Pathological Study on the Expression of Vasohibins in Peripheral Artery Disease

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Abstract

Vasohibin-2 (VASH2) is a gene that promotes local angiogenesis. The tubulin carboxypeptidase activity of vasohibin causes detyrosination of alpha-tubulin and may play an important role in the regulation of various phenomena. Pathological and therapeutic angiogenesis are involved in atherosclerotic lesions. This study aimed to investigate whether the expression of VASH2 is associated with peripheral artery disease (PAD) in relation to angiogenesis, tubulin detyrosination, and severity of atherosclerotic lesions. An analysis of femoral and tibial arteries obtained from 86 patients with PAD or abdominal aortic aneurysm (AAA) was performed. The expressions of cluster of differentiation 31, VASH1, VASH2, and detyrosinated alpha-tubulin (DT-tubulin) were examined by immunohistochemistry, and their association with PAD was analyzed. The counts of VASH2 in the tunica media and adventitia in the tibial artery were significantly higher than those in the femoral artery in the PAD (P = 0.005 and P = 0.008, respectively) and AAA (P = 0.002 and P < 0.001, respectively) groups. In the tunica media and adventitia, VASH2 was significantly correlated with DT-tubulin. There was no significant difference in the expression of VASH2 and DT-tubulin in medial smooth muscle cells (McNemar test, P > 0.999). This study revealed the possible involvements of VASH2 in atherosclerosis by two methods—one maybe related to the progression of atherosclerosis by inducing angiogenesis and the second may be related to the decrease in arterial elasticity by increasing DT-tubulin in medial smooth muscle cells.
Keywords: angiogenesis, detyrosinated alpha-tubulin, peripheral artery disease, smooth muscle cells, vasohibin
Introduction

Peripheral artery disease (PAD) is an ischemic disease of the lower extremities that is primarily caused by atherosclerosis. The advanced symptoms of PAD include rest pain, leg ulcers, and gangrene, defined as chronic limb-threatening ischemia (CLTI) (Conte MS et al. 2019). Approximately 12%–15% of patients with CLTI undergo major amputation 1 year after endovascular or bypass surgery (Wübbeke LF et al. 2020).

Angiogenesis in arteriosclerosis can be divided into pathological angiogenesis involved in plaque progression and therapeutic angiogenesis forming collateral circulation (Chen and Walterscheid 2006). Previous studies on angiogenesis in PAD have mainly focused on angiogenic factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), and hepatocyte growth factor (Lederman et al. 2002; Biscetti et al. 2013; Sanada et al. 2020).

The vasohibin family has been isolated as a family of genes regulating angiogenesis (Watanabe et al. 2004). Vasohibin-1 (VASH1) is produced by vascular endothelial cells in response to angiogenic stimuli and inhibits angiogenesis by negative feedback (Sato 2013); it is mainly expressed in vascular endothelial cells at the termination zone of angiogenesis to halt it. Vasohibin-2 (VASH2), a homolog of VASH1, is expressed in non-endothelial perivascular cells at the sprouting front and promotes local angiogenesis (Shibuya et al. 2006; Kimura et al. 2009). Many previous studies on vasohibins (VASHs) have been conducted for
malignant tumors (Murakami et al. 2014; Kim et al. 2015; Sano et al. 2017; Ninomiya et al. 2018). In particular, VASH2 is overexpressed in various cancer cells and promotes tumor angiogenesis; it stimulates angiogenesis in a paracrine fashion by acting on adjacent endothelial cells. In contrast, some studies assessing non-neoplastic lesions have focused on diabetic nephropathy and aortic aneurysm (Masuda et al. 2018; Ohtaka et al. 2021). However, the precise role of VASH2 in such diseases remains to be clarified.

Both VASH1 and VASH2 were recently shown to have tubulin carboxypeptidase (TCP) activity, causing detyrosination of alpha-tubulin within a cell as posttranslational modification (Aillaud et al. 2017; Nieuwenhuis et al. 2017). Detyrosination of alpha-tubulin (DT-tubulin) by TCP activity is associated with microtubule (MT) stabilization and may play an important role in the regulation of various phenomena (Huebner et al. 2018). Detyrosination of alpha-tubulin in endothelial cells induced by VASH1 impairs endocytosis and trafficking of receptors for angiogenic factors, such as VEGF and FGF2, which results in the stoppage of their signaling and angiogenesis inhibition (Kobayashi et al. 2021).

The presence of VASH1 in the tunica adventitia of human atherosclerotic lesions has been previously demonstrated (Yamashita et al. 2006); however, to the best of our knowledge, its expression in the tunica media and differences in expression depending on the arterial site in the lower limb have not been clarified. Furthermore, whether VASH2 is expressed on the vascular wall remains unclear.
Therefore, this study aimed to investigate whether VASH2 is associated with PAD in relation to its effects, tubulin detyrosination, and angiogenesis and to determine whether VASH1 is expressed not only in the tunica adventitia but also in the tunica media. To this end, we performed pathological examinations to clarify the association between the expressions of VASH1 and VASH2 and PAD.

Materials and Methods

This study was approved by the Institutional Review Board of Kawasaki Medical School (approval #3619) and conducted in accordance with institutional guidelines. Written informed consent was obtained from all patients in this study.

From January 2019 to June 2021, 86 arterial tissue specimens were surgically resected from 86 patients at the Kawasaki Medical School General Medical Center (Okayama, Japan). The femoral artery in patients with PAD (PAD femoral group), the tibial artery in patients with PAD (PAD tibial group), and the femoral artery with no lower extremity hypotension in patients with abdominal aortic aneurysm (AAA) (control group) were compared. The control group comprised patients with AAA whose femoral artery were repaired during the intraoperative process. Indications for the treatment of PAD were intermittent claudication, rest pain, and tissue loss (ulcers) with or without gangrene. The tibial artery was amputated in all the patients due to gangrene.
**Preparation of Tissues and Staining**

The specimens were fixed in 10% formalin and embedded in paraffin. The paraffin blocks were cut into 4-μm-thick sections. Hematoxylin and eosin (H&E)-stained sections were prepared from the obtained arterial tissues. Subsequently, all sections were subjected to Elastica–Masson (Masson–Goldner) (E–M) staining and immunohistochemistry analysis.

**Elastica–Masson Staining**

After deparaffinization and hydration, the sections were immersed in Bouin’s solution for 30 min at 56 °C, and then the slides were rinsed under running tap water. After rinsing with acid alcohol solution (1% HCl in 70% alcohol), Weigert’s staining was performed using resorcin fuchsin solution for 20 min, and followed by rinsing under running tap water. Next, Sections were immersed in 99% reagent alcohol three times and then rinsed with lukewarm water. Next, the slides were stained in Biebrich scarlet-acid fuchsin for 3 min, rinsed with running tap water, incubated in phosphotungstic-phosphomolybdic acid for 10–15 min, dyed with Light Green solution for 5 min, and rinsed with 1% acetic acid for 1–3 min.

Finally, the tissue sections were dehydrated in ethanol and xylene and the slides were covered using dronabinol.

**Immunohistochemistry**
After deparaffinization and hydration, hot-bath antigen retrieval was performed at 95 °C for 40 min in Target Retrieval solution (pH 9.0) (Dako, Glostrup, Denmark) for cluster of differentiation 31 (CD31), a marker of endothelial cells known as PECAM-1 (platelet endothelial cell adhesion molecule), DT-tubulin, and VASH1 staining and in citrate buffer (pH 6.0) for VASH2 staining. Sections were incubated in 3% hydrogen peroxide for 10 min at room temperature to inactivate endogenous peroxidase activity. The following primary antibody reactions were performed overnight at 4°C: (1) anti-CD31 antibody (JC70A, Dako, Glostrup, Denmark) at 1:50 dilution, (2) monoclonal mouse anti-human VASH1 antibody (Watanabe et al. 2004) at 1:500 dilution, positive control was (3) monoclonal mouse anti-human VASH2 antibody (Koyanagi et al. 2017) at 1:200 dilution, and (4) anti-DT-tubulin antibody (ab48389, Abcam, Cambridge, United Kingdom) at 1:200 dilution. The antibodies used are listed in Table 1. VASH antibodies were provided by the Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University. Secondary antibody incubations using EnVision Plus (Dako) were performed at room temperature for 30 min. Subsequently, 3,3′-diaminobenzidine tetrahydrochloride solution (0.05 M Tris–HCl, 3% hydrogen peroxide) was used for staining. Hematoxylin nuclear staining was performed. As positive controls, pancreatic cancer was used for VASH1, ovarian cancer for VASH2, and peripheral nerves for DT-tubulin. Negative controls were stained with buffer instead of primary antibody.
Evaluation of Immunostaining

First, the tunica adventitia and tunica media of each artery were confirmed by E–M-stained sections at 40× magnification. In addition, one field with many CD31-positive blood vessels was selected using the Olympus ocular micrometer WHN10X (1 unit length = 1 m, 100 mm²). Among the CD31-positive vessels, the number of vessels positive for each antibody was counted at 100–200× magnification. Structures without a visible vascular lumen and single-cell positivity were excluded from the count. The number of microvessels less than 100 μm in diameter was counted, as reported in a previous study (Giannoni et al. 2009).

Statistical Analyses

Qualitative variables are expressed as absolute numbers and percentages and quantitative variables as medians and interquartile ranges for nonparametric data. Qualitative data were analyzed using Fisher’s exact test for the entire group, followed by between-group comparisons using Fisher’s exact test. Quantitative data were analyzed using the Kruskal–Wallis test for the entire group, followed by between-group comparisons using the Mann–Whitney U test. Multiplicity of comparisons among groups was accounted using Bonferroni correction. Correlations were assessed with Spearman’s rank correlation coefficients. The presence or absence of VASH2 and DT-tubulin in the smooth muscle was tested for
significant differences using the McNemar test. P-values of \( \leq 0.05 \) were considered statistically significant. All statistical analyses were performed using the Statistical Package for the Social Sciences version 22.0 for Windows (IBM Japan, Tokyo, Japan).

**Results**

The PAD femoral group comprised 37 males and 13 females with ages ranging from 43 to 95 years (median, 76.5 years). The PAD tibial group comprised 6 males and 10 females with ages ranging from 48 to 92 years (median, 74.5 years). The control group comprised 16 males and 4 females with ages ranging from 34 to 94 years (median, 78 years).

Vessel wall pathology of the femoral and tibial arteries is shown in Figures 1 and 2, respectively. H&E staining showed intimal thickening of the femoral (Fig 1A) and tibial arteries (Fig 2A) (40×). Meanwhile, E–M staining showed extensive medial fibroplasia due to increased collagen fiber and intimal thickening in the femoral artery (Fig 1B). In the tibial artery, thinning of the external elastic lamina and part of the smooth muscle bundles were replaced by collagen fibers in the tunica media (40×). Microvascular endothelial cells in the adventitia and tunica media showed positivity for each antibody (100×, 400×).

Figure 3 presents an example of an immunohistochemical pattern, wherein nuclei of medial smooth muscle cells are positive for VASH2 and DT-tubulin (100×, 400×). The results of the three-group comparison are shown in Table 2. Comparison of all groups showed
significant differences in the presence of CD31 (P < 0.001), VASH1 (P = 0.003), VASH2 (P < 0.001), and DT-tubulin (P = 0.003) in the tunica media and in the presence of CD31 (P < 0.001), VASH1 (P < 0.001), VASH2 (P < 0.001), and DT-tubulin (P < 0.001) in the tunica adventitia. The counts of CD31 (P = 0.024), VASH2 (P = 0.005), and DT-tubulin (P = 0.048) in the tunica media and CD31 (P = 0.017), VASH1 (P < 0.001), and VASH2 (P = 0.008) in the tunica adventitia were significantly higher in the PAD tibial group than in the PAD femoral group. The counts of CD31 (P = 0.001), VASH2 (P = 0.047), and DT-tubulin (P < 0.001) in the tunica adventitia were significantly higher in the PAD femoral group than in the AAA group. The counts of CD31 (P = 0.001), VASH1 (P = 0.004), VASH2 (P = 0.002), and DT-tubulin (P = 0.004) in the tunica media and of CD31 (P < 0.001), VASH1 (P < 0.001), VASH2 (P < 0.001), and DT-tubulin (P < 0.001) in the tunica adventitia were significantly higher in the PAD tibial group than in the AAA group.

Results of the correlation analysis are shown in Table 3. VASH2 in the tunica media was significantly correlated with DT-tubulin in the tunica media (ρ = 0.851, P < 0.001) and DT-tubulin in the tunica adventitia (ρ = 0.332, P = 0.002). Meanwhile, VASH2 in the tunica adventitia was significantly correlated with DT-tubulin in the tunica media (ρ = 0.431, P < 0.001) and DT-tubulin in the tunica adventitia (ρ = 0.763, P < 0.001). There were no significant differences in the expressions of VASH2 and DT-tubulin in medial smooth muscle cells (McNemar test, P > 0.999) in Table4.
Discussion

This study revealed that VASH2 is expressed on the vascular wall and is associated with PAD in relation to angiogenesis, tubulin detyrosination, and severity of atherosclerotic lesions. Furthermore, VASH1 is expressed in the tunica adventitia.

Results of a previous quantitative analysis of angiogenesis in the arterial wall of symptomatic atherosclerosis suggest that arteries in patients who experienced cardiovascular events, such as myocardial infarction, cerebral infarction, and PAD, have a hyperplastic vasa vasorum network and intimal thickening with high intimal macrophage content (Fleiner et al. 2004).

PAD, especially in CLTI cases, is characterized by severe atherosclerosis, and the tibial artery is particularly exposed to ischemic conditions. In this study, the tibial arteries of patients with CLTI who were amputated due to gangrene showed more CD31-positive vessels than the femoral arteries. Therefore, we hypothesized that VASH2 is expressed in the tibial artery in response to ischemic conditions.

Here, we showed for the first time that smooth muscle cells were positive for VASH2 at the lesion of PAD. These smooth muscle cells were also positive for DT-tubulin, indicating that smooth muscle cells express VASH2. The expression of VASH2, which might promote angiogenesis, was significantly increased in human PAD arteries. Pathological angiogenesis
in the progression of arteriosclerosis proliferates in the vasa vasorum from the tunica adventitia to the tunica intima (Vasuri et al. 2012). The trigger for this angiogenesis is associated with hypoxia in the vessel wall (Moreno et al. 2006; Sluimer and Daemen 2009). Notably, in this study, the expression of VASH2 was higher in the tibial artery than in the femoral artery. We believe that this result is due to the angiogenesis in response to hypoxia caused by more severe ischemia, although a response due to any kind of inflammatory process cannot be ruled out. In addition, in this study, VASH1 was expressed in endothelial cells of not only the tunica adventitia but also the tunica media. Thus, VASH1 may be responsible for DT-tubulin shown in endothelial cells. A previous study using a mouse arterial wall injury model suggested that VASH1 plays a prophylactic role in the angiogenesis-dependent component of neointimal formation (Yamashita et al. 2006). The expression of VASH1 in the tunica media may be a negative feedback mechanism to prevent intimal thickening due to angiogenesis from the tunica adventitia to the tunica intima.

MTs are composed of alpha beta-tubulins that are essential for cell shape and intracellular organization and transport (Janke and Bulinski 2011). The C-terminus of alpha-tubulin undergoes a detyrosination–tyrosination cycle. In this cycle, the C-terminal tyrosine residue of alpha-tubulin is cleaved from the peptide chain by TCP, which is the role of VASHs, and reattached to the chain by tubulin-tyrosine ligase (Barra et al. 1988; Peris et al. 2006). Importantly, proper tubulin detyrosination has been involved in the regulation and
maintenance of vascular tube morphogenesis (Kim et al. 2013). In contrast, tubulin detyrosination might be enhanced in muscle cells in certain pathological conditions, such as hypertrophic cardiomyopathy and Duchenne muscular dystrophy (Kerr et al. 2015) and may impair the function of muscle cells (Chen et al. 2018). Our study shows another example of enhanced tubulin detyrosination in muscle cells under a pathological condition, particularly in the arterial smooth muscle cells of atherosclerotic lesions.

VASH1 is the negative feedback regulator of angiogenesis induced by angiogenic factors (Watanabe et al. 2004). The presence of VASH1 protein in the atherosclerotic lesion is previously reported by Fukumitsu et al. (Fukumitsu et al. 2015). Our present observation reproduced this notion, and further confirmed the angiogenic force in the atherosclerosis lesion. Angiogenic factors, such as VEGF and FGF-2, are involved in atherosclerosis (Lederman et al. 2002; Giacca and Zacchigna 2012; Parma et al. 2020). Here, we added another factor, namely VASH2, which might also play a pathological role in atherosclerosis. However, the unique feature of VASH2 is that it might affect arterial lesions in two ways: (1) worsening of arterial elasticity by increasing DT-tubulin in smooth muscle cells and (2) worsening of intimal thickening by inducing adventitial angiogenesis.

This study has some limitations. First, there was a bias in the number of patients in each group. Second, it was not possible to set controls for young patients without atherosclerosis, and the expression of VASH in normal tissue groups was unclear.
Our study confirmed the expressions of VASH and DT-tubulin in arterial tissues. However, it is necessary to consider whether these proteins are actually produced and secreted in the same place and whether VASH is involved in therapeutic angiogenesis.

In conclusion, the present study disclosed two possible involvements of VASH2 in atherosclerosis—one maybe related to the progression of atherosclerosis by inducing angiogenesis and the second maybe related to the decrease in arterial elasticity by increasing DT-tubulin in medial smooth muscle cells. Further investigation is required to verify the role of VASH2 in atherosclerosis in future. In addition, the expression of VASH1 in the tunica media may play a prophylactic role as a negative feedback mechanism to prevent intimal thickening due to angiogenesis from the tunica adventitia to the tunica intima. These findings may provide new therapeutic targets for the treatment and inhibition of atherosclerosis progression in PAD.

Acknowledgments

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Conflicts of Interest

The authors declare that they have no potential conflicts of interest.

References


Figure Legends

**Figure 1.** Vessel wall pathology of the femoral artery. (A) Hematoxylin and eosin staining showing intimal thickening of the femoral artery (40×) EC, endothelial cells; TI, tunica intima; TM, tunica media; TA, tunica adventitia. (B) Elastica–Masson staining showing extensive medial fibroplasia due to increased collagen fiber and intimal thickening (40×). (C–F) Microvascular endothelial cells in the adventitia and tunica media showing positivity for each antibody (100×, 400×) (arrows).

**Figure 2.** Vessel wall pathology of the tibial artery. (A) Hematoxylin and eosin staining showing partial intimal thickening of the tibial artery (40×). (B) Elastica–Masson staining showing thinning of the external elastic lamina and part of the smooth muscle bundles are replaced by collagen fiber in the tunica media (40×). (C–E) Microvascular endothelial cells in the adventitia and tunica media showing positivity for each antibody (100×, 400×).

**Figure 3.** Example of an immunohistochemical pattern, wherein nuclei of identical medial smooth muscle cells are positive for vasohibin-2 and detyrosinated alpha-tubulin (100×, 400×).
Table 1. Antibody list in this study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Clone</th>
<th>Supplier</th>
<th>Dilution</th>
<th>Incubation</th>
<th>Antigen retrieval</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>JC70A</td>
<td>Dako, Glostrup, Denmark</td>
<td>1:50</td>
<td>Overnight at 4°C</td>
<td>TRS (pH 9.0)</td>
<td>Water bath for 40 min (95°C)</td>
</tr>
<tr>
<td>VASH1</td>
<td>monoclonal mouse anti-human</td>
<td>Tohoku University, Japan</td>
<td>1:500</td>
<td>Overnight at 4°C</td>
<td>TRS (pH 9.0)</td>
<td>Water bath for 40 min (95°C)</td>
</tr>
<tr>
<td></td>
<td>VASH1 (Watanabe et al. 2004)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VASH2</td>
<td>monoclonal mouse anti-human</td>
<td>Tohoku University, Japan</td>
<td>1:200</td>
<td>Overnight at 4°C</td>
<td>citrate buffer (pH 6.0)</td>
<td>Water bath for 40 min (95°C)</td>
</tr>
<tr>
<td></td>
<td>VASH2 (Koyanagi et al. 2017)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DT-tubulin</td>
<td>ab48389</td>
<td>abcam, Cambridge, UK</td>
<td>1:200</td>
<td>Overnight at 4°C</td>
<td>TRS (pH 9.0)</td>
<td>Water bath for 40 min (95°C)</td>
</tr>
</tbody>
</table>

CD31, cluster of differentiation 31; VASH1, vasohibin-1; VASH2, vasohibin-2; DT-tubulin, detyrosinated alpha-tubulin; TRS; Target Retrieval solution
### Table 2. Pathological examination results for each of the three groups

<table>
<thead>
<tr>
<th></th>
<th>① PAD femoral group (n = 50)</th>
<th>② PAD tibial group (n = 16)</th>
<th>③ AAA group (n = 20)</th>
<th>P-value for all</th>
<th>① vs. ②</th>
<th>① vs. ③</th>
<th>② vs. ③</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31 in TM</td>
<td>0.0 [0.0, 3.0]</td>
<td>4.5 [0.0, 11.0]</td>
<td>0.0 [0.0, 0.0]</td>
<td>&lt; 0.001*</td>
<td>a 0.024*</td>
<td>0.105</td>
<td>0.001* b</td>
</tr>
<tr>
<td>CD31 in TA</td>
<td>10.0 [5.0, 15.0]</td>
<td>16.0 [11.3, 27.5]</td>
<td>3.0 [0.0, 5.8]</td>
<td>&lt; 0.001*</td>
<td>a 0.017*</td>
<td>0.001*</td>
<td>&lt; 0.001* b</td>
</tr>
<tr>
<td>VASH1 in TM</td>
<td>0.0 [0.0, 0.0]</td>
<td>0.0 [0.0, 4.0]</td>
<td>0.0 [0.0, 0.0]</td>
<td>&lt; 0.001*</td>
<td>a 0.065</td>
<td>0.179</td>
<td>0.004* b</td>
</tr>
<tr>
<td>Prevalence of VASH1 in TM</td>
<td>8 [16.0%]</td>
<td>7 [43.8%]</td>
<td>0 [0.0%]</td>
<td>0.003*</td>
<td>c 0.110</td>
<td>0.284</td>
<td>0.004* d</td>
</tr>
<tr>
<td>VASH1 in TA</td>
<td>0.0 [0.0, 0.3]</td>
<td>4.5 [0.0, 8.0]</td>
<td>0.0 [0.0, 0.0]</td>
<td>&lt; 0.001*</td>
<td>a 0.001*</td>
<td>0.052</td>
<td>&lt; 0.001* b</td>
</tr>
<tr>
<td>VASH2 in TM</td>
<td>0.0 [0.0, 0.0]</td>
<td>2.5 [0.0, 4.0]</td>
<td>0.0 [0.0, 0.0]</td>
<td>&lt; 0.001*</td>
<td>a 0.005*</td>
<td>0.371</td>
<td>0.002* b</td>
</tr>
<tr>
<td>VASH2 in TA</td>
<td>4.0 [2.0, 7.0]</td>
<td>8.0 [5.3, 14.5]</td>
<td>0.5 [0.0, 5.3]</td>
<td>&lt; 0.001*</td>
<td>a 0.008*</td>
<td>0.047*</td>
<td>&lt; 0.001* b</td>
</tr>
<tr>
<td>DT-tubulin in TM</td>
<td>0.0 [0.0, 1.0]</td>
<td>2.0 [0.0, 5.5]</td>
<td>0.0 [0.0, 0.0]</td>
<td>&lt; 0.001*</td>
<td>a 0.048*</td>
<td>0.309</td>
<td>0.004* b</td>
</tr>
<tr>
<td>DT-tubulin in TA</td>
<td>6.0 [3.0, 10.0]</td>
<td>9.0 [5.3, 13.8]</td>
<td>1.5 [0.0, 3.0]</td>
<td>&lt; 0.001*</td>
<td>a 0.361</td>
<td>&lt; 0.001*</td>
<td>&lt; 0.001* b</td>
</tr>
</tbody>
</table>

Values are presented as medians [interquartile range], except for prevalence of VASH1 in the tunica media, which is presented as counts of VASH1 divided by the total number of specimens in each group.
PAD, peripheral artery disease; AAA, abdominal aortic aneurysm; n, number of examinations; CD31, cluster of differentiation 31; VASH1, vasohibin-1; VASH2, vasohibin-2; DT-tubulin, detyrosinated alpha-tubulin; TM, tunica media; TA, tunica adventitia.

*Statistically significant (P ≤ 0.05). P-value for all determines whether the difference in expression between two groups is statistically significant.

a, Kruskal–Wallis test; b, Mann–Whitney U test with Bonferroni correction; c, Fisher’s exact test for all; d, Fisher’s exact test with Bonferroni correction.
Table 3. Correlation analysis between each pathological examination: Spearman

<table>
<thead>
<tr>
<th></th>
<th>VASH2 in TM</th>
<th></th>
<th>VASH2 in TA</th>
<th></th>
<th>DT-tubulin in TM</th>
<th></th>
<th>DT-tubulin in TA</th>
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</thead>
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<tr>
<td></td>
<td>ρ</td>
<td>P-value</td>
<td>n</td>
<td>ρ</td>
<td>P-value</td>
<td>n</td>
<td>ρ</td>
<td>P-value</td>
</tr>
<tr>
<td>VASH2 in TM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.458</td>
<td>&lt; 0.001*</td>
<td>86</td>
<td>0.851</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>VASH2 in TA</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.431</td>
<td>&lt; 0.001*</td>
</tr>
<tr>
<td>DT-tubulin in TM</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>/</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DT-tubulin in TA</td>
<td>/</td>
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<td>/</td>
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</tr>
</tbody>
</table>

VASH2, vasohibin-2; DT-tubulin, detyrosinated alpha-tubulin; TM, tunica media; TA, tunica adventitia; ρ, Spearman’s rank correlation coefficient; n, number of examinations.

*Statistically significant (P ≤ 0.05). If P ≤ 0.05, the observed correlation is unlikely to be due to chance.
Table 4. Correlation analysis between each pathological examination: McNemar test

<table>
<thead>
<tr>
<th></th>
<th>DT-tubulin in smooth muscle</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>VASH2 in smooth muscle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>Positive</td>
<td>9</td>
<td>54</td>
</tr>
</tbody>
</table>

VASH2, vasohibin 2; DT-tubulin, detyrosinated alpha-tubulin;

Data are represented as numbers. P-value: McNemar test.

*Statistically significant (P ≤ 0.05). There was no significant difference in the expressions of VASH2 and DT-tubulin in medial smooth muscle cells (McNemar test, P > 0.999). i.e., there is a correlation between the expressions of VASH2 and DT-tubulin.
Figure 2

A. H&E

B. E-M

C. CD31

D. VASH1

E. VASH2

F. DT-tubulin