ORIGINAL PAPER

Lymphatic vessels identified with podoplanin. Comparison of immunostaining with three different detection systems

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Abstract

The aim of the study was to find the optimal immunostaining protocol for monoclonal mouse anti human podoplanin antibody clone 18H5, which is less studied. We tested three detection systems for different antibody dilutions and antigen retrieval methods applied on normal and tumor breast tissues, lip squamous cell carcinoma, kidney tumors and testis carcinoma. The interpretation was linked to the background staining, specificity for the lymphatic endothelium and cross-reactivity. The ABC method gave a high background even the antibody dilution was increased. No immunostaining was obtained with Envision detection system for any dilution or type of antigen retrieval. We obtained the best results using LSAB+ working system for an antibody dilution of 1:500 with no background and podoplanin expression was restricted to the lymphatic endothelium. We consider that the standardization of immunostaining protocol is the first step for an optimal interpretation of the podoplanin expression using less studied antibody clones.

***Keywords*:** podoplanin, gp36, immunohistochemistry, detection system, antigen retrieval.

Background

Podoplanin is a mucin type transmembrane glycoprotein found into the podocyte of normal rat glomeruli and is expressed in lymphatic endothelium but not vascular, endothelial cells in culture [1, 2]. Podoplanin was reported to be expressed in lymphatic endothelial cells, epithelial cells of the choroid plexus, alveolar type I cells, osteoblasts, and peritoneal mesothelial cells [3].

It is also homologous to the mouse glomerular membrane protein gp 38 P [4] the OTS-8 gene product in cultured mouse osteoblast [5] and the 45-kD protein PA2.26, identified as a cell-surface antigen induced in keratinocytes during mouse skin carcinogenesis and in dermal fibroblast-like cells during wound healing [6].

Together with vascular endothelial growth factor receptor 3 (VEGFR-3) and the hyaluronan receptor LYVE-1, podoplanin constitute an important marker for lymphatic vessels in tumors [7, 8].

Podoplanin has also been shown to be strongly expressed in seminoma, epithelioid mesothelioma, and hemangioblastoma and immunostaining for this marker can assist in the diagnosis of these tumors. Moreover, podoplanin is usually upregulated in the invasive front of the human cancers and induces tumor cell migration and invasion by induction of filopodia formation and modulation of the activities of Rho-family GTP-ases [9, 10].

A good immmunohistochemical expression depends on the type of antibody, optimal dilution, and compatible detection system used [11]. The standardization of immunohistochemical protocol is one of the most important objectives of each immunohistochemistry lab. The aim of this study is to describe different aspect of podoplanin expression according to the different antibody dilution, antigen retrieval methods and different detection systems. Finally, we will describe the protocol, which we consider to be compatible with the podoplanin antibody, mouse monoclonal type, clone 18H5.

Material and methods

We included in our standardization process three cases of lip tumors, four cases of breast tumors (which also contained adjacent normal mammary tissue) three kidney specimens with normal and tumor tissue and one case with tumor of the testis.

The biopsies were fixed in 10% neutral buffered formalin and paraffin embedded using standard protocol. Five micrometers sections from each case were obtain and mounted on silanized slides.

We performed Hematoxylin and Eosin stain for the pathological diagnosis and morphologic identification of blood and presumed lymphatic vessels. Adjacent sections from the same cases were used to identify lymphatic vessels using mouse-anti human monoclonal antibody against podoplanin (gp 36), clone 18H5 (aggrus T2alpha).

The lyophilized form of 100 µg antibody (Abcam) was reconstituted with 1 ml antibody diluent from Dako and we obtained a stock solution with o concentration of 100 µg/ml. From this concentrate, we made progressive dilutions between 1:5 and 1:2000 using phosphate buffered saline solution as antibody diluent. The slides were dewaxed and rehydrated in two bath of xylene and decreased concentration bath of ethanol.

We applied heat induced epitope retrieval for unmask the antigen testing the citrate buffer pH 6 and epitope retrieval solution pH 9 for ten minutes and separately pretreatment with Proteinase K for five minutes at room temperature.

The incubation time with primary antibody varied between 60 minutes and 12 hours at room temperature in a humid chamber. We applied independently on the slides from each case three working systems: ABC Elite system from Vectastain, Envision and LSAB+ Systems from Dako. The visualization of the final product used 3,3'-diaminobenzidine and the reaction appeared as a brown staining. The slides were counterstained using modified Lille's Hematoxylin for three minutes. The intensity of the reaction and background staining were scored.

We used as positive control the brown staining of the lymphatics from the vasa vasorum of the great vessels. We choose the final protocol according to the expression of podoplanin which was restricted on lymphatic endothelium from normal and tumor tissues.

Results

On routine Hematoxylin and Eosin stain, we established the histopathologic diagnosis for each case and we tried to identify lymph vessels according to the morphologic features. The four selected cases from the breast were ductal invasive carcinoma. In addition, we included in our study three squamous cell carcinoma of the lip, three renal cell carcinomas and one testicular tumor.

For a dilution between 1:5–1:200 applied on each slide, we used three different detection systems. We started with Vectastain ABC detection method and we observed a high background for all types of tissues. The background was persistent even after incubation of the slides with 1% bovine serum albumin for one hour at room temperature.

The positive reaction was not limited to the endothelial cells of the lymphatics. Both blood and lymph vessels were positive for podoplanin. The connective tissue fibroblasts were intensely stained and we cannot differentiate the lymphatic vessels inside it. Also at this dilution, we found false positive reaction to podoplanin into the perivascular smooth muscle cells and perycites around the blood vessels (Figure 1).

The squamous cells of the lip tumors were intensely stained and in the deeper portion of the same tissues almost all nuclei of sero-mucous glands expressed podoplanin with weak to moderate intensity (Figure 2).

In the normal renal tissue, for the same method and antibody dilution, the background was also present and the intense brown staining was registered in the epithelial cells of the tubular system, endothelial cells of the capillaries inside the glomeruli and podocytes. The renal tumors had positive cells with weaker intensity than that from normal tissue. Ductal invasive carcinoma cells and terminal ductal lobular unit (TDLU) cells of normal adjacent breast tissue were intensely positive for podoplanin with fine granular cytoplasmatic pattern.

For antibody dilutions between 1:200–1:750 in PBS and ABC Vectastain detection system the background decreased for all types of tissues but did not disappear. It was possible to detect the lymphatics from the adventicia of the great vessels but an inconstant weak staining was also registered in the blood vessels endothelial cells (Figure 3).

The smooth muscle perivascular cells showed a slightly positive staining but another types of muscle cells from the lip structure expressed podoplanin with the same intensity like for the previous method. We detected lymphatics grouped around the squamous areas of lip tumors. No positive staining was found when we applied Envision detection system for the same antibody dilutions on the selected tissues.

We also tried to find the optimal antigen unmasking method for the studied antibody. The pretreatment with proteinase K for five minutes at RT and microwave antigen retrieval using high pH solution did not show any positive results. We obtained best results with microwave antigen retrieval method using citrate buffer pH 6 for 2 × 5 minutes cycles.

We obtained the best immunohistochemical results with LSAB+ working system for an antibody dilution of 1:500 in PBS. No background was observed for the studied normal and tumor tissues. All nuclear, connective tissues components and muscular structures were negative for podoplanin staining. The positive reaction was restricted to the endothelial cells of the lymphatic vessels (Figure 4a) and podocytes. Weak, inconstant positive reaction was found in renal tubular epithelial cells (Figure 4b) and normal breast tissue inside the TDLU. No blood vessels endothelial cells expressed podoplanin when we applied the following protocol:

- Dewax and rehydrate the slides.
- Antigen retrieval: microwave method, 10 minutes, citrate buffer pH 6.
- Cool at room temperature for 20 minutes.
- Endogenous peroxidase blocking: 5 minutes with 3% H_2O_2 in distillated water.
- Primary antibody: one hour incubation at RT with mouse monoclonal antibody anti podoplanin, clone 18H5, dilution 1:500 in PBS.
- Detection system: LSAB+ Universal System, HRP
- Chromogen: 3,3'-diaminobenzidine, 10 minutes at room temperature.
- Counterstain: modified Lille Hematoxylin, 3 minutes at room temperature.
- Permanent mounting media.

Discussions

Podoplanin, a glomerular filtration-regulating protein that was originally identified as a podocyte membrane protein in the renal corpuscle was found to be specific for lymphatic endothelium [12]. The expression of T1α/podoplanin is regulated by the lymphatic-specific homeobox gene. Podoplanin is found specifically in the lymphatic endothelium in many organs, including the skin, kidney and lungs in humans, and coexists with VEGFR-3 in murine tissue [13].

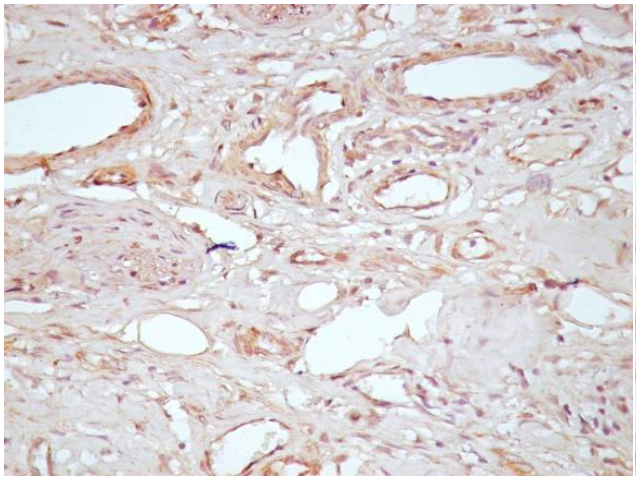

Figure 1 – ***Positive staining for podoplanin in blood and lymphatic endothelium. Note the false positive staining for podoplanin in the perivascular cells and connective tissue cells (Podoplanin staining, dilution 1:5–1:200, ABC method, ×20)***

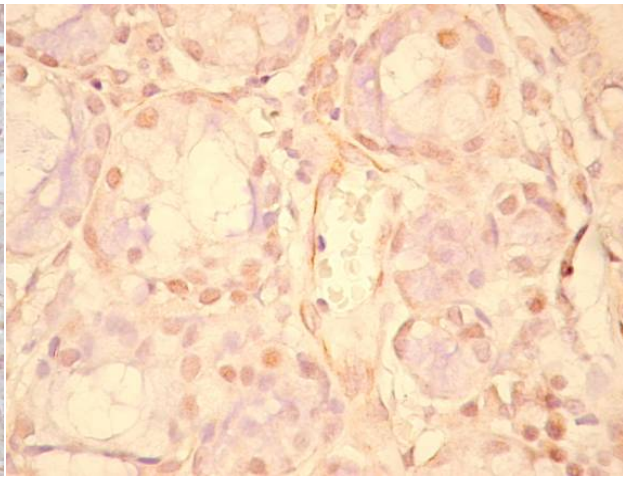

Figure 2 – ***Unspecific nuclear and cytoplasmatic staining of glandular epithelial cells (Podoplanin staining, dilution 1:5–1:200, ABC method, ×40)***

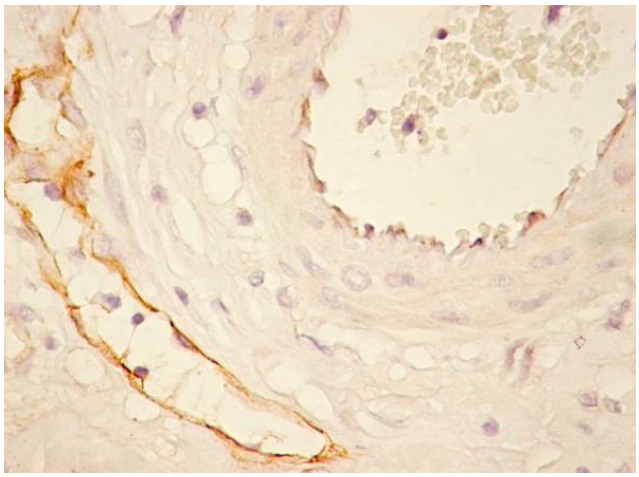

Figure 3 – ***Reduced background and blood endothelial cell staining at a dilution of 1:500. Note the persistence of a weak immunostaining of smooth muscle cells around the blood vessels and inconstant staining of the blood vessel endothelium (Podoplanin staining, dilution 1:500, ABC method, ×40)***

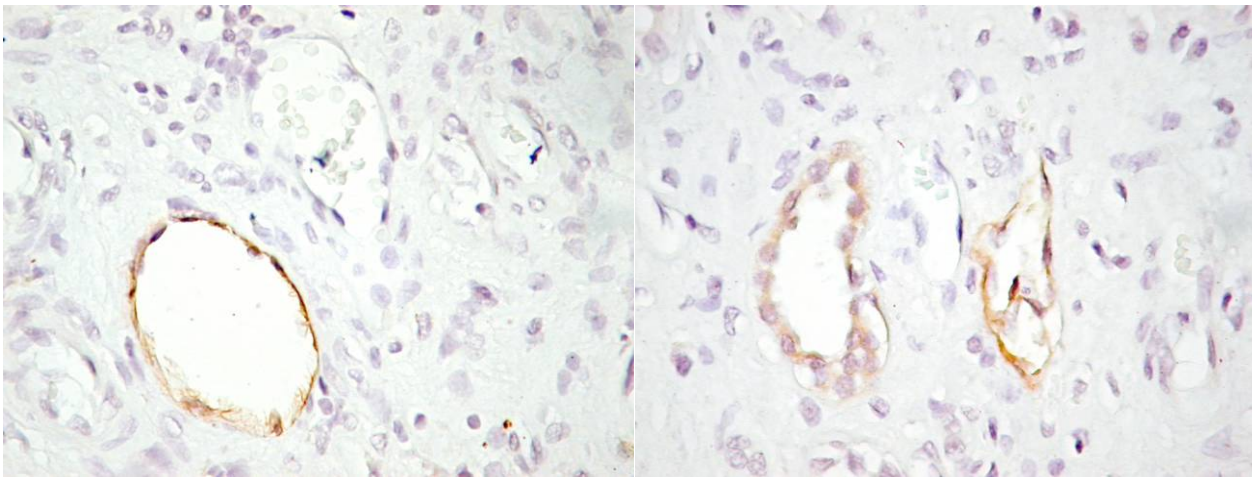

Figure 4 – ***No background registered. The lymphatic endothelial cells are positive for podoplanin compared with endothelium of the neighboring blood vessel negative for this antibody. Correct immunostain (a). Note the weak and persistent staining of the tubular epithelial cells of the kidney even at a higher dilution (b) (kidney, podoplanin, 1:500, LSAB+ method, DAB, ×40)***

Podoplanin (Aggrus) plays a key role in tumor cell-induced platelet aggregation [14].

There are a lot of tumors were podoplanin expression is restricted to the lymphatic endothelium and represents o marker for counting lymphatic microvessel density related with lymph node metastasis and an adverse outcome [15].

Identification of lymphatic vessels by podoplanin immunostaining provides an accurate evaluation of lymphatic involvement. Lymphatic vessel density is an useful predictor of lymph node metastasis of submucosal colorectal cancer [16].

A correct appreciation of lymphatic microvessel density depends on the clone of the antibody, dilution and a proper immunostaining with no background or another false positive reaction. Mouse monoclonal antibody anti human podoplanin clone 18H5 is little used in the literature. The dilution of the antibody is an important step of immunostaining method. The use of antibody diluent may influence the staining of the specimens by cross-reactivity with endogenous proteins.

A proper preparation of the antibody dilution from stock solution using PBS, pH 7.2 reduced background and false positive reactions. ABC staining method even after incubation with 1% BSA for one hour, at room temperature gave high unspecific results. For this reason, we consider that for monoclonal mouse antibody anti human podoplanin clone 18H5 (Abcam), LSAB+ working system is the most reliable detection method. This is an important aspect for a right interpretation of immunostaining of such tumors that also express podoplanin into the neoplastic cells like primary central nervous system germ cell tumors [17], malignant astrocytic tumors [18], and germ cell tumors of the gonads [19].

For all of these tumors the lymphatic marker used was D2-40. We consider that podoplanin expression study may include other clones for a better characterization of the tumors cells. The investigation of podoplanin expression like a target for future therapy may be carefully interpreted according to the type of the antibody and detection system.

Conclusions

Monoclonal mouse antibody anti human podoplanin clone 18H5 is less used. For this reason a correct protocol and standardization of immunostaining is important for a correct interpretation.

Our study suggests that for this type of marker we can use LSAB+ detection system to reduce background and other false positive reaction.

For an optimal dilution the human anti podoplanin clone 18H5 can be used as a specific marker for lymphatic endothelium and for counting lymphatic microvessel density.

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Received: January 20th, 2007

Accepted: March 20th, 2007