be used to remove the bias of heterogeneous tissue samples (5), other techniques can also be used. Billings *et al.* (1) describe a quantification system based on absolute pixel count. While this method of quantification would allow for accurate comparison amongst homogenous tissue sources, heterogeneous tissue specimen sizes would undoubtedly affect the total pixel count. The use of pixel density rather allows for comparison independent of tissue size.

Pixel density accounts both for the colour density or luminosity, as defined in the histogram from 0 to 255, with lower numbers indicating more colour density. These dense signals correspond to the greatest areas of chromophore deposition. The following equation can be used to weigh colour density and account for tissue size using data outputted from the luminosity histogram using the ImageJ program (Link: rsbweb.nih.gov/ij).

$$\frac{\sum(255 - \text{Value}) \times \text{Count}}{\text{Width(pixels)} \times 100}.$$

Data should be taken only from the first peak, as this corresponds with the peak chromophore signal. These values can be calculated for each slide and then compared between two independent samples using a student t-test allowing for determination of statistical significance between two sample groups. A sample of this process is provided in Data S1.

Billings *et al.* provide a useful method for using open-source software to quantify immunohistochemical stains. The above methods can improve the precision of using Billings *et al.*'s method when required experimentally.

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Conflicts of interest

The authors have declared no conflicting interests.

Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Data S1. Instructional on creating a luminosity histogram and processing its data.

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Letter to the Editor

Evaluation of the *CAV1* gene in clinically, sonographically and histologically proven morphea patients

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Background

The inflammatory skin disorder morphea (circumscribed cutaneous scleroderma) is characterized by significant fibrosis (collagen deposition) of the dermis, evident throughout all of its pathologic phases (1). Morphea association with fibrosis may even extend to other fibrotic diseases such as lichen sclerosus, which has been reported to coexist with localized scleroderma within the genetic background of female monozygotic twins (2). Besides its typical dermal involvement, ultrasound of morphea has revealed alteration of the underlying subcutaneous tissue consisting of inflammatory signs (i.e. increased echogenicity and vascularity) during the active phase, and decrease or even total absence of the subcutaneous fatty lobules, suggestive of lipodystrophy, in the atrophic phase (3). Within the context of tissue fibrosis, recent research has identified an important pathogenic mechanism: the deficiency of caveolin 1 (CAV1) the main coating protein of caveola (plasma membrane invaginations

rich in the cell surface receptors critical for the initiation of intracellular signalling cascades) (4,5). Thus, CAV1 (determined as mRNA, by immunofluorescence, or directly using chemiluminescence) has been found decreased in affected lungs and skin of patients with systemic sclerosis (scleroderma; SSc), as well as in lungs and fibroblasts of patients with idiopathic pulmonary fibrosis. Experimentally, the CAV1 knockout mouse develops striking pulmonary and skin fibrosis (4,6), while the CAV1 protein has antifibrotic actions from the inhibition of transforming growth factor-β (TGFβ) receptor internalization (7). In addition, the effect of CAV-1 interfering with intracellular signalling by vascular endothelial growth factor A (VEGF-A, hereafter referred to as VEGF) may also be important for the production of fibrosis in SSc. In this disease, fibrosis may result from vascular alterations via dedifferentiation of endothelial cells and pericytes towards myofibroblasts. CAV 1 deficiency would induce the profibrotic phenotype through suppression of VEGF signalling, by default stimulation (derepression) of VEGF receptor 2 degradation. Such mechanism is consistent with the reportedly low levels of CAV-1 with upregulation of VEGF signalling in bone marrow SSc mesenchymal stem cells, a pericyte surrogate (8).

Question addressed

Is *CAV1* gene positive in morphea?

Experimental design

We therefore analysed the coding sequence of the *CAV1* gene in a population of well-characterized patients with morphea, fully assessed for disease activity, both clinically and by skin ultrasound. The genetic study with DNA sequencing was performed at Columbia University (from 1 February 2014 to 30 July 2014) in patients recruited from among all cases with morphea diagnosed by dermatologic examination and histologic evaluation from 1 March 2008 to 30 July 2013 (n = 138), sonographically positive and seen at a single referral centre (Clinical Servet, Santiago, Chile). For this study, we selected the first ten patients enrolled, while excluding cases with the Parry–Romberg (PR) syndrome (facial morphea with ipsilateral hemiatrophy of the face). The test group consisted of nine females and one male, with mean age of 24.8 $\pm$ 13.1 years (SD); they had a mean of 1.9 morphea lesions. The distribution of the cutaneous lesions was as follows: 40% truncal, 30% facial and 30% extremities. On ultrasound, the lesions were predominantly inactive in one patient; mostly in the atrophic phase in three patients; and active, with subcutaneous fat involvement, in six patients. More data are available in the supplemental material.

For the genetic studies, genomic DNA was extracted from whole blood with PureGene reagents (Gentra Systems Inc., Minneapolis, MN, USA). Genomic DNA was extracted from saliva using Oragene kits (Omnigene-Discover OM-501 kits; Genotek Inc., Ottawa, ON, Canada), according to the manufacturer's instructions. More data are available in the supplemental material. All participants signed an informed consent form, and the study was approved by the Institutional Review Board at Clinica Servet, Santiago, Chile.

Results

None of the patients had positive family history for morphea; eight had the normal, reference *CAV1* sequence, while the remaining two subjects (# 1 and # 7) had novel variants. Patient #1 was heterozygous for two variants: c. 66 C>A (p. C22Ter) and c. 284 C>T (p. T95M). Patient #7 was heterozygous for one variant: 284 C>T (p. T95M). Segregation analysis showed that the unaffected father of patient # 1 was heterozygous for the c. 284 C>T (p. T95M) variant, and the unaffected mother was heterozygous for c. 66 C>A (p. C22Ter). The C22Ter variant is predicted to be pathogenic at the protein level and to result in haploinsufficiency. However, as it is also carried by the clinically unaffected mother, if pathogenic, the variant has incompletely penetrance.

In the family of patient #7, the mother had the wild-type sequence, and an unaffected sister was heterozygous for c. 284 C>T (p. T95M) (Table 1). Therefore, T95M is, in all likelihood, an inherited normal variant.

Conclusions

The present work represents the first attempt at evaluating genetic contributions to morphea pathogenesis using a candidate gene approach. While the results of our genetic analysis do not support the involvement of *CAV1* in morphea, there are some limitations to our study. (i) The sample size is small, although the age, distribution of location and activity of the lesions are representative of morphea. (ii) We did not quantify CAV1 protein, nor performed assessment of the gene regulatory sequences. (iii) We purposely restricted the study to patients with the most common form of the disease and excluded those with the clinically more homogeneous Parry–Romberg form. (iv) It is possible that some of the asymptomatic relatives of patients harbouring *CAV1* variants (who did not undergo sonographic examination) could have cryptic lesions that were not symptomatic, because patients with morphea can have subclinical lesions evident only on ultrasound (3).

Table 1. Morphea study: clinical characteristics of the patient population and genetic results

Patient	Age	Sex	Affected area	Ethnicity	Genetic results	Family member testing
1	38	F	Dorsum (1 lesion)	Spanish Native	c. 66 C>A (p. C22Ter) and c. 284 C>T (p. T95M)	Father: c. 284 C>T (p. T95M) Mother: 66 C>A (p. C22Ter)
2	4	F	Right upper extremity (2 lesions)	Spanish Native	Wild type	n/a
3	31	F	Right frontal region (1 lesion)	Spanish Native	Wild type	n/a
4	21	F	Left frontal and glabellar regions (2 lesions)	Spanish Native	Wild type	n/a
5	10	F	Left upper extremity (5 lesions)	Romani Gipsy	Wild type	n/a
6	44	F	Left lower extremity (2 lesions)	Spanish Native	Wild type	n/a
7	32	F	Lower extremities (2 lesions)	Spanish Native	c. 284 C>T (p. T95M);	Mother: Wild-type Sister: c. 284 C>T (p. T95M)
8	35	F	Right inframammary (1 lesion)	Spanish Native	Wild type	n/a
9	18	M	Chin (1 lesion)	Spanish Native	Wild type	n/a
10	15	F	Abdominal wall (1 lesion)	Spanish Native	Wild type	n/a

M, male; F, female; n/a, not available.

While morphea is not a Mendelian disorder, excessive fibrosis is common to morphea, scleroderma, idiopathic pulmonary hypertension, lipodystrophy, and even allograft failure after renal transplantation (2–7,9) S1–S3. TGFβ-responsive gene signature has been found in a subset of diffuse scleroderma patients (S4). Given the effects of TGFβ, CAV1 and VEGF in fibrosis and antifibrosis, it becomes increasingly important to determine the role of these proteins in these diseases to develop novel therapies. Within this context, our examination of the *CAV1* gene in the commonest form of morphea may represent an important step in that direction.

Author contributions

Ximena Wortsman, MD, worked in the design, gathering and analysis of the data, and writing of the manuscript. Lijiang Ma, PhD, worked in the gathering and analysis of the data as well as the revision of the manuscript. Wendy K. Chung, MD, PhD, worked in the design, gathering and analysis of the data, and writing of the manuscript. Jacobo Wortsman, MD, worked in the design, analysis of the data and writing of the manuscript.

Ethical approval

All participants signed an informed consent form, and the study was approved by the Institutional Review Board at Clinica Servet, Santiago, Chile.

Conflict of interest

None for all authors.

Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Data S1 Material.

Supplementary References - S1-S4.

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Letter to the Editor

Filaggrin-2 barrier protein inversely varies with skin inflammation

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Background

Atopic dermatitis (AD) is a common chronic, episodic inflammatory ailment (1). Recent studies have shown that AD is a life-long disease that often begins in early childhood and that persists into adulthood. AD is at least in part associated with a dysfunctional skin barrier often due to loss of function (LOF) mutations of filaggrin (*FLG)* (2). Although the impact of FLG LOF on skin barrier function is less certain (3). While mutations of *FLG* are relatively common in Asian and European children with AD, they were very infrequently found in those of African ancestry (2,4).

The *FLG* gene is part of the epidermal differentiation complex (EDC) (5). The EDC contains more than 60 genes many of which are involved in skin barrier function, filaggrin-2 (*FLG2*) (6). We have shown that variation of the *FLG2* gene is associated with the persistence of AD in African Americans (7). The *FLG2* gene is structurally similar to the *FLG* gene, and the FLG2 protein is has similar biochemical and biophysical properties to the FLG protein (8). Like FLG, the production of FLG2 protein has also been shown to be diminished in those with AD and in the presence of inflammatory mediators (3,6). (s1).

Question addressed

Does FLG2 immunohistochemical staining vary with skin inflammatory response in those with AD?

Experimental design

Thirty-eight archived and anonymized skin tissue specimens with a clinical diagnosis of AD were evaluated. The staining protocol and assessment are presented in the supplement.