallis test	p-value
Neuroblastoma (19)	513.2±145.2 (598.3, 244-727)	2.045	0.36
Wilms' tumor (13)	344.8±178.8 (368.6, 75-582)		
Rhabdomyosarcoma (8)	429.3±208.9 (379.3, 81-671)		
Others (5)	495.2±279.4 (634.2,62.8-713.7)		

Table 3: Mean Micro vessel Count (MVC/X400 field) among studied cancer groups

Group (No.)	MVC/X400 field Mean±SD (median, range)	Kruskal-Wallis test	p-value
Neuroblastoma (19)	18.9±12.7 (17.7, 3.3-38)	930.	0.63
Wilms' tumor (13)	11.8±7.2 (8.7, 3-26)		
Rhabdomyosarcoma (8)	16.8±13.5 (13.2, 3-44)		

Table 4: Angiogenic markers in Neuroblastoma versus Ganglioneuroma

	Serum VEGF (pg mL^{-1}) Mean±SD (median, range)	MVC/X400 field Mean±SD (median, range)	Mann-Whitney test	p-value
Neuroblastoma (19)	513.2±145.2 (598.3, 244-727)	18.9±12.7 (17.7, 3.3-38)	2.67	0.005
Ganglioneuroma (6)	237.7±145.3 (299.9, 76-399)	12.0±5.5 (9.8, 5.3-39)	1.2	0.257

undifferentiated in 2. Those with hepatoblastoma were both of fetal subtype.

Tumor markers: Neuroblastoma patients; serum neuron specific enolase was available for 17 patients at diagnosis with a median of 81 (range, 5.8-522.9) ng mL^{-1}. Serum Lactate dehydrogenase was determined for 15 NB patients with a median of 1620 (761-1707.6) U mL^{-1}. In GN, median NSE was 13 (11-14) ng mL^{-1} and LDH was 732 (504-940) U mL^{-1}. Hepatoblastoma patients were tested for alpha fetoprotein levels (αFP) which were 1710 and 3200 m I U mL^{-1}.

Table 5: Serum Vascular Endothelial Growth Factor (VEGF) in patients with metastatic versus non-metastatic disease

Group (No.)	Mean±SD (median, range)	Mann Whitney test	p-value
VEGF (pg mL^{-1})			
Non metastatic disease (30)	432.9±203.4 (379.9, 62.8-726.6)	-0.619	0.54
Metastatic disease (15)			
MVC/X400 field	481.8±174.1	-0.855	0.4
Non metastatic disease	(598.3, 81.3-656.5)		
Metastatic disease			
	11.2±7.1 (8.7, 2.3-27)		
	13.99±10.3 (9.5, 3-44)		

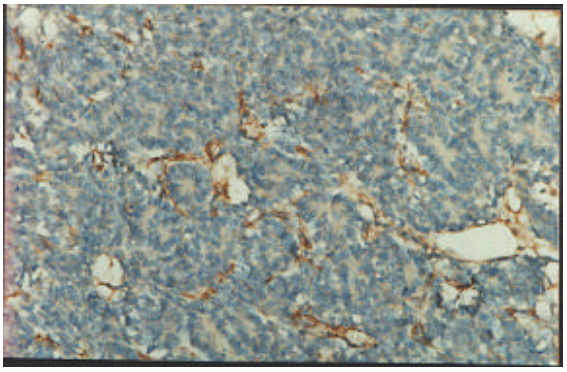

Fig. 1: High MVC in favorable histology Wilms' tumor, average count 32/X400 field

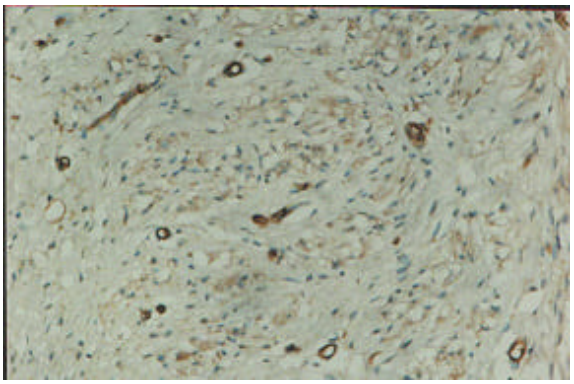

Fig. 2: Low MVC in stroma-rich neuroblastoma, average count 11/X400 field

Angiogenic markers

Serum angiogenesis: Before therapy, median concentration of serum VEGF was highly significantly increased in solid tumor patients when compared to control group ($p<0.001$). Yet, there was non significant difference in the mean serum VEGF between patients' subgroups ($p = 0.36$) (Table 2).

Tissue angiogenesis (MVC): Examples of tumors with high and low micro vessel count (Table 3) as delineated

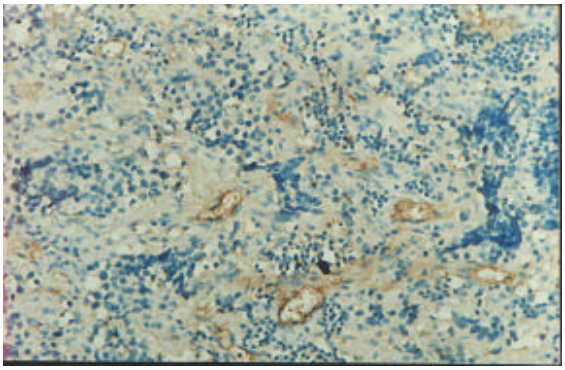

Fig. 3: Intermediate MVC in hepatoblastoma, average count 19 /X400 field

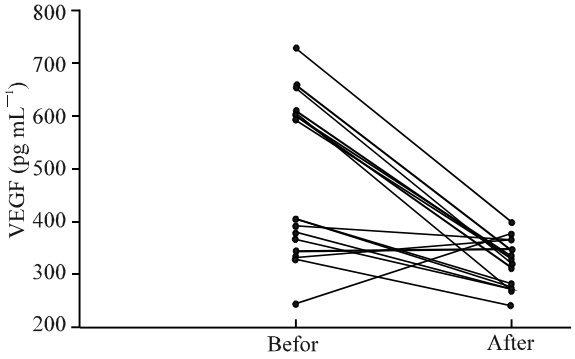

Fig. 4 : Serum VEGF before and after therapy

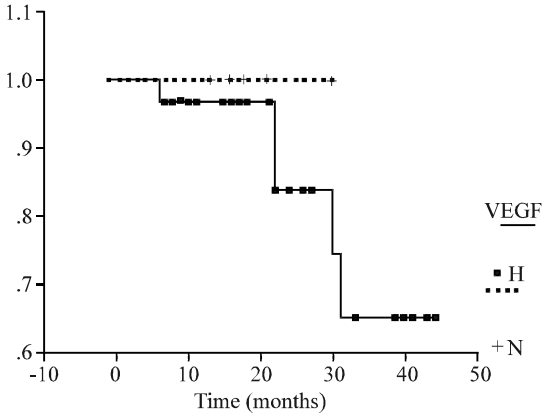

Fig. 5: Overall survival of malignant solid tumors' patients in relation to serum VEGF levels

by fVIII-related antigen staining are shown in Fig. 1-3. Patients with different diagnoses had comparable median MVC.

On comparing NB and GN patients, serum VEGF was found to be significantly elevated in the former group (Table 4). With respect to MVC, there was non significant difference between patients with NB and those with GN. When patients with metastatic cancer (n =15) were

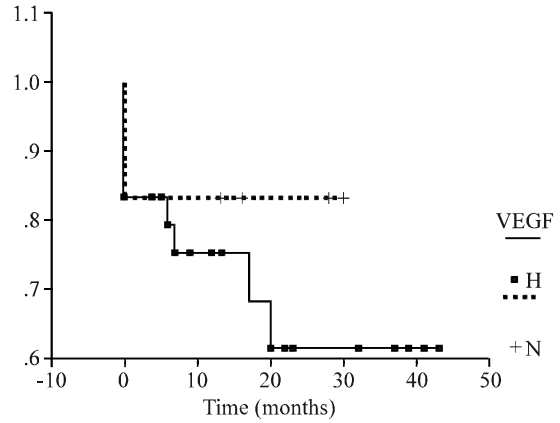

Fig. 6: Event-free survival of malignant solid tumors' patients in relation to serum VEGF levels

compared to those with non metastatic disease (n = 30), median VEGF levels and MVC tended to be higher in the former group, the difference did not reach statistical (Table 5).

After treatment (chemotherapy and/or surgery), mean serum concentrations of VEGF in 17 patients (11 NB and 6 W T) dropped significantly (from Mean±SD, median of 484.3±149.3, 403.6 pg mL^{-1} to 320.1±44.8, 328.9 pg mL^{-1}; $p<0.001$) as shown Fig. 4 but still significantly higher than control values ($p<0.001$).

CORRELATIONS

In healthy children, serum VEGF concentrations did not show any correlation with age (r = 0.09, p = 0.65). No significant correlation was observed between VEGF and either age (r = 0.106, p = 0.46) or tumor size (r = 0.08, p = 0.62) or metastasis number (r = 0.144, p = 0.32). In all cancer patients (n = 45), VEGF values correlated with MVC (r = 0.33, p = 0.022). In NB patients, VEGF correlated with LDH levels (r = 0.58, p = 0.014) and MVC correlated with NSE (r = 0.527, p = 0.02).

Follow up and survival analysis: Median follow up of the whole group was 18 months (range 6-44 months). At time of survival analysis, 31 patients were alive, with a mean follow up of 38 months (95th confidence interval 33.6-42.5) and five patients had died 6-30 months after diagnosis. Estimated OS and EFS of the whole group with malignant solid tumors patients were 69 and 64%, respectively.

Serum VEGF did not correlate with outcome for the whole group of patients; OS and EFS for patients with normal serum VEGF (n = 8) were 100 and 64.5% in comparison to 83.3% and 62% for those with elevated values; p = 0.4 and 0.5, respectively (Fig. 4-5).

Cox regression revealed that MVC ($p<0.001$) and stage of cancer ($p<0.001$) were highly associated with EFS of patients with embryonal tumors (all patients but rhabdomyosarcoma). When patients with RMS were included, such association was not observed, but the probability for serum VEGF was borderline ($p = 0.65$) (Fig. 6).

DISCUSSION

Currently, the prognosis of patients with cancer is judged by knowledge of probable length of survival and of incidence of recurrence for given stage. Therefore, the search for some marker that is invariably associated with good or poor prognosis is continuing, so that therapy can be still more accurately and appropriately adjusted to needs of a particular patient. This was considered while planning for the current study. To date, little is known regarding the role of angiogenic factors in pediatric solid tumors, a matter extensively investigated in adult cancer. To our knowledge, this is the first research registered in Egypt depicting the value of angiogenesis assessment in some pediatric solid tumor with respect to outcome. In the current cohort, serum VEGF concentrations were increased in pediatric patients with solid tumors when compared to healthy subjects ($p<0.001$). This result goes in agreement with others; Bredel *et al.* (1997), Tabone *et al.* (2001), Pavlakovic *et al.* (2001), Bentas *et al.* (2003), Barthlen *et al.* (2003), El-Houseini *et al.* (2004), Sampath *et al.* (2004), Dina *et al.* (2005) and Sun *et al.* (2005).

Vascular endothelial growth factor promotes growth of malignant cells in several ways: by enhancing delivery of oxygen and nutrients through new vessel formation; by enriching the extracellular matrix through increased vessel permeability; and, in some circumstances, by a direct receptor-mediated effect. It is a mitogen for endothelial cells that promotes as well their survival *in vitro* and *in vivo* (Parikh and Ellis, 2004).

Serum VEGF levels were elevated in the present cohort with lack of correlation with type of malignant solid tumor ($p = 0.36$). This is supported by the finding of a non significant difference in median micro vessel count (MVC, $p = 0.63$) in various histologic subtypes of those patients. Similarly, El-Houseini *et al.* (2004) (Ewing's sarcoma, osteosarcoma, neuroblastoma and rhabdomyosarcoma), Yamamoto *et al.* (1996) (breast and gastric cancer) and Dirix *et al.* 1997 (colorectal, breast, ovarian and renal carcinoma) found that the rate of elevation of serum VEGF was not related to the tumor type.

To further explore the significance of angiogenesis in malignant solid tumors, we compared patients with NB versus those with GN regarding serum and tissue angiogenic markers. Those with NB displayed higher serum VEGF but MVC was comparable to those with GN ($p = 0.005$, $p = 0.257$, respectively). The latter result might be due to lower MVC in patients with NB as tissue specimens were obtained after chemotherapy effect. Our result might indicate the importance of serum VEGF as a tumor marker differentiating benign from malignant solid tumors. In addition to its effect on angiogenesis, VEGF may affect neuroblastoma cell growth directly and could be an autocrine growth factor (Langer *et al.*, 2000).

The capacity of tumor cells to induce angiogenesis does not always correlate with malignancy and there is considerable confusion in the literature about this fact. Adrenal adenoma, for example, is a benign tumor that is highly angiogenic (Folkman, 1990). Contrary to other types of cancer, pancreatic endocrine tumours are highly vascularised, but poorly angiogenic tumours. As they progress, VEGF expression is lost and MVD significantly decreases. The regulation of hypoxia-inducible factor signaling appears to be specific in pancreatic endocrine tumors (Couvelard *et al.*, 2005). However, patients with high grade malignancies, being characterized by fast progression, were found to have about five times higher VEGF serum levels than patients with low-grade sarcomas or lymphomas (Dirix *et al.*, 1997). The levels of VEGF were significantly higher in high-grade astrocytomas than in nonastrocytic tumors in the series of Sampath *et al.* (2004).

A clinically relevant observation in the current work was that patients with metastatic disease exhibited higher serum VEGF and MVC than those with localized disease ($p = 0.54$ and $p = 0.4$, respectively), although difference did not reach statistical significance. The percentage of elevated serum VEGF above cut-off value among patients with metastatic disease (87%) was comparable to that of localized disease (87%, $p = 0.58$). This result was not surprising because most studied patients with localized disease (80%) belonged to late stages (stage III in 16 out of 20). This observation supports the role of angiogenic factors in advanced cancer, not only metastatic but also locally disseminated disease.

Kayton *et al.* (1999) reported that absolute serum VEGF levels increased as primary tumors grow and VEGF production was significantly associated with tumor metastasis in both clinical and experimental Wilms' tumor. Significantly higher expression levels of VEGF were found in advanced-stage neuroblastoma (stages 3 and 4) compared to low-stage tumors (stages 1 and 2) (Eggert *et al.*, 2000). Serum VEGF was suggested to serve as a diagnostic tool in advanced stage NB (Fakhari *et al.*, 2002). Similar results were reported

by El-Houseini *et al.* 2004 in a group of children with malignant solid tumors (NB, RMS, Ewing's sarcoma, and osteosarcoma). A tendency of higher vessel densities in retinoblastoma presenting with metastasis (stage IV) at time of diagnosis was observed by Rossler *et al.* (2004). VEGF was expressed in 70% of Langerhans cell histiocytosis patients. All the multisystem lesions were VEGF producers (Dina *et al*., 2005). Clinically aggressive favorable histology Wilms tumor displayed progressive alteration in p53/TSP-1 status and upregulation of VEGF. Such alteration was observed in the unfavorable histology tumor, but was absent from the standard-risk favorable histology tumor. Xenografts from clinically aggressive tumors displayed brisk neoangiogenesis and yielded lung metastases (Huang *et al*., 2002).

There is a direct correlation between the number of capillaries visible in histologic sections of tumors and their metastatic behavior. Reports indicate that counting the number of capillaries per square millimeter in the most highly vascularized areas of a tumor section provides an important independent variable predicting prognosis of patients with lung cancer (Macchiarini *et al*., 1992) or breast cancer (Bosari *et al.,* 1992). In patients with node-positive breast carcinoma, MVC were considerably higher in tumors from patients who experienced distant recurrence than in those who did not, although the difference did not reach statistical significance (Bosari *et al*., 1992).

In the current study, after successful therapy and attaining a state of complete remission, levels of VEGF decreased significantly to values ($p = 0.003$) which were still higher than controls ($p<0.001$). Drop of serum VEGF after therapy suggests that the source of which might be present in the tumor tissue. Similarly, results of Pavlakovic *et al.* (2001), showed that elevated pretherapy levels of b-FGF and vascular endothelial growth factor (serum and urine) declined, but to levels present in healthy subjects. Lin *et al.* (1995) found that high postoperative b-FGF level may indicate recurrence or persistence of WT. Thus, monitoring the levels of VEGF and possibly other angiogenic peptides, in the urine, serum, or CSF of patients with cancer may prove to be a useful marker for tumor status and perhaps prognosis. Tumor cells may overexpress angiogenic factors, may mobilize angiogenic proteins from the extracellular matrix, may recruit host cells such as macrophages which then produce their own angiogenic molecules/or may engage in a combination of these processes (Folkman, 1997). Measurement of various angiogenic factors in body fluids will most likely be useful as a monitor of therapy and/or predictor of outcome (Nguyen, 1997).

In adult cancer, removal of a primary colorectal adenocarcinoma clearly decreased individual serum levels of VEGF (Dirix *et al*., 1997). The same observation was made by Yamamoto *et al.* 1996 in primary breast cancer. Furthermore, serum VEGF appears to be a useful marker for monitoring the clinical course after surgery for breast (Yamamoto *et al*., 1996) and ovarian cancers (Yamamoto *et al*., 1997).

Contrary to what was expected, no correlation was observed in the current study between tumor size (by computed tomography) and either VEGF or MVC whether in solid tumor group or each patients' group individually. The limited number of patients evaluated in this pilot study must be borne in mind, but solid tumor heterogeneity of vascularity could not be ignored.

A significant correlation was observed in the current work between serum VEGF and tissue MVC ($r=0.330$) which might implicate that serum VEGF determination could be used as a surrogate marker of tumor angiogenesis. Ghanem *et al*., 2003 concluded that VEGF and its receptor Flt-I protein expression were closely related to MVC in nephroblastoma patients. Serum VEGF concentration in 137 breast cancer patients was associated with microvessel density wherein patients with less than 100 microvessels per mm^2, 2.9% had elevated serum VEGF. In those with more than 150 microvessels per mm^2 42.9% had an elevated VEGF serum levels (Yamamoto *et al*., 1996). Indirectly, VEGF serum levels might thus be related with prognosis as high MVC has been associated with shortened survival in many studies (Gasparini and Harris, 1995).

Present study has demonstrated that serum VEGF did not correlate with outcome for the whole group of patients. In the study of Tabone *et al.* (2001) conducted on children with solid tumors (NB, WT, osteosarcoma and Ewing's sarcoma), the estimated event-free survival was 71% in case of normal serum VEGF ($n = 20$) versus 38% ($n = 19$) for those with high levels.

Cox regression analysis revealed that MVC and stage of cancer were the only among prognostic factors that were independently highly associated with event-free survival of current patients with embryonal tumors (NB, WT, HB and RB, $N = 37$). Such an association was not observed when patients with RMS were included. The independence of MVC from several other prognostic factors could further enhance its clinical usefulness. The practical implication is that, the microvessel quantitation will have to be weighed with the other prognostic indicators.

In the study of Abramson *et al.* (2003), Kline and Sevier (2003) increased MVC was found to identify

Wilms' tumor patients at risk for relapse, especially those with favorable histology tumors. In the latter study, MVC was the only predictor of relapse when compared to age, sex, tumor weight and histology. Ghanem *et al.* (2003) reported that MVC was predictive of clinical progression in nephroblastoma patients. Intratumoral vessel density was also positively related with Event Free Survival (EFS) in childhood embryonal rhabdomyosarcoma (RMS); mean EFS was 20.8 months (Diniz *et al.*, 2004). Children with malignant liver tumors, especially with hepatocellular carcinoma, may have extensive angiogenesis, reflected by significant expression of VEGF and high MVC, that induced a rapid tumor growth and led to a poor prognosis (Sun *et al.*, 2005).

In pediatric patients with another solid tumor, complete and partial response rate to therapy was significantly higher in VEGF-negative patients than in the VEGF-positive non Hodgkin lymphoma patients (Hazar *et al.*, 2003). However, vascular parameters adequately determined by a computerized system in neuroblastoma were not predictive of survival and neither disseminated nor local relapses were influenced by the angiogenic characteristics of the tumors as reported by Canete *et al.* (2000).

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