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RESEARCH

Lymphatic Differentiation in Classic Kaposi's Sarcoma: Patterns of D2-40 Immunoexpression in the Course of Tumor Progression

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Abstract The recent development of lymphatic endothelium-specific immuno-indicators has given rise to research on the histogenesis of Kaposi sarcoma (KS), specifically focusing on its lymphatic root and differentiation. D2-40 is a new lymphatic marker that recognizes podoplanin and is easily applied to formalin-fixed paraffin-embedded human tissues. This study examined D2-40 immunoexpression in 178 classical KS lesions using immunohistochemical methods. D2-40 immunoexpression was also examined in 63 non-KS soft tissue lesions to test the reliability of D2-40 monoclonal antibody in the pathological diagnosis of KS. D2-40 immunoreactivity was detected at all of the KS lesions and in lymphangioma and nonneoplastic lymphatic endothelium. There was no significant relationship between the extent of D2-40 staining and histopathological stage; however, there was a positive correlation between the staining intensity and histopathological stage in KS cases. D2-40 immunoreactivity was detected at all histopathological stages of KS and may be added to the routine immunohistochemical panel used for the differential diagnosis of KS. Widespread D2-40 protein expression is evidence of a lymphatic origin or the differentiation of neoplastic cells in KS, and D2-40 expression increases with tumor progression.

Keywords D2-40 · Immunohistochemistry · Kaposi sarcoma · Lymphatic · Podoplanin

Abbreviations

ABC-DAB	Avidin biotin complex-diaminobenzidine
AIDS	Acquired Immune Deficiency Syndrome
factor VIII-ra	factor VIII-related antigen
H&E	hematoxylin and eosin
HHV-8	human herpesvirus 8
IHC	immunohistochemistry
KS	Kaposi sarcoma
LNA-1	latent nuclear antigen-1
mAb	monoclonal antibody
SCs	spindle cells

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Introduction

Kaposi's sarcoma (KS) is a slowly progressing multifocal vascular neoplasia [1]. Although cutaneous lesions are the most common symptoms, the lymph nodes and visceral organs may become involved during the course of the disease [2]. The progression of lesions, characterized by an increase in spindle cells (SCs), is composed of early, intermediate, and late stages [3]. The four known epidemiological forms of KS (classic, endemic, acquired immune deficiency syndrome [AIDS]-related, and iatrogenic) have common histopathological features [4–6]. Human herpesvirus 8 (HHV-8) is a common etiological factor for all epidemiological forms of KS [7–9].

The histogenesis of KS has been debated for many years. When the disease was first defined, the tumors were thought to originate from blood vessel endothelium [10–17]. Recent trials, however, have shown that lymphatic origin or differentiation may play a key role in the histogenesis of KS [18–24]. Some researchers have claimed that the neoplastic cells of KS originate from endothelial progenitor cells, and that blood and lymphatic vessel endothelium may each differentiate into both cell types [25].

Many vascular endothelial markers are conventionally used to diagnose KS. The most commonly used indicators include factor VIII-related antigen (factor VIII-ra), CD34, CD31, Flt-1, and vascular endothelial growth factor receptor [1–3, 13–16, 21]. Recently, the production of lymphatic endothelium-specific antibodies that can be applied to formalin-fixed and paraffin-embedded tissues has accelerated studies on lymphangiogenesis. Lymphatic endothelium-specific markers include lymphatic vessel endothelial receptor-1, Prox1, desmoplakin, and podoplanin [18, 19, 21]. D2-40, a monoclonal antibody (mAb) with an IgG2a structure, has been produced against an oncofetal antigen (M2A) that is present in fetal testis and is re-expressed on germ cell neoplasms. The D2-40 antibody specifically recognizes podoplanin, a glomerular podocyte membrane protein. It has been shown to be very sensitive to lymphatic endothelium in normal and neoplastic lesions [17–21]. D2-40 immunoreactivity has been reported for the vascular tumors of KS, Dabska tumors, and some angiosarcomas [26–28]. Only a limited number of trials have assessed the lymphatic origin and differentiation of KS. Furthermore, most trials have focused on AIDS-related KS [13–16, 21–24].

This study used the D2-40 mAb to assess the relationship between tumor progression and lymphatic differentiation in a large series of classic KS cases. We also tested the reliability of the D2-40 mAb for the histopathological diagnosis of KS.

Materials and Methods

Sample Selection

The study sample included 178 classic cutaneous KS cases (43 early, 64 intermediate, and 71 late stages) in 75 patients who were diagnosed in the pathology departments of the University of Karaelmas, Atatürk State Hospital, and Dr. Lütfi Kırdar Training and Research Hospital between 2001 and 2010. All patients with KS were HIV-1 seronega tive. The study group also included 33 non-KS vascular tumors or reactive vascular proliferations (5 lymphangiomas, 6 hemangiomas, 4 angiosarcoma, 5 pyogenic granulomas, 3 acroangiodermatitis, 3 spindle cell hemangioma, 2 Kaposiform hemagioendothelioma, 3 vascular malformations, 1 hemangiopericytoma, and 1 hemangioendothelioma) and 30 nonvascular soft-tissue tumors (5 leiomyomas, 2 leiomyosarcomas, 4 neurofibromas, 5 dermatofibromas, 5 dermatofibrosarcoma protuberans, 4 schwannomas, and 5 gastrointestinal stromal tumors).

The clinical data were obtained from patient files. The study was performed in accordance with the Helsinki Declaration, and the privacy of patients was protected by decoding of data, according to the privacy regulations of the Zonguldak Karaelmas University Hospital (Zonguldak, Turkey), Ataturk State Hospital (Zonguldak, Turkey), and Dr. Lutfi Kirdar Training and Research Hospital (İstanbul, Turkey).

Histopathological Evaluation and Formation of Research Groups

The histopathological diagnoses of all cases were confirmed through the examination of archived hematoxylin and eosin (H&E) slices and immunohistochemical survey results. KS cases were divided into three groups (early, intermediate, and late stages) on the basis of histopathological lesion stage [29] (Fig. 1). Non-KS soft-tissue lesions were divided into two groups, vascular and non-vascular lesions.

Immunohistochemical Analysis

Immunohistochemical observations of vascular lesions employed D2-40, CD34, CD31, factor VIII-ra, and HHV-8 mAbs. The observations of non-vascular soft-tissue lesions used only D2-40 mAb. All other immunoreactions needed for histopathological diagnosis were obtained from archived materials. Immunohistochemical studies were performed using archival materials. A set of 4-μm-thick tissue sections was prepared from formalin-fixed and paraffin-embedded blocks, deparaffinized in xylene, rehydrated, and microwaved for 10 min at 30% power in citrate

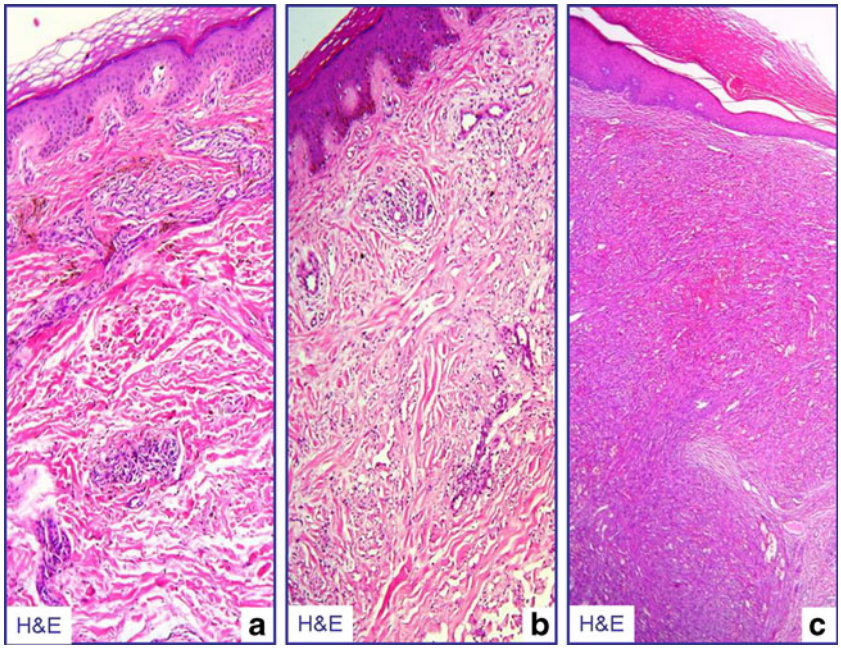

Fig. 1 Histological aspects of early **a**, intermediate **b** and late **c** stages in KS (a-c, H&E, ×100)

buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide.

The slides were then incubated at room temperature for 1 h with D2-40 [1:100 dilution, Clone D2-40; BioCare Medical, Concord, CA, USA), CD31 (1:50 dilution, Clone JC/7A; NeoMarkers, Freemont, CA, USA), CD34 (1:100 dilution, Clone QBEnd/10; NeoMarkers), factor VIII-ra (1:50 dilution, Clone F8/86; NeoMarkers), and HHV-8 (LNA-1) (1:50 dilution, Clone 13B10; Novocastra, Newcastle, UK), using the avidin biotin complex-diaminobenzidine (ABC-DAB) technique for detection. Negative controls were processed in parallel without primary antibody. The D2-40 immunoreactivity of lymphatic endothelial cells and the CD31, CD34, and factor VIII-ra immunoreactivity of capillaries in the upper dermis were used as internal positive controls.

Immunohistochemical Evaluation

KS Cases

The D2-40 immunoreactions of all KS cases were evaluated. We examined SCs, neoplastic vascular splitting of lesions at the intermediate and late stages, and neoplastic vascular structures of lesions at the early stage. The D2-40 immunoreactivity of each lesion compositional element [e.g., angiomatoid focus, lymphangiomatoid area, and sieve- and slit-like structures) was separately observed. The extent of D2-40 immunoreactivity was semiquantitatively graded according to the percentage of positive tumor cells: 0, indicated that fewer than 1% of tumor cells were reactive; 1+, 1–25% of cells; 2+, 26–50% of cells; 3+, 51–75% of cells; and 4+, >75% of cells. An additional score was assigned according to the intensity of staining: 0, colorless; 1, weak; 2, moderate; and 3, intense. The predominant staining patterns of D2-40 (membranous and/or cytoplasmic) were also recorded.

Non-KS Vascular and Non-vascular Lesions

The D2-40 immunoreactivity of the lesion cells in this category was assessed using the same criteria as described for the KS cases. Immunoreactions of non-neoplastic vascular components that took place within and around the lesions were evaluation.

Statistical Analysis

Statistical analysis was used to examine the relationships between KS histopathological stage and the extent of labeling, intensity of labeling, and predominant staining pattern of D2-40 immunoreactivity. The relationships between tumor histopathological stage and the extent of D2-40 labeling and predominant staining pattern were examined using a chi-square test ($\chi 2$), or Fisher's exact test when one or more cells contained less than five respondents. The correlation between tumor histopathological stage and D2-40 staining intensity was examined using a Pearson correlation test. The data were analyzed using SPSS for Windows, version 12. Values of $P<0.05$ were considered to indicate statistical significance.

Results

Non-neoplastic Lymphatics

D2-40 immunoreactivity was observed in the endothelia of dermal lymphatics adjacent to all lesions. These lymphatic vessels exhibited structural collapse and lacked erythrocytes within the lumen. Some vessels contained small numbers of lymphocytes. Mild to moderate densities of D2-40 immunoreactivity were observed on non-neoplastic lymphatic endothelia. The staining pattern was cytoplasmic. Capillary structures and small-diameter arterioles showed no D2-40 immunoreactions. The non-neoplastic lymphatic vessel endothelia did not react with antibodies to factor VIII-ra, CD34, CD31, or HHV-8.

KS Cases

The lesion cells of all KS cases showed various degrees of CD31, CD34, factor VIII-ra, and HHV-8 immunoreactivities. Positive D2-40 immunoreactivity (1+ or greater) was detected in all KS cases at the early (43/43) (Fig. 2), intermediate (64/64) (Fig. 3), and late (71/71) stages (Fig. 4). We found no significant relationship between histopathological stage and the extent of labeling of neoplastic cells for D2-40 immunoreactivity ($P>0.05$). However, the mean staining score was lower in early-stage lesions (1.9±0.5) than in intermediate-stage (2.5±0.5) and late-stage lesions (3.1±0.5). Staining intensity was significantly related to tumor histopathological stage ($r=0.40$, $P=0.016$). The D2-40 immunoreactivities of all stages of the KS lesions are shown in Table 1.

Cytoplasmic staining was observed in the neoplastic vascular structures of early- and intermediate-stage lesions, while membranous reactions were absent. Cytoplasmic staining patterns were dominant in the SCs of late-stage lesions. Weak membranous staining accompanied the cytoplasmic reaction in 10% (7/71) of late-stage cases. The SCs with membranous staining exhibited a notably wider cytoplasm, a vesicular nucleus, and prominent nucleoli compared with SCs in which cytoplasmic staining was detected (Fig. 5). We found no significant relationship between cellular localization of the D2-40 immunoreactivity and the histopathological stage of the lesions ($P>0.05$).

No D2-40 immunoreactions were observed on the well-developed central vessels at angiomatoid foci, but did occur in the capillary-like neoplastic vessel structures around the central vessels. Strong reactions to D2-40 were also observed on neoplastic endothelia protruding toward the lumen in lymphangiomatoid components, on sieve- and slit-like structures composed of SCs, and on neoplastic vessel structures with autolumination (paranuclear vacuoles containing erythrocytes) (Table 2, Fig. 6).

Non-KS Soft-Tissue Lesions

Vascular Lesions

Strong D2-40 immunoreactivity was observed on the endothelia covering sinusoidal elements in all lymphangioma cases (5/5), and D2-40 also stained one of four angiosarcomas. No D2-40 immunoreactions were observed on the lesion cells of hemangiomas (0/6), pyojenic granulomas (0/5), acroangiodermatitis (0/3), spindle cell hemangioma

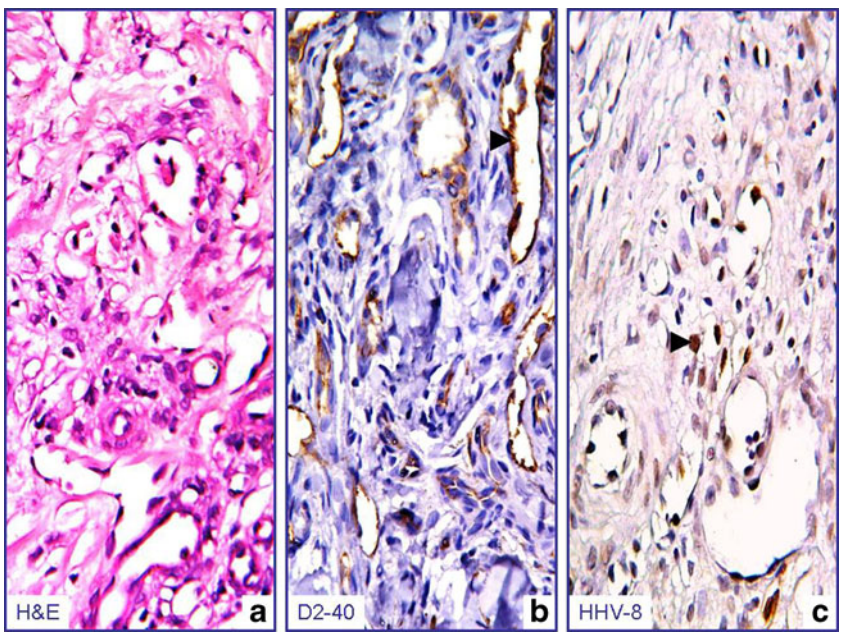

Fig. 2 Microphotographs of angiomatoid focus in early-stage KS. Well-developed capillary-like vascular structures standing back to back and adjacent to the central vessel are shown (a, H&E, ×400). A cytoplasmic immunoreaction with D2-40 (arrow) and nuclear immunoreaction with HHV-8 (arrow) are seen at the neoplastic vascular canals (b, D2-40; c, HHV-8; Immunohistochemistry [IHC], Avidin biotin complex-diaminobenzidine [ABC-DAB], ×400)

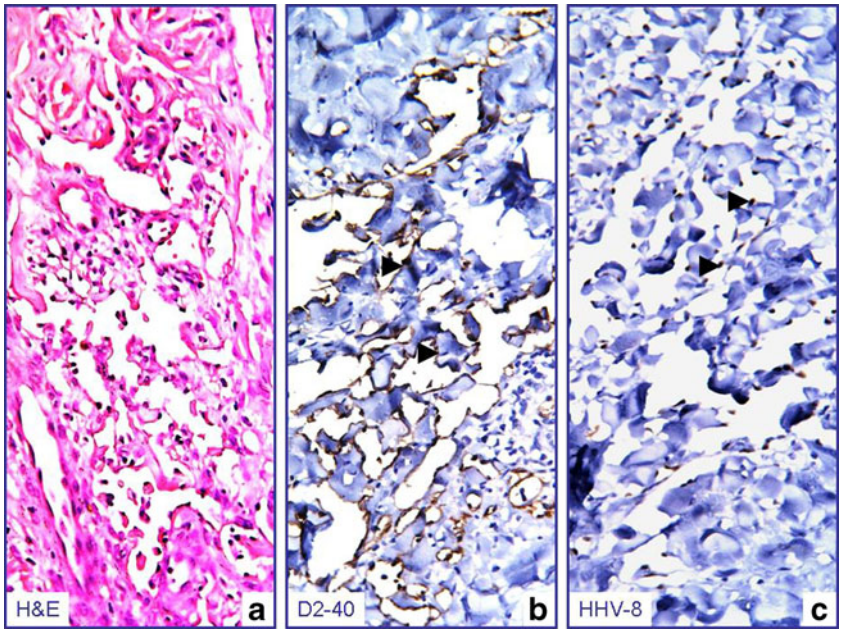

Fig. 3 Microphotographs of lymphangiomatoid components in intermediate-stage KS. Lymphatic-like vascular canals splitting dermal collagen and laid down by marked endothelial cells are shown (**a** H&E, ×400). A cytoplasmic immunoreaction with D2-40 (arrow) and nuclear immunoreaction with HHV-8 (arrow) are seen at endothelial cells (**b** D2-40; c, HHV-8; IHC ABC-DAB, ×400)

(0/3), Kaposiform hemagioendothelioma (0/2), vascular malformations (0/3), hemangiopericytoma (0/1), or hemangioendothelioma (0/1).

Non-vascular Soft-Tissue Tumors

No D2-40 immunoreactivity was observed in the neoplastic cells of leiomyomas (0/5), leiomyosarcomas (0/2), neurofibromas (0/4), dermatofibrosarcoma protuberans (0/5), schwannomas (0/2), or gastrointestinal stromal tumors (0/5) in the study samples. However, D2-40 stained two of five dermatofibromas.

Discussion

The classification of neoplastic lesions depends on first determining the correct immunohistochemical markers and cellular phenotypic features. The use of the correct indicators is particularly important for the diagnosis of soft-tissue lesions with atypical histomorphology. Although many endothelial indicators contribute to the diagnosis of vascular lesions, it has only recently become possible to discriminate the origin or differentiation of blood vessel and lymphatic endothelia [1, 26, 28–31]. D2-40 is a new lymphatic endothelium-specific marker that reacts with an O-linked sialoglycoprotein

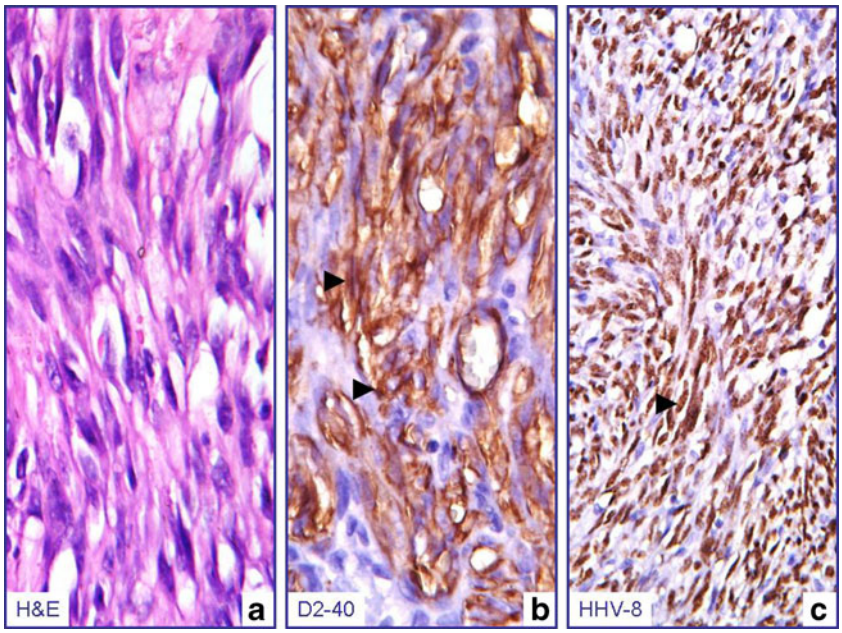

Fig. 4 Slit-like areas containing rare erythrocytes in which spindle cells dominate during late-stage KS (**a** H&E, ×1000). A cytoplasmic immunoreaction with D2-40 (arrows) and nuclear immunoreaction with HHV-8 (arrow) are seen in neoplastic cells (**b** D2-40; c, HHV-8; IHC ABC-DAB, b×1000, cx400)

Table 1 Distribution of D2-40 immunoreactivity according to histopathological stage of in KS cases

Stage	Extent of D2-40 reactions n (%)				Intensity of D2-40 reactions n (%)			
	1+	2+	3+	4+	Weak	Moderate	Strong	Total, n (%)
Early	13 (30)	9 (21)	11 (25)	10 (24)	23 (53)	16 (37)	4 (43)	43 (100)
Intermediate	7 (11)	16 (25)	24(37)	17 (27)	0 (0)	23 (36)	41 (64)	64 (100)
Late	0 (0)	0 (0)	43 (60)	28 (40)	0 (0)	21 (29)	50 (71)	71 (100)
Total	20 (11)	25 (14)	78 (44)	55 (31)	23 (13)	60 (34)	95 (53)	178 (100)

(Mr 40000) representing a fixation-resistant epitope of lymphatic endothelium. The D2-40 antibody specifically recognizes podoplanin, a glomerular podocyte membrane protein. Studies have shown that podoplanin plays a role in the organization of Prox1, a lymphatic-specific homeobox gene that regulates the evolution of lymphatic progenitors from embryonic veins [17, 19, 20, 26].

The histogenesis of neoplastic cells in KS has been studied for many years [10–12, 19, 22, 25, 30, 31]. These cells were traditionally considered to originate from blood vascular endothelial cells, a theory supported by their reactivity with pan-endothelial markers [1, 13–16, 22, 32]. Alternatively, KS cells may originate from lymphatic endothelium, as suggested by ultrastructural studies showing neoplastic KS cells that lack vascular endothelial cell characteristics and do not express blood vessel endothelium-specific antibodies such as the PAL-E antigen [10, 12, 18, 19, 23, 33–40]. Histogenetic studies have recently focused on lymphatic origins, made possible by the successful staining of neoplastic KS cells with lymphatic markers such as vascular endothelial growth factor receptor-3 and podoplanin (Clone D2-40). These results support the existence of a lymphatic origin and/or differentiation in neoplastic KS cells and suggest that D2-40 is a more specific marker than other vascular endothelial indicators for the diagnosis of KS [23, 26, 30–40].

Few studies have examined D2-40 immunoreactivity in KS, and most of those have investigated predominantly AIDS-related and epidemic forms of KS using a small number of samples. Reliable data on D2-40 expression in classic KS cases are limited. D2-40 staining ratios ranged between 90 and 100% in neoplastic cells of KS [17, 19–24, 26]. Fukunaga et al. reported that D2-40 immunoreactivity was positively correlated with histological stage in KS [30]. Dubina et al. detected D2-40 immunoreactivity in both neoplastic endothelium-floored vascular canals and spindle cells [23]. Kahn et al. showed that D2-40 immunoreactivity could be detected at all histological stages of KS, with an immunoreaction pattern similar to that of CD31 [20]. In our study, KS lesions at all histological stages showed D2-40 immunoexpression. We found no significant relationship between the extent of labeling and histological stage. This result agrees with those of other studies and indicates that D2-40 immunoexpression in all KS cases occurs independently of disease progression.

Our study also investigated the relationship between D2-40 staining density and histological stage. We found predominantly weak- to moderate-density staining in early-stage KS lesions, similar to patterns observed for non-neoplastic lymphatic endothelium. Strong staining was observed in 71% of late-stage KS lesions, but in only 10% of early-stage lesions. Staining density typically increases with the histological progression of a tumor. We observed extended and strong D2-40 immunoreactions in SCs, particularly those of late-stage cases. This result indicates that D2-40 protein expression increases with the progression of KS lesions.

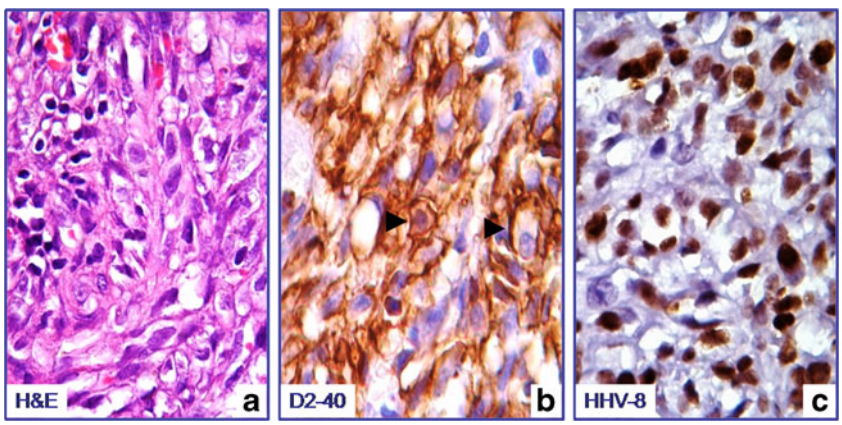

Fig. 5 Slit-like areas in late-stage KS (**a** H&E, ×1000). Focal membranous immunoreaction accompanied by cytoplasmic staining with D2-40 (arrows) is observed within neoplastic cells (**b** D2-40, IHC ABC-DAB, ×1000). A nuclear immunoreaction with HHV-8 is seen within spindle cells (**c** HHV-8, IHC ABC-DAB, ×1000)

Table 2 D2-40 immunostaining profile in different morphological components of KS

Component	D2-40 immunoreactivity (n)		Total n (%)
	Positive	Negative	
Early/Intermediate			
Lymphangiomatoid Angiomatoid	11	0	11 (6)
Central vessels	0	28	28 (16)
Capillaries	28	0	28 (16)
Early spindle cells	66	0	66 (37)
Late			
Sieve-like areas	27	0	27 (15)
Slit-like areas	65	0	65 (37)

Cellular localizations of immunohistochemical markers may vary according to cell type and neoplasia, and these differences provide pathogenic information and contribute to differential diagnoses. Podoplanin staining patterns differ among various normal and neoplastic tissues [17, 20, 41–43]. Our study examined D2- 40 staining patterns in neoplastic KS cells. We detected only cytoplasmic staining in early-stage lesions characterized by immature SCs and splitting neoplastic vascular canals. A weak membranous reaction accompanied cytoplasmic staining in 10% of late-stage lesions. The morphology of SCs with membranous staining included a wider cytoplasm, large vesicular nuclei, and prominent nucleoli. A membranous localization of D2-40 has been reported in some sarcomas with epithelioid morphology (e.g., angiosarcoma and synovial sarcoma) [17, 20, 26, 44]. Our study showed focal membranous reactions in SCs with different morphological features, which may indicate epithelioid differentiation.

KS is composed of heterogenous lesions containing different morphological components [1, 29]. In KS, the expression of pan-endothelial markers may change according to the histological stage of the lesion or structural components of the tumor [13–16, 32]. This status can be explained by antigenic heterogeneity of the human vascular endothelium [9, 10, 13, 15]. However, Johns et al. suggested that the loss of differentiation within neoplastic cells is the reason for this difference [11]. We did not detect D2-40 immunoreactivity in well-developed central vessels, but consistently detected D2-40 immunoreactivity within spindle cells, lymphangioma-like areas, and vascular structures showing autolumination. Our findings show the presence of D2-40 expression, thereby the lymphatic immunophenotype, in true neoplastic components of KS. Central vessels, which lacked D2-40 immunoreactivity in our study, morphologically and immunophenotypically resemble normal blood vessels. Thus, we hypothesize that these structures are reactive or non-neoplastic in nature. However, some researchers have suggested that this situation is the result of stem cell differentiation playing a role in the development of KS [25, 38, 39].

The most widely used immunohistochemical markers for the diagnosis of vascular tumors include factor VIII-ra, CD34, and CD31. Although factor VIII-ra is specific to blood vessel endothelial cells, it has low sensitivity. CD34 is sensitive to blood vessel endothelium, but its specificity is low because it is expressed in many non-vascular neoplasms. CD31, also known as platelet/endothelial cell adhesion molecule-1 (PECAM-1), is expressed primarily by vascular endothelial cells. Weak expression by lymphatic endothelial cells (e.g.

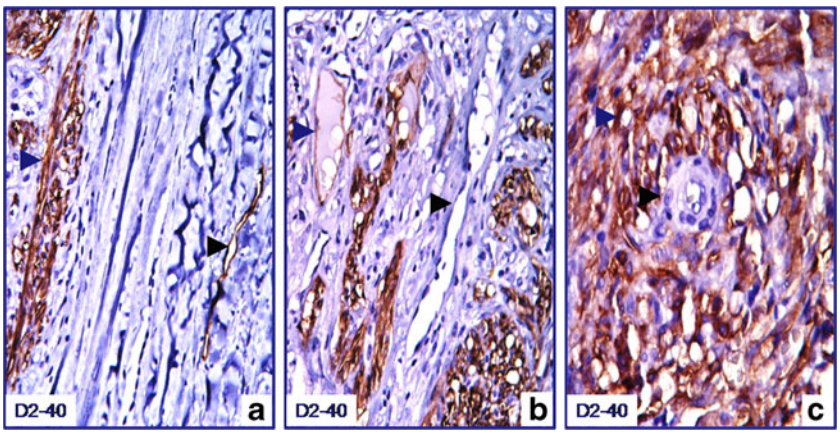

Fig. 6 In late-stage KS, a strong immunoreaction with D2-40 is observed in tumor cells (blue arrow) and non-neoplastic lymphatic vessels (*black arrow*) around the tumor (**a** D2-40, IHC ABC-DAB, ×400). Within the tumor tissue, immunoreaction with D2-40 (*blue arrow*) is seen in lymphatic vessels, whereas no immunoreaction is observed in blood vessels (*black arrow*) (**b** D2-40, IHC ABC-DAB, ×400). A strong immunoreaction with D2-40 is seen within spindle cells (*blue arrow*), whereas no immunoreaction with D2-40 was detected in central vessels (*black arrow*) within the tumor (**c** D2-40, IHC ABC-DAB, ×1000)

clone EN4/CD31) has also been reported and is considered to be evidence that lymphatic endothelial cells originate from primitive vascular endothelial cells. Additionally, CD31, which functions as an adhesion molecule for lymphocytes, may play a critical role in lymphatic vessel function. Hence, it is argued that CD31 is not downregulated in lymphatic differentiation, as are other vascular endothelial markers [45]. D2-40, a vascular indicator expressed in certain capillary endothelium types, has a proven high sensitivity to lymphatic endothelium [17, 20, 26].

Our study detected various degrees of immunostaining for factor VIII-ra, CD34, and CD31 in KS cases at all histological stages. Nevertheless, because these markers are expressed in other vascular lesions originating from blood vessel endothelium, their contribution to the discrimination of KS is minimal. We detected D2-40 immunoexpression in all KS and lymphangioma cases, some angiosarcoma and dermatofibroma cases, and non-neoplastic lymphatic vessels. No other soft-tissue tumors showed D2-40 immunoreaction. These results suggest that D2-40 mAb can be used to determine lymphatic origin and/or differentiation and support the theory that neoplastic KS cells exhibit lymphatic differentiation. The present study, like previous studies, has demonstrated that D2-40 immunoreactivity can be detected in all stages and epidemiological types of KS. However, it must be kept in mind that D2-40 can also be expressed in some tumors other than KS.

In this study, to the best of our knowledge, D2-40 immunoreactivity was examined in the largest classical KS series reported to date. Our study shows that D2-40 mAb can be used to determine lymphatic origin and/or differentiation and support the theory that neoplastic KS cells exhibit lymphatic differentiation. Additionally, D2–40 immunoexpression can be detected at all stages of KS and that D2–40 may be a useful addition to the immunohistochemical panel for the differential diagnosis of KS. The main limitation of our study is the search of D2-40 expression only among markers regarding lymphatic endothelium and inclusion of classical KS cases only. Studies involving molecular analyses of stem cell markers are needed to definitively identify the origin of neoplastic cells in KS.

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