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The prognostic value of blood and lymph vessel parameters in lichen sclerosus for vulvar squamous cell carcinoma development: an immunohistochemical and electron microscopy study

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OBJECTIVE: The objective of the study was to quantify vessel type and density in lichen sclerosus (LS) to find a marker for its malignant potential.

STUDY DESIGN: Quantitative analysis was performed on paraffin-embedded tissue samples of 28 patients with LS (7 adjacent to vulvar squamous cell carcinoma, 21 solitary) and immunohistochemical staining for CD34 (vascular and lymphangiogenic lymph endothelial cells), D2-40 (lymphatic-specific marker), and α-SMA (pericyte marker). Electron microscopy was performed on fresh tissue.

RESULTS: No significant differences in vessel density or other vessel parameters could be demonstrated between the 2 groups. In hyalinized lesions, vessel diameter, and α-SMA positivity was reduced compared with nonhyalinized lesions. Electron microscopy revealed detachment of pericytes from vascular endothelial cells and increased thickening of basement membrane, whereas endothelial cell function did not appear strongly impaired.

CONCLUSION: Malignant potential of LS cannot be predicted by vessel characteristics. Hyalinization in LS is associated with pericyte detachment from the basal lamina of vascular endothelial cells.

Key words: lichen sclerosus, malignant potential, microvessel density, pericytes, vulvar cancer


Lichen sclerosus (LS) is a chronic inflammatory skin disease that may occur in any cutaneous surface but has a distinct preference for the anogenital area. It is more commonly found in women than men, and extragenital LS can be found in up to 20% of all women suffering from vulvar LS. The etiology of LS remains elusive.

Multiple associations with autoimmune disorders, sexual hormones, infection, or repeated trauma (itch and scratch hypothesis/Koebner phenomenon) have been reported, and genetic as well as immunologic factors are thought to play a role. The classic histological features of lichen sclerosus are epidermal thinning, decreased rete ridge length, band-like dermal inflammation of varying intensity, with or without edema and/or hyalinization.

LS is diagnosed in all age groups, including infancy, but is most prevalent in postmenopausal women. An incidence of 1:300-1000 in gynecological and dermatological female patients is estimated, but the exact numbers are unknown. Women suffering from genital LS have a 4-6% risk of developing vulvar squamous cell carcinoma (SCC). Furthermore, in 20-60% of the cases of vulvar SCC, LS can be found in adjacent areas.

Because of the risk of malignant progression, it is current practice that all patients with vulvar LS undergo regular check-ups, although there is no evidence that this follow-up prevents the development of vulvar SCC or results in earlier detection of a malignancy.

Until now, there is no biomarker available that can identify vulvar LS lesions in which SCCs are more likely to develop. Microvessel density (MVD) has been suggested to be of predictive value for tumor development and progression in skin tumors and multiple types of gynecological tumors. The induction of the angiogenic response is considered a key step in the transition from a premalignancy toward an invasive neo-
plasm,\textsuperscript{14-16} and high MVD is related to poor survival in vulvar cancer patients.\textsuperscript{17} Furthermore, Raspollini et al.\textsuperscript{18} suggested that MVD could identify those cases of LS that have the potential to evolve to vulvar SCC.

In addition to vascular endothelial cells, pericyte number and function could have a prognostic value. Pericytes envelop microvascular endothelial blood vessels and are key regulators of vessel homeostasis.\textsuperscript{19,20} In bladder and colorectal tumors, loss of pericyte function is associated with poor prognosis.\textsuperscript{21,22} Pericytes have not been studied in vulvar lesions.

Edema and hyalinization may be considered surrogate markers for the (dys)functioning of microvasculature: edema and deposition of proteins (hyalinization) may be the results of leaky microvessels and/or inadequate drainage. Although edema and hyalinization are both manifestations of vascular mal-function, hyalinization is thought to occur after the edematous phase in LS.\textsuperscript{23,24}

The aim of this study was to analyze MVD and pericyte characteristics in vulvar LS associated with vulvar SCC and

\begin{table}[h]
\centering
\caption{Hyalinization vs edema}
\begin{tabular}{|c|c|c|c|}
\hline
Variable & Edema & No edema & Total \\
\hline
Hyalinized & 7 & 13 & 20 \\
Nonhyalinized & 0 & 8 & 8 \\
Total & 7 & 21 & 28 \\
\hline
\end{tabular}
\end{table}

Fisher's exact test, \( P = .065 \). In lichen sclerosus, edema was found only in the hyalinized cases.


\begin{table}[h]
\centering
\caption{Hyalinization vs association with carcinoma}
\begin{tabular}{|c|c|c|c|}
\hline
Variable & Solitary & SCC associated & Total \\
& LS & LS & \\
\hline
Hyalinized & 15 & 5 & 20 \\
Nonhyalinized & 6 & 2 & 8 \\
Total & 21 & 7 & 28 \\
\hline
\end{tabular}
\end{table}

Fisher's exact test, \( P = .673 \). LS, lichen sclerosus; SCC, squamous cell carcinoma.

solitary vulvar LS to establish a possible diagnostic significance in the identification of malignant potential.

**MATERIALS AND METHODS**

**Sample selection**
Paraffin-embedded tissue of 28 LS patients were selected from the archives of the Department of Pathology, Radboud University Nijmegen Medical Centre (Nijmegen, The Netherlands). Two distinct groups of patients were distinguished: solitary LS (n = 21), and LS directly adjacent to vulvar squamous cell carcinoma (n = 7). LS was considered to be solitary when the patient had no history of differentiated vulvar intraepithelial neoplasia (VIN) and/or vulvar SCC prior to or after the biopsy. All hematoxylin and eosin (H&E)–stained slides were reexamined by an expert gynecopathologist (John Bulten). In each lesion, the presence of edema and hyalinization was scored.

Because the stored tissue samples were anonymously studied, this part of the study was exempt from institutional review board approval.

For electron microscopy, fresh 4 mm biopsies of LS lesions were obtained and split in 2. Half was processed for H&E staining and the other fixed in 2.5% glutaraldehyde dissolved in 0.1 M sodium cacodylate buffer (pH 7.4). Tissues were obtained according to local ethical guidelines and approved by the local regulatory committee.

**Immunohistochemistry of CD34, D2-40, and α-SMA**
Paraffin sections (4 μm) were mounted onto Superfrost slides (Menzel-Gläser, Braunschweig, Germany) and dried overnight at 37°C. Tissues were dewaxed in xylene, rehydrated through graded alcohol baths, and rinsed 3 times in phosphate-buffered saline (PBS; pH 7.4) for 10 minutes. Following an antigen retrieval step (sodium citrate [0.01 M; pH 6.0], 95°C, 10 minutes), tissue sections were preincubated with 20% normal goat serum in PBS and subsequently incubated with the primary antibodies for CD34 (Dako, Glostrup, Denmark, 1:750), D2-40 (Dako, 1:100), or α-SMA (1:15,000; NeoMarkers, Fremont, CA) for 60 minutes at room temperature. All antibodies were diluted in PBS containing 1% bovine serum albumin.

Slides were rinsed in PBS for 10 minutes and incubated with biotinylated anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. A biotin-avidin alkaline phosphatase complex was generated according to standard procedures (Vector Laboratories). Alkaline phosphatase was visualized with Fast Blue (20 ml Tris HCl [pH 8.2], 4 mg Naphtol AS-MX, 4.8 mg levamisole, and 20 mg Fast Blue BB salt; Sigma-Aldrich, Steinheim, Germany) and counterstained with Nuclear Fast Red (Vector Laboratories). Slides were mounted with Imsol (Klinipath, Duiven, The Netherlands) and subsequently with Permount (Fisher Scientific, Fairlawn, NJ). Negative controls (buffer only) and positive controls (normal skin) were applied in each run.

**Quantification of CD34, D2-40, and α-SMA staining**
Image acquisition was performed using a 3CCD color video camera (Sony.
DXC-950P; Sony Corp, Tokyo, Japan) mounted on a conventional light microscope (Axioskop 2 plus; Carl Zeiss AG, Jena, Germany) and attached to a personal computer with frame grabber card (Matrox Meteor-II Multichannel; Matrox Imaging, Dorval, Canada). Images were acquired using a 20 objective (Plan Neofluar, NA 0.5, resulting in specimen level pixel size of 0.39 μm²).

Prior to analysis of the immunohistochemical staining, an image of an empty microscopic field was acquired, which was used for correction for unequal illumination. Image acquisition and analysis were performed using a custom written macro in KS400 image analysis software (Carl Zeiss).

Thresholds were determined from a set of training slides and were found adequate for almost all slides analyzed in this study. When the initial thresholds led to unrealistic patterns, adjustment was performed by the operator (data not shown). For each patient, MVD, mean vessel area, and mean vessel perimeter in the lichenoid area between the band-like inflammatory infiltrate and epidermis was calculated per surface area in multiple nonoverlapping images (range, 3–13; mean, 6.4).

Statistical analysis
Calculation of vessel parameters per patient was performed using Statistical Package for the Social Sciences (version 15.0.1; SPSS, Chicago, IL). Because the vessel variables were not normally distributed, the nonparametric Mann-Whitney U test was used to analyze differences between the groups. The relationship between the presence of edema or hyalinization in the 2 groups of LS was calculated according to the Fisher’s exact test. P < .05 was considered to be statistically significant.

To test our hypothesis that no differences exists for MVD between SCC-associated and solitary lesions and also between nonhyalinized and hyalinized lesions, equivalence testing was used next to the commonly performed testing for differences between groups. The null hypothesis for equivalence testing is that the difference between the means of 2 groups exceeds a certain threshold, with alternative hypothesis that no such difference exists. The critical t value for rejecting the null hypothesis may be computed from the inverse noncentral F distribution, with noncentrality parameter A = n1 n2 ε²/(n1 + n2). In the present study, the constant ε was fixed at a value of 20% of the average MVD for the respective experiment, which we consider an appropriate threshold for a difference of physiological significance.
RESULTS

Clinicopathological features

The histopathological analysis of all cases of LS showed hyperkeratosis, a thin epidermis, and a variable chronic inflammatory cell infiltrate. The presence of edema and hyalinization was variable and recorded for each patient. We found no cases of edema without hyalinization and half of the cases showed hyalinization without edema, confirming that hyalinization occurs after the edema (Table 1). The presence of hyalinization was not related to the presence of SCC adjacent to the LS (Table 2; \( P = .673 \)).

Immunohistochemistry

LS lesions were immunohistochemically stained for CD34, D2-40, and \( \alpha \)-SMA (Figure 1) on consecutive sections. In addition to vascular endothelial cells, CD34 also stains lymphangiogenic but not quiescent lymph vessels.28 We did not observe CD34-positive vessels that were also positive for the specific lymph vessel marker D2-40 (Figure 1, B and C), which indicates that lymph vessel growth is not evident in these LS samples. Subsequently the respective vessels number, area, and perimeter were quantified. No significant differences in the total number of CD34, D2-40, or \( \alpha \)-SMA-positive vessels could be demonstrated between solitary LS and SCC-associated LS (Figure 2). In addition, no differences in CD34, \( \alpha \)-SMA, or D2-40-positive vessel area and vessel perimeter were observed in both LS groups (data not shown).

We found a significantly reduced number of \( \alpha \)-SMA-positive vessels in hyalinized LS, whereas the number of CD34-positive and D2-40-positive vessels was not reduced in hyalinized LS (Figure 3). However, all vessels were significantly smaller in hyalinized LS (Figure 4). A trend toward smaller vessels and less SMA positivity in edematous lesions was observed, but these differences were not statistically significant (data not shown).

Electron microscopy

Loss of \( \alpha \)-SMA expression suggested impaired pericyte function or even lack thereof. To further analyze pericyte characteristics in LS, transmission electron microscopy was performed on fresh LS specimens with and without hyalinization. Microvessels in LS are enveloped by pericytes in nonhyalinized LS (Figure 5, A and B) but are detached from the basal lamina of microvascular endothelial cells in hyalinized regions of LS (Figure 5, C and D). At higher magnification (Figure 5, E), thickening of the basement membrane is evident. Furthermore, interepithelial junctions (IEJs) are closed and vesiculovacuolar organelles can be seen, which suggests that endothelial cell function is not strongly impaired.

COMMENT

Our study shows that vessel parameters in LS are not associated with development of vulvar SCC. Neither did we find a correlation between edema or hyalinization and the presence of SCC adjacent to LS. However, vessels were significantly smaller in LS with hyalinization, and reduced \( \alpha \)-SMA positivity was observed. Electron microscopy revealed that pericytes are detached from the basal lamina and thickening of the basement membrane in hyalinized regions of LS. Hyalinization was observed in both types of
LS, and we did not find a significant difference between hyalinization and tumor development (Table 2). Therefore, although pericyte detachment occurs in hyalinized LS, we propose that this does not increase risk for tumor development. The role of angiogenesis in vulvar oncogenesis is still unclear, and studies focusing on the different vulvar premalignancies give conflicting results. Raspollini et al described that expression of vascular endothelial growth factor (VEGF) was highest among the group of LS that evolved to vulvar SCC. However, MacLean et al showed that VEGF was not expressed in normal vulva, solitary LS, or LS adjacent to SCC. Likewise, Bamberger and Perrett could not demonstrate a predictive value of VEGF expression. Furthermore, it has been suggested that increased density of subepithelial CD34-positive microvessels could identify those LS that are at greater risk for SCC development.

Our study does not corroborate these findings. We did not find differences in vessel density, area, or perimeter, nor did we detect lymphangiogenesis because we have not observed D2-40 positive vessels that also expressed CD34. Our data confirm a previous report that micro vessel density in LS does not predict its malignant potential. Our study does not corroborate these findings. We did not find differences in vessel density, area, or perimeter, nor did we detect lymphangiogenesis because we have not observed D2-40 positive vessels that also expressed CD34. Our data confirm a previous report that micro vessel density in LS does not predict its malignant potential. Our study does not corroborate these findings. We did not find differences in vessel density, area, or perimeter, nor did we detect lymphangiogenesis because we have not observed D2-40 positive vessels that also expressed CD34. Our data confirm a previous report that micro vessel density in LS does not predict its malignant potential.

Unexpectedly, we found a correlation between pericyte coverage of vascular endothelial cells and the presence of hyalinization: Reduced α-SMA positivity and detachment of mural cells was observed in the hyalinized lichenoid areas in both types of LS. Pericytes are generally considered to be contractile cells that stabilize vessel walls and participate in the regulation of blood flow in the microcirculation. Pericytes may also influence endothelial permeability, proliferation, survival, migration, and maturation. Regulation of blood vessel homeostasis and response to angiogenesis or vascular permeability-inducing stimuli involves a complex spatiotemporal regulation through several molecules.

This provides a balance between the activation of endothelial cells to expand the vasculature and the interaction of endothelial cells with pericytes to ensure a stable, functional vasculature. This interaction is in part under control of the VEGF-VEGF receptor system. Currently we do not know whether VEGF receptor activity is altered in vessels with detached pericytes, which is the subject of our future investigations. However, despite detachment of pericytes and loss of their α-SMA expression in hyalinized...
LS, these vascular endothelial tubes do not display widening of the IE. Vesicular-vacuolar organelles that are involved in transcellular transport from vessel lumen to the interstitium can be readily detected in these vessels. This suggests that vascular function might be still intact and that the thickening of the basement membrane prevents the vessels from leaking.

The probability that LS is a vulvar SCC precursor lesion is debatable. The number and dilation of vessels in solitary LS is identical to that in LS directly adjacent to SCC. In tumors, however, MVD is strongly increased (shown by Bancher-Todesca et al. and our unpublished results).

Our data demonstrate the lack of an angiogenic switch in LS juxtaposed to SCC. Since the induction of an angiogenic program is considered to be required for progression of a premalignant lesion towards an invasive tumor, we argue that LS is not a vulvar SCC precursor lesion. However, it has to be noted that the number of patients included in this study may have been too small for this statement to be unequivocal.

An a priori power analysis using vessel density values (n, average, SD, α, β) as input data indicates that at least 1400 samples need to be analyzed to detect a significant difference. However, the opposite statistical approach (i.e., testing that absolute difference in blood vessel density in the 2 groups is <20%) demonstrated no differences in the density of CD34 and α-SMA–positive vessels in solitary and SCC-associated LS (P < .05). However, because of the low number of D2-40–positive vessels, we cannot exclude a difference in lymph vessel density.

It is tempting to speculate that human papillomavirus (HPV)–negative differentiated vulvar intraepithelial neoplasia (dVIN), which can be found in the edges of the tumor, is the true precursor. MVD has been reported to be of value for determining the potential malignant progression of HPV–associated usual VIN to SCC. Whether this also holds true for dVIN remains elusive and is subject of further studies.

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REFERENCES


