

# D2-40 labeling in lymphangiomyoma/lymphangiomyomatosis of the soft tissue: further evidence of lymphangiogenic tumor histogenesis

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**Abstract** We examined ten cases of extrapulmonary lymphangioliomyoma/lymphangioliomyomatosis (LAM; all patients female; median age 46.5 years) for immunohistochemical labeling with a monoclonal antibody against podoplanin (D2-40), which is specific for lymphatic endothelial lining. We found positive staining in thin-wall branching vessels reflecting the lymphatic nature of tumor vessels in all cases tested. In contrast, perivascular (HMB-45 positive) myoid cells were not detected by D2-40. The D2-40 labeling confirms the current concept of lymphangiogenic origin of the tumor vessels in LAM. In addition, this study makes a further contribution to the immunohistochemical mapping of this antibody in vascular tumors. Finally, the use of this commercially available antibody provides an additional help in the differential diagnosis of LAM from other soft tissue tumors.

**Keywords** Lymphangi(leiomyoma) · Lymphangi(leiomyomatosis) · *Podoplanin* · D2-40 · Lymphatic tumor vessels

## Introduction

Lymphangi(leiomyoma) is a rare disorder that nearly always affects women, mostly in the premenopausal period

[5, 17]. This tumor may occur as a localized form or as a widespread multisystem disease, then called lymphangi(leiomyomatosis) (LAM). Lymphangi(leiomyoma)/LAM belongs to the tumor family of so-called perivascular epithelioid cell tumors (PEComas), i.e., they show perivascular epithelioid cell differentiation [6, 10]. Besides LAM, this group comprises angiomyolipoma as well as pulmonary and extrapulmonary clear cell “sugar” tumors. The concept of this tumor family is based on perivascular epithelioid cells with myomelanocytic origin [6, 10, 17]. Therefore, these cells show a characteristic positivity for smooth-muscle actin and variably desmin as well as for the (pre-)melanosomal marker HMB-45 and/or melan-A [6, 10].

LAM is different from the other PEComas in that it always contains large numbers of thin-walled branching vessels, sometimes surrounded by lymph follicles [6, 16, 17]. It was therefore proposed that these vessels are of lymphatic origin. This was confirmed by the fact that especially extrapulmonary LAMs are very often closely associated with lymph nodes or large lymphatic vessels such as the thoracic duct [16]. In addition, Kumasaka et al. [13] showed strong expression of vascular endothelial growth factor receptor-3 (VEGFR-3) in the vessels of LAM. This molecule has been demonstrated to be a marker for lymphatic endothelial cells in human tissues. Kumasaka et al. furthermore showed extensive lymphangiogenesis in association with the myoid cells [13].

Besides VEGFR-3, a new monoclonal antibody, D2-40, plays an increasing role in the routine diagnostics of vascular soft-tissue tumors. D2-40 is directed against an oncofetal antigen (M2A antigen) [15]. This protein was subsequently identified on the surface of rat glomerular podocytes, and because it was found to be involved in the flattening of foot processes in puromycin-induced nephro-

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sis, it was named podoplanin [18]. Recently, D2-40 has been shown to react with a fixation-resistant epitope on lymphatic endothelium [12]. It is now considered to be the best commercially available antibody for the delineation of lymphatic endothelial cells, and has been tested for several vascular tumors [12]. Although positivity has been found for lymphangioma [8, 11], Kaposi's sarcoma [8, 11], kaposiform hemangioendothelioma [4], hobnail hemangioma [7], and a subset of angiosarcomas [8, 11], labeling could not be demonstrated for hemangiomas, epitheloid hemangioendotheliomas, angioliipomas, and glomus tumors [8, 11]. Recently, sinonasal-type hemangiopericytoma could be further separated from the family of solitary fibrous tumors/hemangiopericytomas by the fact that the former commonly revealed strong labeling, whereas the latter did not at all [9]. However, until now, there are no reports concerning D2-40 labeling in LAM. We therefore analyzed LAM for D2-40 staining to contribute to immunohistochemical mapping of D2-40 in soft-tissue tumors and to confirm the lymphatic origin of this distinct tumor entity.

## Materials and methods

The records from the Soft Tissue Tumor Registry of the Institute of Pathology in Jena and the records of the Institute of Pathology in Mainz were searched for cases coded as LAM of the soft tissue from 1995 to the present. We found ten cases (listed in Table 1) with a median age of 46.5 years. All patients were female. Tumors were mostly localized in the retroperitoneum ( $n=8$ ), whereas one case was found in subcutaneous fat tissue of the neck, and one case was from the mediastinum. All tumors except one (patient no. 3) were found to diffusely involve soft tissues leading to the diagnosis of "lymphangioleiomyomatosis."

All specimens were prepared for routine histology using hematoxylin and eosin staining according to standard

protocols. In brief, all tumors evaluated showed bundles of myoid cells, which were mostly arranged in a fascicular pattern. These bundles were surrounded by thin-walled and branching vessels (Fig. 1). Thus, these tumors showed the common and recently recapitulated morphological features of LAM [10].

Immunohistochemical staining was performed according to previous protocols [9] using the alkaline phosphatase-antialkaline phosphatase method. Besides D2-40, tissue specimens were investigated for expression of CD31, HMB-45, and  $\alpha$ 1-smooth-muscle actin (ASMA). Primary antibodies applied for immunohistochemical analysis are summarized in Table 2. D2-40 (Signet, Dedham, USA) was applied in a 1:400 dilution. Pretreatment by microwave (600 W,  $3 \times 5$  min) was performed before the immunohistochemical procedure. Labeling was semiquantitatively analyzed and scored by one investigator (T.H.) as follows: no vessels/cells positive (=0), less than 20% of vessels/cells positive (=1), greater than 20% and less than 50% of vessels positive (=2), and greater than 50% of vessels positive (=3). Slides were evaluated and photographed by a Zeiss microscope (type Axiophot, Zeiss, Jena, Germany; digital camera Camedia, Olympus, Hamburg, Germany).

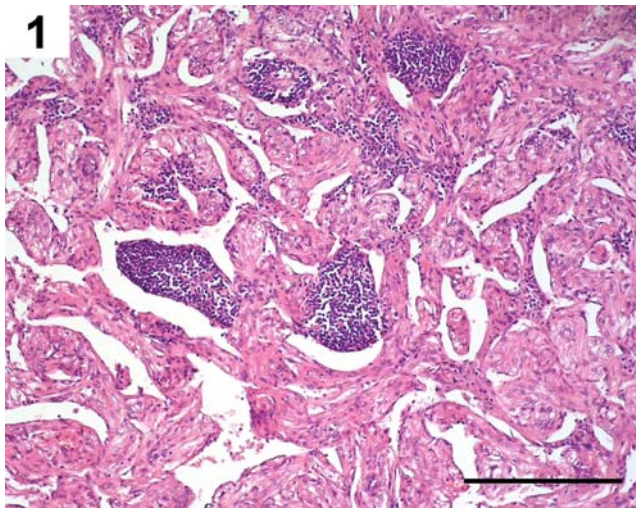
## Results

In all cases, the typical morphological features of LAM were found as summarized above and shown in Fig. 1. Analysis of the immunohistochemical staining is summarized in Table 1. Strong expression of ASMA was demonstrated in the majority of myoid cells in nearly all patients (with more than 50% of cells positive in eight cases). In addition, this molecule was also strongly labeled in smooth muscle cells in the blood vessels (Fig. 2). By contrast, staining for HMB-45 was less intensive in the myoid cells. Most commonly, a diffuse staining pattern

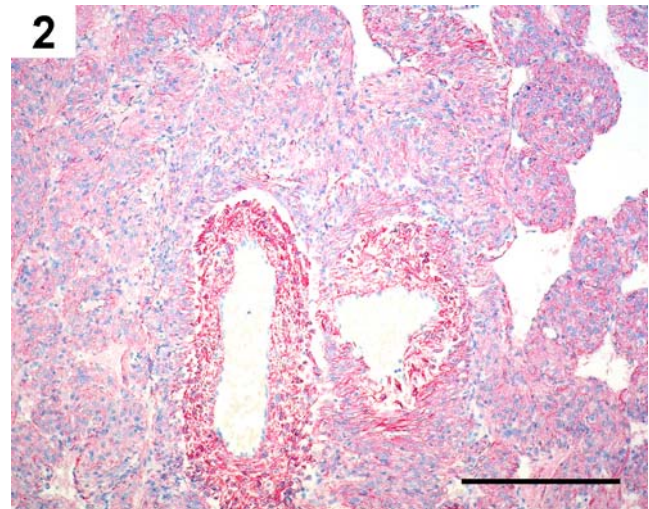
**Table 1** Patients' characteristics and scoring of the immunohistochemical labeling

Patient no.	Age	Sex	Localization	D2-40	CD31	ASMA	HMB-45
1	35	F	Retroperitoneum (neighborhood of vena cava)	2	2	3	1
2	75	F	Retroperitoneum (neighborhood of A. ilicia com)	3	3	3	ND
3	43	F	Retroperitoneum (NFS)	1	3	3	1
4	35	F	Retroperitoneum (NFS)	2	2	2	2
5	41	F	Mediastinum (between esophagus and parietal/visceral pleura)	2	ND	3	2
6	ND	F	Retroperitoneum (neighborhood of iliacal lymph nodes)	3	ND	3	2
7	51	F	Retroperitoneum (neighborhood of the left renal hilus)	2	ND	3	1
8	32	F	Retroperitoneum (NFS)	3	3	3	1
9	41	F	Retroperitoneum (lateral pelvis)	3	1	3	1
10	66	F	Right neck	3	2	2	1

ASMA  $\alpha$ 1-smooth-muscle actin, F female, ND no data; NFS not further specified



**Fig. 1** Histological overview shows multiple branching vessels arranged around bundles of myoid cells as well as lymph follicles (HE; patient no. 9; bar=200  $\mu$ m)



**Fig. 2** Immunohistochemical staining of smooth-muscle actin (red) reveals strong positivity in the myoid cells as well as in the media of two larger blood vessels (APAAP method; center of the picture; patient no. 8; bar=200  $\mu$ m)

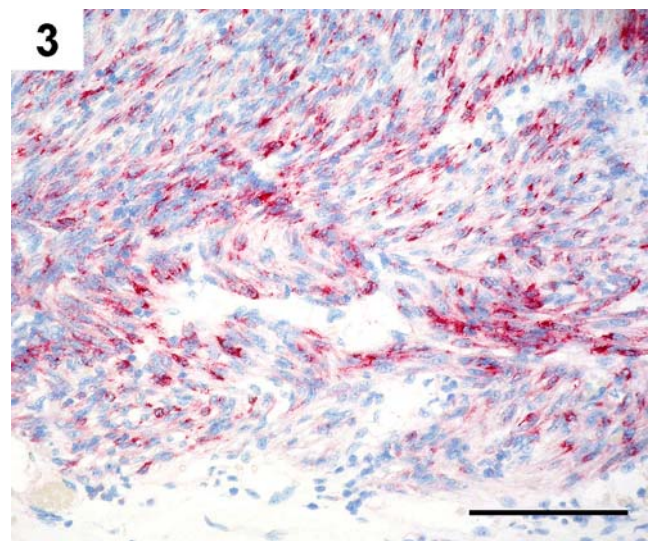
occurred in bundles of myoid cells, whereas vascular smooth muscle cells generally did not display HMB-45 positivity (Fig. 3). Moreover, CD31 was detected in the majority of thin-walled branching vessels between the myoid cells (see Table 1). Typically, linear staining pattern was seen. In addition, positivity for CD31 was also found in numerous blood vessels again exhibiting a linear staining (Fig. 4).

D2-40 labeled vessels occurred in all cases examined. In particular, the staining reaction was found exclusively in the thin-walled branching vasculature (Fig. 5). By contrast, neither fascicular bundles of myoid cells nor lymphocytes arranged in follicles in the vicinity of the bundles revealed any positivity. In the vessels, we commonly observed a linear staining of the thin cytoplasm of the endothelial cells, although nuclei were typically spared (Fig. 5). Some blood vessels were interspersed between myoid bundles and thin-walled vessels. These did not reveal positivity for D2-40 (Fig. 5). Semiquantitative analysis revealed five LAMs with more than 50% of vessels positive; four cases were found with more than 20% and less than 50% of vessels positive, whereas there was only one patient (no. 3) with less than 20% of the vessels positive. In the vicinity of the tumors,

there were some residual thin-walled vessels without intraluminal erythrocytes, which were interpreted as lymphatic vessels, and which revealed D2-40 labeling as well (Fig. 6). However, residual blood vessels did not show any positivity for this marker.

## Discussion

In contrast to the other tumors comprising the group of the so-called “PEComas,” LAM shows large numbers of thin-walled branching vessels, which have been suggested to be of lymphatic origin [6, 16, 17]. D2-40 is a (relatively novel) monoclonal antibody directed against podoplanin, which



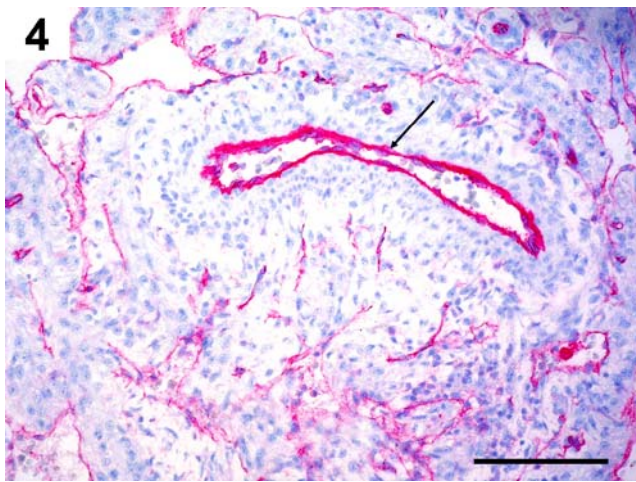
**Fig. 3** HMB-45 (red) reveals a focally distributed strong expression in the myoid cells (APAAP method; patient no. 5; bar=100  $\mu$ m)

**Table 2** Data on the primary antibodies used for this study

Marker (manufacturer)	Clone	Dilution	Pretreatment
ASMA (Dako, Glostrup, Denmark)	Clon 1A4	1:600	None
CD31 (Dako)	JC70A	1:50	MW
D2-40 (Signet)	D2-40	1:400	MW
HMB-45 (Dako)	HMB-45	1:200	Trypsin

ASMA  $\alpha$ 1-smooth-muscle actin, MW microwave pretreatment (600 W, 3 $\times$ 5 Min)

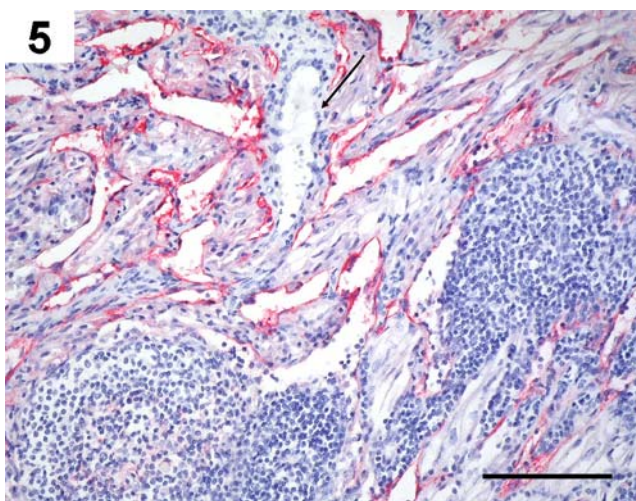




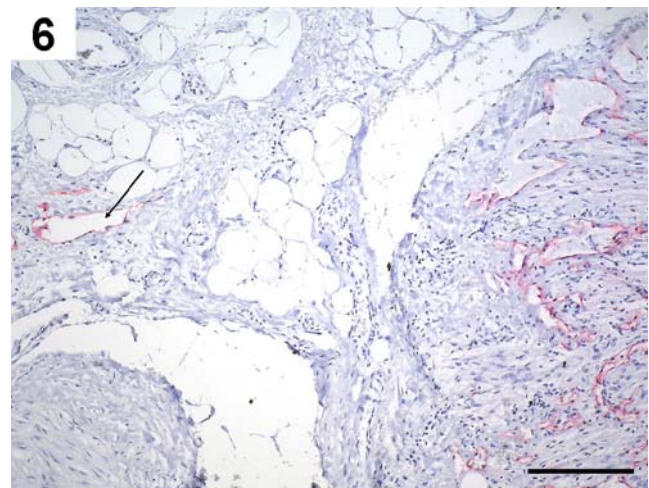
**Fig. 4** CD31 (red) is detected in numerous vessels: Besides the thin-walled branching vessels between myoid cell bundles, there is also a blood vessel (arrow) with a strong linear positivity at the luminal side reflecting the endothelial lining (APAAP method; patient no. 8; bar=100  $\mu$ m)

has been shown to be specific for lymphatic endothelial lining [8–11]. In the present study, we investigated the D2-40 in LAMs to further contribute to the immunohistochemical mapping of this antibody for vascular tumors, and moreover, to confirm the lymphatic origin of this distinct tumor entity. We examined (extrapulmonary) cases of LAM of the soft tissue only, because previous examinations by Matsui et al. [16] showed distinct differences between pulmonary and extrapulmonary LAMs, principally regarding the histological features.

In nearly all LAM cases, D2-40 strongly labeled a large numbers of vessels both in the tumor areas as well as in surrounding vessels in the neighborhood of the tumor. Histological differences between negative and positive



**Fig. 5** Immunohistochemical labeling of D2-40 (red) demonstrates cytoplasmic staining in numerous positive thin-walled vessels. Note negative blood vessel (arrow; APAAP method; patient no. 9; bar=100  $\mu$ m)



**Fig. 6** D2-40 (red) is also labeled in the residual lymphatic vessels (arrow). LAM is revealed at the right part of the image (APAAP method; patient no. 7; bar=200  $\mu$ m)

vessels were not found. It is known that D2-40 in some cases does not reveal a homogenous labeling of lymphatics. Furthermore, D2-40 does not detect lymph vessels only, but also reacts with several other cell types such as mesothelial cells, myofibroblasts, and some kinds of gonadal tumors (for review see Ordonez [18]). However, in comparison with the other commercially available vascular markers, D2-40 is generally accepted as an antibody that does react with lymphatic-derived vascular tumors with the highest sensitivity. In our study, CD31 showed strong expression with lymph vessels, but on the other hand, this molecule was also labeled in numerous blood vessels (which is a typical staining pattern as described previously [19]). Taken together, these firstly presented data of D2-40 in LAM contribute to the immunohistochemical mapping of this marker in vascular soft-tissue tumors. They are in line with the general view that lymphangiogenic origin of vascular tumors can be ascertained by this marker.

The LAM cases presented in our study show a similar local distribution as those in the study of Matsui et al. [16]. Thus, extrapulmonary LAMs can be observed in three major locations: the mediastinum, the upper retroperitoneum, and the pelvic cavity (a cervical localization, as presented in our study, has been described only in one prior case [1]). In general, the specific histological features of LAM lead to the correct diagnosis. Because of the presented restricted locations, a few distinct tumor entities have to be excluded: In the case of the localization in the upper retroperitoneum (especially in the neighborhood of the kidneys), one has to bear in mind that leiomyomas of the kidney, particularly of the renal capsule, can show reactivity for HMB-45 [2], and thus can be misinterpreted as a tumor of the PEComa family. In contrast to LAM, lymph vessels do not comprise major portions of renal

leiomyomas, and therefore, D2-40 can aid in the differentiation between both diseases.

Because extrapulmonary LAMs are closely associated or even embedded in the regional (pelvic or iliacal) lymph nodes, metastasis of leiomyosarcoma is a further differential diagnosis. In fact, data concerning the incidence of lymph node metastasis in patients with uterine sarcomas are controversial, reaching from 45% (9/20 patients) of uterine stage I sarcomas in the study of Chen [3] to 0% (0/27 patients) of lymph node metastases of stage I/II uterine leiomyosarcomas in the study of Leitao et al. [14]. Nevertheless, for purposes of microscopical diagnosis, one should consider this possible pitfall, which can be solved by careful examination for signs of atypia (as a common finding for leiomyosarcoma) on the one hand, and numerous D2-40 positive branching lymphatic vessels arranged between myoid bundles without signs of atypia (as a common finding for LAM) on the other hand.

In summary, we examined ten cases of extrapulmonary LAM for labeling of D2-40 and contributed to the further immunohistochemical mapping of this marker. D2-40 positivity was revealed in all LAMs tested and confirms the current concept of lymphangiogenic origin of the tumor vessels in this neoplasm. Furthermore, the use of this commercially available antibody provides an additional help in the differential diagnosis of LAM from other soft-tissue tumors.

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## References

- Betlejewski S, Burduk D, Szukalski J (2004) Lymphangioma and lymphangiomyomatosis—the same lesion? *Otolaryngol Pol* 58:903–906
- Bonsib S (1996) HMB-45 reactivity in renal leiomyomas and leiomyosarcomas. *Mod Path* 9:664–669
- Chen SS (1989) Propensity of retroperitoneal lymph node metastasis in patients with stage I sarcoma of the uterus. *Gynecol Oncol* 32:215–217
- Debelenko LV, Perez-Atayde AR, Mulliken JB, Liang MG, Archibald TH, Kozakevich HP (2005) D2-40 immunohistochemical analysis of pediatric vascular tumors reveals positivity in kaposiform hemangioendothelioma. *Mod Path* 18:1454–1460
- Fiore MG, Sanguedolce F, Lolli I, Piscitelli D, Ricco R (2005) Abdominal lymphangioliomyomatosis in a man with Klinefelter syndrome: the first reported case. *Ann Diagn Pathol* 9:96–100
- Folpe AL (2002) Neoplasms with perivascular epithelioid cell differentiation (PEComas). In: Fletcher CDM, Unni KK, Mertens F (eds) WHO classification of tumours of soft tissue and bone. IARC, Lyon, pp 221–222
- Franke FE, Steger K, Marks A, Kutzner H, Mentzel T (2004) Hobnail hemangiomas (targetoid hemosiderotic hemangiomas) are true lymphangiomas. *J Cutan Pathol* 31:362–367
- Fukunaga M (2005) Expression of D2-40 in lymphatic endothelium of normal tissues and in vascular tumors. *Histopathology* 46:396–402
- Hansen T, Katenkamp K, Katenkamp D (2006) D2-40 staining in sinonasal-type hemangiopericytoma—further evidence of distinction from conventional hemangiopericytoma and solitary fibrous tumor. *Virchows Arch* 448:459–462
- Hornick JL, Fletcher CDM (2006) PEComa: what do we know so far? *Histopathology* 48:75–82
- Kahn HJ, Bailey D, Marks A (2002) Monoclonal antibody D2-40, a new marker of lymphatic endothelium, reacts with Kaposi's sarcoma and a subset of angiosarcomas. *Mod Path* 15:434–440
- Kaiserling E (2004) Immunohistochemical identification of lymph vessels with D2-40 in diagnostic pathology. *Pathologe* 25:362–374
- Kumasaka T, Seyama K, Mitani K, Sato T, Souma S, Kondo T, Hayashi S, Minami M, Uekusa T, Fukuchi Y, Suda K (2004) Lymphangiogenesis in lymphangioliomyomatosis. Its implication in the progression of lymphangioliomyomatosis. *Am J Surg Pathol* 28:1007–1016
- Leitao MM, Sonoda Y, Brennan MF, Barakat RR, Chi DS (2003) Incidence of lymph node and ovarian metastases in leiomyosarcoma of the uterus. *Gynecol Oncol* 91:209–212
- Marks A, Sutherland DR, Bailey D, Iglesias J, Law J, Lei M, Yeger H, Banerjee D, Bauml R (1999) Characterization and distribution of an oncofetal antigen (M2A antigen) expressed on testicular germ cell tumors. *Br J Cancer* 80:569–578
- Matsui K, Tatsuguchi A, Valencia J, Yu ZX, Bechtel J, Beasley MB, Avila N, Travis WD, Moss J, Ferrans VJ (2000) Extrapulmonary lymphangioliomyomatosis (LAM): clinicopathologic features in 22 cases. *Human Pathol* 31:1242–1248
- Miettinen M (ed) (2003) Diagnostic soft tissue pathology. Churchill Livingstone, Philadelphia, pp 248–249
- Ordóñez NG (2006) Podoplanin: a novel diagnostic immunohistochemical marker. *Adv Anat Pathol* 13:83–88
- Pusztaszeri MP, Seelentag W, Bosman FT (2006) Immunohistochemical expression of endothelial markers CD31, CD34, von Willebrand factor, and flt-1 in normal human tissues. *J Histochem Cytochem* 54:385–395