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From Cell Biology to Tissue Engineering

Smoothelin and WT-1 expression in glomus tumors and glomuvenous malformations

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Summary. Background: Smoothelin is a specific marker for smooth muscle cells with contractile capacity which has not been widely studied in glomus lesions. In the same way, the expression for Wilms tumor 1 (WT1) has only been studied occasionally in the endothelial cells of glomovenous malformations and in the glomus cells of glomus tumours. Objective: We studied the significance of immunohistochemical expression of smoothelin and WT1 in 25 glomus lesions. Methods: We assessed 9 cases of solid glomus tumors (SGT), 8 cases of glomus tumors with vascular ectasia (VEGT), 2 cases of glomangiomyomas (GMM) and 6 cases of glomuvenous malformation (GM). Immunohistochemistry was performed, evaluating the expression of WT1, smoothelin, smooth muscle actin (SMA), smooth muscle myosin (SMM), h-caldesmon and desmin. Results: Glomic cells showed cytoplasmic positivity for smoothelin, and WT1 expression was present in all studied cases. SGT showed WT1 positivity in all endothelia. However, in regarding VEGT and GMM, WT1 endothelial expression was positive in some areas, but not in others. GM did not show endothelial cell positivity for WT1. Conclusions: Smoothelin expression in glomic cells indicates that they are contractile smooth muscle cells, and thus its role in routine diagnosis should be considered. The absence of WT1 expression in the endothelium of the vascular structures of the GM is a differential characteristic between SGT, VEGT and GMM.

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Introduction

Glomus lesions that reproduce the neuromyoarterial glomus (glomus body), comprise three variants: solid glomus tumor (SGT), glomuvenous malformation/ glomangioma (GM) and glomangiomyoma (GMM) (Gombos and Zhang, 2008). "The World Health Organization (WHO) includes glomangioma as a variant of the typical glomus tumor. However, we believe this is misleading, given that glomangioma is in fact a glomovenous malformation, and this is better reflected in the classification proposed by the International Society for the Study of Vascular Anomalies (ISSVA) (Fletcher et al., 2013; Wassef et al., 2015).

The glomus tumor is a benign lesion that appears in adults age range between 40 to 60 years, as a nodule or red-blue papule, usually located in the distal portion of the extremities, and may be painful in response to changes in pressure or temperature (Tomak et al., 2003; Mravic et al., 2015). The glomuvenous malformation usually appears in children and adolescents, tends to present as bluish-red nodules or multiple papules that may be scattered or form plaque-like structure (Iqbal et al., 1998; Requena et al., 1998; Monteagudo et al., 2007).

GM can be inherited in an autosomal dominant pattern with incomplete penetrance and variable expressivity, associated to a mutation of the GLMN gene, located in chromosome 1p21-p22 (Bomm et al., 2004; Brouillard et al., 2005). Glomulin, its gene

product, is expressed in vascular smooth muscle cells and seems to be implicated in late–stage maturation.

GMM are glomus lesions with smooth muscle differentiation that have been considered as a variant of SGT or GM (Ezinguer and Weiss, 2014).

The glomus tumors and malformations present immunoreactivity for smooth muscle differentiation markers, the most used being smooth muscle actin (SMA), muscle specific actin and h-caldesmon (Boon et al., 2004; Gombos and Zhang, 2008; Mravic et al., 2015). However, few studies exist assessing the cytoplasmic expression of smoothelin and Wilms tumor 1 (WT1) in glomus cells (Wong et al., 2014; Galfione et al., 2014; Borroni et al., 2014). Also, one study has described the absence of expression for WT1 in the endothelial cells of glomovenous malformations (Al Dhaybi et al, 2010). Smoothelin is a protein specifically expressed in smooth muscle cells, and is used as a marker to assess contractile function. It has two tissue-specific isoforms; the long isoform is exclusive to vascular muscle cells (Krämer et al., 1999, 2001; Niessen et al., 2005). However, the expression of smoothelin in smooth muscle tumors is little known (Coco et al., 2009; Wong et al., 2014).

WT1 was originally described as a tumor suppressor gene based on its mutational inactivation in a subset of Wilms tumor, with a traditional nuclear staining pattern (Haber et al., 1990; Anuchapreeda et al., 2006). More recently, the cytoplasmic expression of WT1 was demonstrated in a variety of tumors of the gastrointestinal tract, lung, breast, bladder, soft tissues, etc (Nakatsuka et al., 2006; Trindade et al., 2011; Galfione et al., 2014).

The aim of this study is to assess the expression of WT1 and smoothelin in SGT, GMM and GM, and its potential diagnostic role.

Materials and methods

A total of 25 cases of glomus tumor and malformations were selected and included in our study. The cases were obtained from the routine caseload of the Provincial Clinical Intercenter Management Unit of Pathology, Granada, Spain.

Histopathological and immunohistochemical studies

For histopathological analysis, skin samples fixed in 10% buffered formalin for 24 hours, dehydrated with alcohol, embedded in paraffin in an automatic tissue processor Excelsior ES (Thermo Scientific, CA, USA). 4 micron sections were stained with hematoxylin and eosin (H&E). Histopathological changes were graded on a 0-2 scale in a blinded manner.

Table 1. Clinical features and results of immunohistochemical assessed on glomus tumors and glomuvenous malformation.

Cases	Age (year)	Sex	Location	Clinical Lesions	Family History	Pain	Clinical appearance	Diagnosis	SMA	SMM	h-caldesmon	Desmin	Smoothelin	WT1	WT1 (Endothelial cells)
1	56	M	Thigh	Solitary	-	+	GT	SGT	3*	3	3	0	1	3	++
2	77	F	Forearm	Solitary	-	+	GT	SGT	3	2	3	0	1	3	++
3	60	M	Leg	Solitary	-	+	Leimyoma	SGT	3	3	3	0	1	3	++
4	61	F	Hand	Solitary	-	+	GT	SGT	3	3	3	0	1	2	++
5	77	F	elbow	Solitary	-	+	Keratosis	SGT	3	3	3	0	2	1	++
6	64	M	Hand	Solitary	-	NR	GT	SGT	3	3	3	0	2	3	++
7	37	M	Shoulder	Solitary	-	+	Leiomyoma	SGT	3	3	3	0	1	3	++
8	54	M	Leg	Solitary	-	+	Leiomyoma	SGT	3	3	3	0	3	3	++
9	58	M	Forearm	Solitary	-	+	GT	SGT	3	3	3	0	1	2	++
10	56	M	Arm	Solitary	-	+	GT	VEGT	3	3	3	0	2	3	+
11	62	M	Thigh	Solitary	-	+	Hemangioma	VEGT	3	3	3	0	2	3	+
12	74	F	Forearm	Solitary	-	+	GT	VEGT	3	3	3	0	1	3	+
13	56	M	Forearm	Solitary	-	+	GT	VEGT	3	3	3	0	3	3	+
14	37	M	Thigh	Solitary	-	NR	GT	VEGT	3	3	3	0	3	3	+
15	46	M	Hand	Solitary	-	+	Leimyoma	VEGT	3	3	3	0	3	3	+
16	50	M	Hand	Solitary	-	NR	Hemangioma	VEGT	3	3	3	0	2	3	+
17	32	M	Forearm	Solitary	-	+	GT	VEGT	3	3	3	0	1	1	+
18	37	M	Thigh	Solitary	-	+	GT	GMM	3	3	3	1	1	3	+
19	69	M	Forearm	Solitary	-	NR	Hemangioma	GMM	3	3	3	1	3	3	+
20	13	M	Forearm	Multiple	+	NR	Malformation	GM	3	3	3	0	1	1	-
21	11	F	Arm	Multiple	-	-	GM	GM	2	2	2	0	1	2	-
22	1	M	Forearm	Multiple	-	-	GM	GM	3	3	3	0	1	2	-
23	16	M	Thigh	Multiple	-	+	VM	GM	3	3	3	0	1	2	-
24	8	M	Arm	Multiple	+	-	GM	GM	3	3	3	0	1	1	-
25	18	M	Arm	Multiple	-	+	NR	GM	3	3	3	0	1	1	-

M, Male; F, Female; SGT, Glomus Tumor with solid pattern; VEGT, Vascular Ectasia Glomus Tumor; GMM, Glomangiomyoma; GM, glomuvenous malformation; VM, Venous Malformation; NR, Not report; SMA, Smooth muscle actin; SMM, Smooth muscle myosin; WT1, Wilms Tumor.

For immunohistochemical analysis, sections were dewaxed, hydrated, and heat-treated in 1 mM EDTA pH 8 for antigenic retrieval using a PT module (Thermo Fisher Scientific Inc., Waltham, MA) at 95°C for 20 minutes. These sections were incubated for 10 min at room temperature with prediluted monoclonal antibodies against smooth muscle actin (SMA) (1A4), h-caldesmon (H-CD) and smooth muscle myosin (SMM) (SMMS-1), desmin (D33) WT1 (6F-H2) and smoothelin (R4A). All antibodies used were supplied by Master Diagnostica, Granada, Spain. An appropriate isotype for each antibody was used as negative control.

The immunohistochemical staining was conducted in an automatic immunostainer (Autostainer 480, LabVision Fremont, CA) using the micropolymer-peroxidase-based method (Ultravision Quanto, Master Diagnostica, Granada, Spain), followed by development by diaminobenzidine. The degree of immunoreactivity for SMA, SMM, h-caldesmon, desmin, smoothelin and WT1 in the glomus cells was assessed in a semiquantitative manner, using a scale of 0 to 3: 0 [absence], 1 [<25% positive cells], 2 [25-75% positive cells], 3 [>75% positive cells]. The level of immunoreactivity for WT1 in the endothelial cells was also assessed in a semiquantitative manner, but using a scale of - to ++: - [negativity in all endotelial cells], + [positive and negative endothelial cells], ++ [positivity in all endothelial cells]

Statistical analyses

A statistical software package SPSS 20.0 (IBM Inc., Chicago, IL) was used for the statistical analysis. Kruskal-Wallis and Mann Whitney U-test for the analysis of non-parametric variables to analyze the differences in morphological and immunohistochemical variables between different glomus lesions were used. Spearman coefficient (rho) test to analyze the correlation

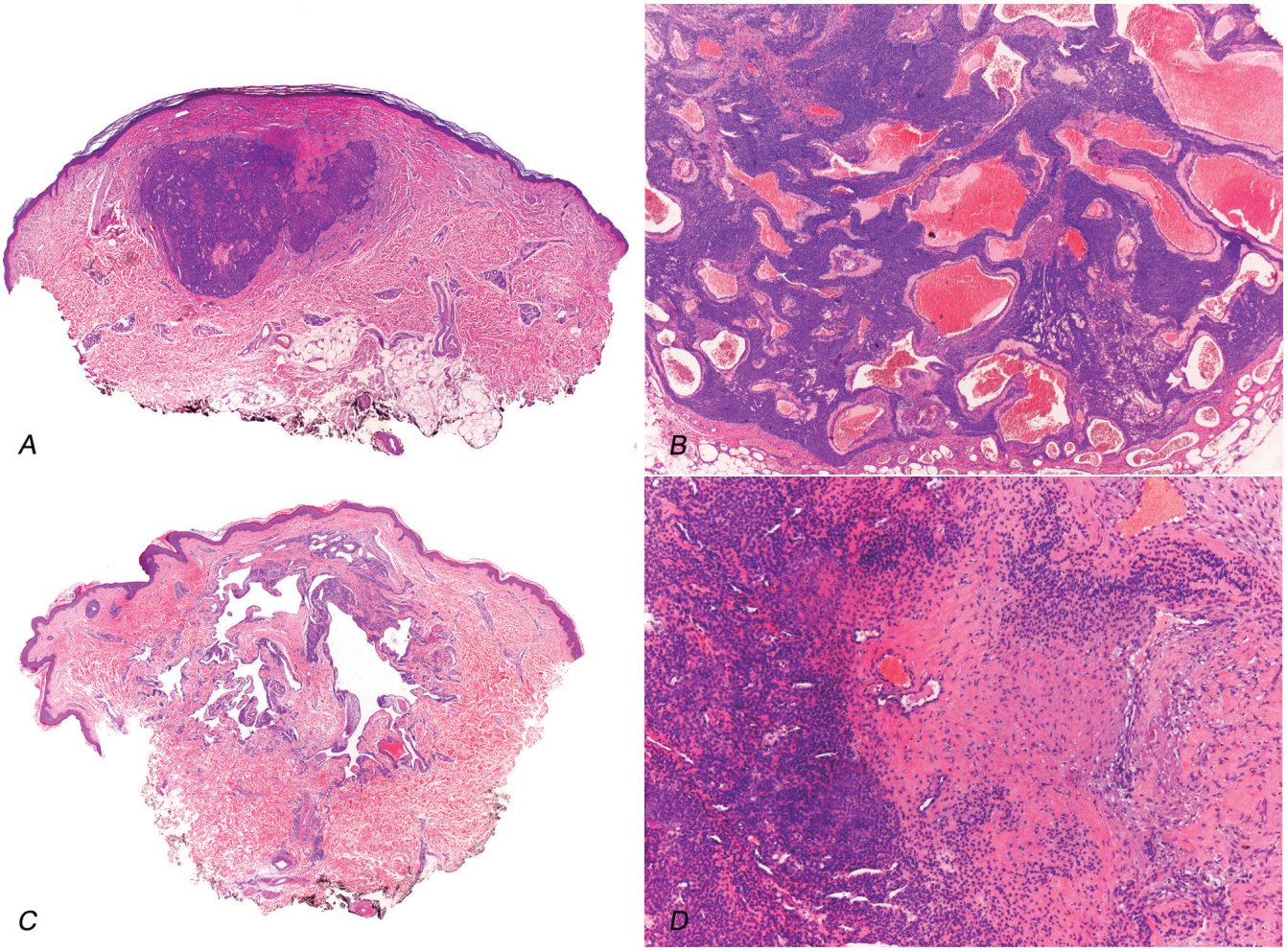

Fig. 1. A. Solid glomus tumor with diffuse pattern located in the dermis. **B.** Glomus tumor with vascular ectasia. **C.** Glomuvenous malformation in dermis with vascular channels. **D.** Glomangiomyoma with proliferation of smooth muscle cells and glomus cells. (Hematoxilin & Eosin). A, C, x 1; B, x 2; D, x 4

between variables was also used. A P-value of 0.05 was accepted for statistical significance threshold.

Results

Clinical findings

Glomic tumor patients had a mean age of 56.29±12 years with an age range between 32 to 77 years, showing a male gender predominance (13M/4F). The lesions were more frequently located in the upper limb (11/17). Patients with glomovenous malformation had an average age of 11.16±2, with a male gender predominance (5M/1F), and the lesions were also more frequently located in the upper limb (5/6) (Table 1).

Pathology analysis

The lesions studied had a mean size of 0.5±0.21 cm and ranged from 0.20 to 1 cm. They were solitary in all cases and located in the dermis (48%), dermo-hypodermis (28%) and hypodermis (24%). Histopathological diagnosis was made according to the amount and distribution of glomus cells, the presence of vascular channels and the typical smooth muscle differentiation. Thus, the diagnosis of SGT was made when the lesions were well delimited with diffuse pattern and showed vascular structures that, when ectatic, were classified as glomus tumors with vascular ectasia (VEGT) (Fig. 1A,B). One case of SGT showed myxoid component. The GMM had the same morphological features as VEGT but had smooth muscle areas that connected with vascular structures (Fig. 1D). GM had irregular margins and showed vascular channels that were surrounded by several layers of glomus cells (Fig. 1C). We thus classified our 25 lesions into four histopathological groups: SGT (9/25), VEGT (8/25), GMM (2/25) and GM (6/25).

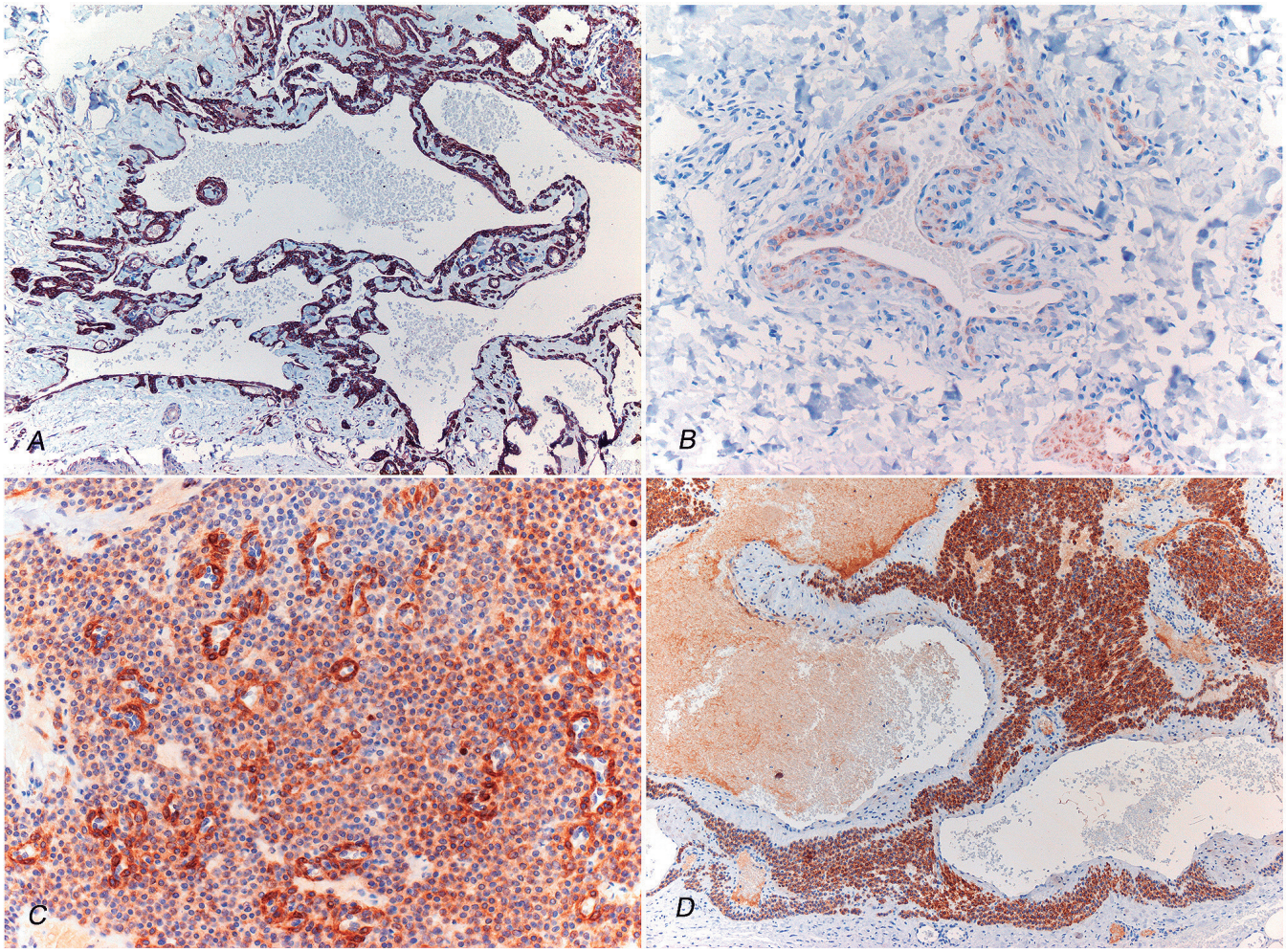

Fig. 2. A. Glomuvenous malformation positive for h-caldesmon in glomus cells (h-Caldesmon). **B.** Glomuvenous malformation with low positive smoothelin in glomus cells (smoothelin). **C.** Solid glomus tumor with smoothelin positive in glomus cells (smoothelin). **D.** Glomus tumor with vascular ectasia with smoothelin expression in glomus cells (smoothelin). A, x 2; B, C, x 10; D, x 4

Immunohistochemical analysis

The immunohistochemistry results are displayed in Table 1. All cases showed intense cytoplasmic positivity for SMA, SMM and h-caldesmon (Fig. 2A). Smoothelin also showed cytoplasmic positivity though less intensely in GM (Fig. 2B-D). Desmin showed only focal positivity in those areas with the typical smooth muscle differentiation of GMM. In all cases, WT1 was expressed in the cytoplasm of the glomus cells, with lesser expression in GM (Fig. 3). Positive staining for WT1 in endothelial cells of the vascular structures of SGT was present in all cases. (Fig. 3A). WT1 expression in EVGT and GMM was observed in the majority of endothelial cells, but in some of them was negative (Fig. 3B,C). In GM, endothelial WT1 expression was negative in all instances (Fig. 3D).

Statistical analyses

Of all the markers studied, only the cytoplasmic positivity for WT1 (p=0.010), smoothelin (p=0.049) and endothelial cytoplasmic positivity for WT1 (p=0.000) were statistically significant (Kruskall-Wallis test). GM had lesser cytoplasmic expression of WT1 and smoothelin (Fig. 4). Also, no endothelial WT1 positivity was observed in GM. Cytoplasmic expression in glomic cells of WT1 was positively correlated with the endothelial expression of WT1 (Spearman's rank correlation coefficient, rho=0.475) and smoothelin expression (rho=0.476) (p = 0.016).

Discussion

We assessed smoothelin expression in normal skin,

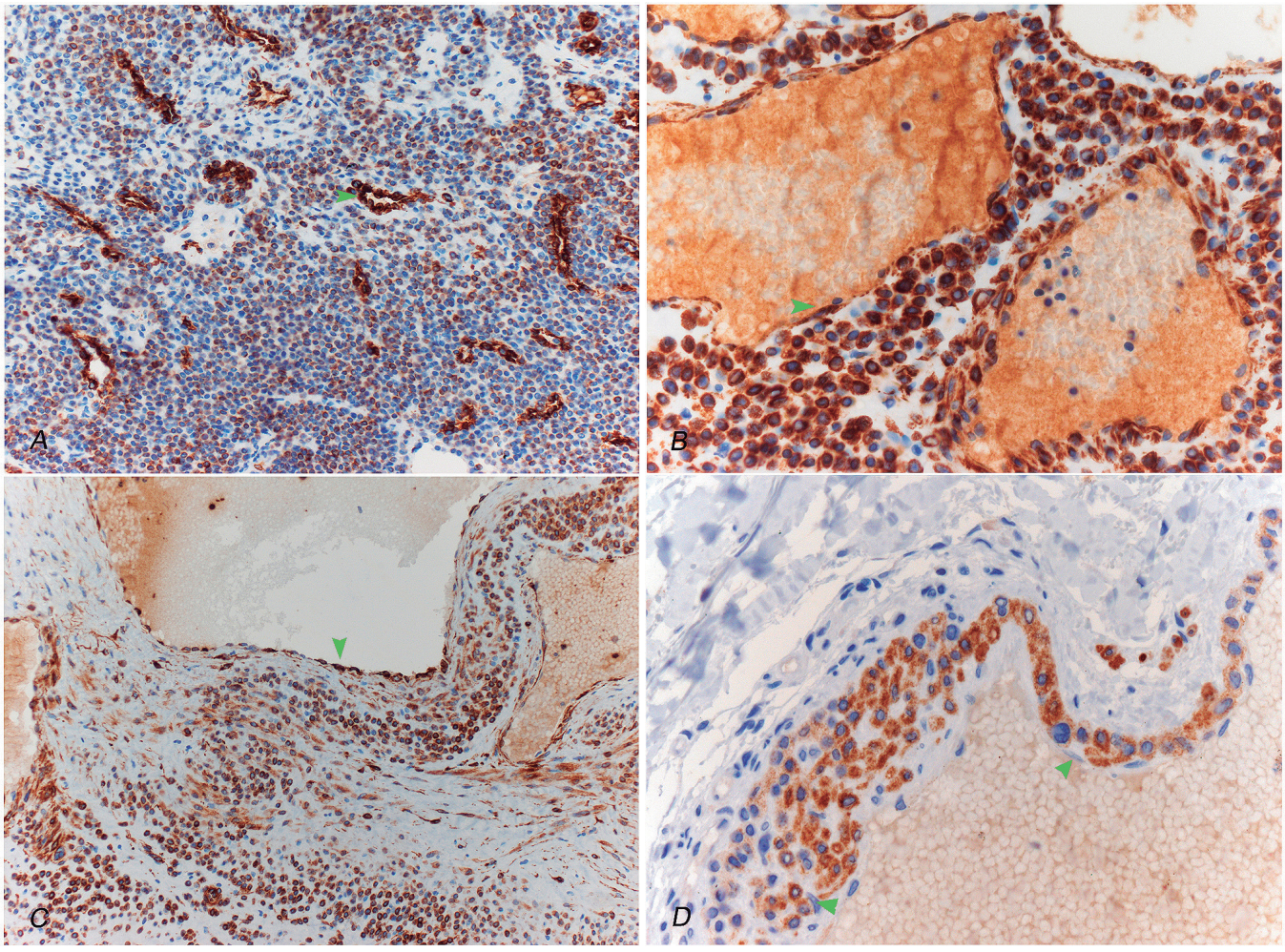

Fig. 3. A. Solid glomus tumors with WT-1 expression in glomus cells and endotelial cells (green arrow) (WT-1). **B.** Glomus tumor with vascular ectasia with WT-1 positivity in glomus and endotelial cells (green arrow) (WT-1). **C.** Glomangiomyoma with expression in glomus cells, smooth muscle cells and endotelial cells (green arrow) (WT1-1). **D.** Glomuvenous malformation positive for WT-1 in the glomus cells and negative for endotelial cells (green arrow) (WT1). A, C, x 10; B, D, x 20

and found cytoplasmic positivity in the smooth muscle cells of the deep vascular plexus and the erector muscle of hair, not appreciating glomus body in the skin biopsies studied (Aneiros-Fernandez et al., 2011). We previously discussed smoothelin positivity in cutaneous hamartoma (Espiñeira-Carmona et al., 2012). There is only one previous report describing weak cytoplasmic reactivity for smoothelin in four of the seven glomic tumors studied (Wong et al., 2014). However, all lesions of our case series presented variable cytoplasmic positivity for smoothelin, this reactivity more being evident in those glomic cells closer to the vessels in GM. This positivity indicates that they are cells with contractile smooth muscle phenotype. SMA, SMM and h-caldesmon were more intense than smoothelin. However, in our experience, smoothelin is the most specific smooth muscle differentiation marker, so It could be routinely used in the diagnosis of glomus lesions. Desmin was negative in the glomus cells of all cases studied, although presented only focal positivity in areas with typical smooth muscle differentiation of GMM. The expression of desmin in this study is consistent with previous reports (Dervan et al., 1989; Mentzel et al., 2002). Some authors have recently described GM associated with prominent smooth muscle component and eccrine glands (Borroni et al., 2014). However, these findings have not been observed in our work.

Two previous studies have reported WT1 expression in glomic tumors, showing focal weak staining in 2/8 cases and 2/2 cases of GM, but this finding has not been fully explained (Borroni et al., 2014; Galfione et al., 2014).

In the present study, we show cytoplasmic positivity for WT1 in glomus cells in all of our cases. WT1 has a pattern of nuclear staining that is usually seen in Wilms tumor, acute leukemia, ovarian and urothelial carcinoma, desmoplastic small round cell tumor, leiomyosarcoma, etc. It shows a cytoplasmic pattern in different carcinomas, such as lung, breast, kidney etc., as well as soft tissue tumors (rhabdomyosarcoma, angiosarcoma, angiomas, etc.) (Anuchapreeda et al., 2006; Nakatsuka et al., 2006; Lee et al., 2009; Trindade et al., 2011; Galfione et al., 2014). An explanation for the cytoplasmic WT1 staining may lie in its presence as a major component of polysomes as a cytoplasmic translational regulator (Galfione et al., 2014). The importance of WT1 in the SGT, VEGT, GMM and GM may be due to the fact that WT1 plays an important role in angiogenesis, regulating vascular endothelial growth factor, angioproteins and nestin (Mokry et al., 2004; Cohen, 2006; Small et al., 2006; Hanson et al., 2007). It is also considered that WT1 may be involved in vascular smooth muscle proliferation (Small et al., 2006). We have also observed cytoplasmic endothelial expression of WT1 in the tumors studied. However, in VEGT and GMM, endothelial WT1 was negative in some vascular structures. Also, immunostaining for WT1 in the endothelium of the vascular channels of the GM was negative in all cases. There are few studies of endothelial WT1 expression in benign vascular tumors and vascular malformations showing endothelial positivity in tumors and negativity in malformations, except for arteriovenous malformations that were positive, and vascular malformations with re-endothelialized neovessels within thrombi (Lawley et al., 2005; Al Dhaybi et al., 2010; Trindade et al., 2011). Al Dhaybi et al describe five cases of MG with negativity for WT1 in the endothelial cells of the vascular channels with no mention of expressión for WT1 in the endothelial cells of SGT.

The positivity for WT1 in arteriovenous malformations could be considered in relation to the proliferative stage of the malformation. Thus, endothelial positivity for WT1 is related to the ability of endothelial cells to remodel and proliferate, while negativity for WT1 would indicate that endothelial cells are static and hence do not have the capacity to proliferate, as would occur in malformations. The inhibition or loss of the endothelial ability to proliferate could happen in some VEGT or GMM endothelial cells that present some vessels with WT1 negative endothelia. Taking these facts into account, the assessment of WT1 expression in vascular endothelial cells of the glomus lesions may help in the differential diagnosis of glomus tumors and malformations.

The classification of glomus tumors in the literature is controversial, WHO include glomangioma as a type of glomus tumor, whereas ISSVA consider glomangioma to be a glomangiovenous malformation (Fletcher et al., 2013; Wassef et al., 2015).

To avoid confusion, the term glomangioma should not be used as a synonym for glomangiovenous malformation. We propose that the term glomangioma should apply to those lesions similar to the solid glomus

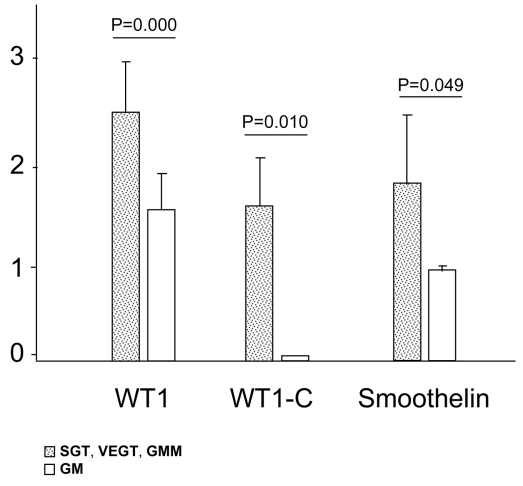

Fig. 4. Comparison between the semiquantitative assessment of the expression of GM vs SGT, GMM, VEGT (Mann-Whitney).

tumor with vascular ectasia and with WT1 expression in the majority of the endothelial component. Conversely, the term glomuvenous malformation would correspond to glomus lesions with WT1 negativity in endothelial cells of the vascular channels. More research with larger sample size is warranted to clarify these findings.

In summary, in this study we describe that smoothelin expression indicates that glomic cells are smooth mucle cells with contractile capability, and should be used in the routine diagnosis. We also demonstrate the presence of WT1 in glomus cells, which may be implicated in cell proliferation and angiogenesis of glomus lesions. Furthermore, we show that the absence of WT1 expression in endothelial cells differentiates malformations from glomus tumors. Thus, the expression of smoothelin and WT1 help to better define the diagnosis of glomus lesions.

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