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Cell junction protein armadillo repeat gene deleted in velo-cardio-facial syndrome is expressed in the skin and colocalizes with autoantibodies of patients affected by a new variant of endemic pemphigus foliaceus in Colombia

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ABSTRACT

Background: We previously described a new variant of endemic pemphigus foliaceus in El Bagre, Colombia, South America (El Bagre-EPF, or pemphigus Abreu-Manu). El Bagre-EPF differs from other types of EPF clinically, epidemiologically, immunologically and in its target antigens. We reported the presence of patient autoantibodies colocalizing with armadillo repeat gene deleted in velo-cardio-facial syndrome (ARVCF), a catenin cell junction protein colocalizing with El Bagre-EPF autoantibodies in the heart and within pilosebaceous units along their neurovascular supply routes. Here we investigate the presence of ARVCF in skin and its possibility as a cutaneous El Bagre-EPF antigen.

Methods: We used a case-control study, testing sera of 45 patients and 45 controls via direct and indirect immunofluorescence (DIF/IIF), confocal microscopy, immunoelectron microscopy and immunoblotting for the presence of ARVCF and its relationship with El Bagre-EPF autoantibodies in the skin. We also immunoadsorbed samples with desmoglein 1 (Dsg1) ectodomain (El Bagre-EPF antigen) by incubating with the positive ARVCF samples from DIF and IIF.

Results: ARVCF was expressed in all the samples from the cases and controls. Immunoadsorption with Dsg1 on positive ARVCF immunofluorescence DIF/IIF cases showed that the immune response was present against non-desmoglein 1 antigen(s). Overall, 40/45 patients showed colocalization of their autoantibodies with ARVCF in the epidermis; no controls from the endemic area displayed colocalization.

Conclusions: We demonstrate that ARVCF is expressed in many areas of human skin, and colocalizes with the majority of El Bagre-EPF autoantibodies as a putative antigen.

Introduction

Endemic pemphigus foliaceus (EPF) is an autoimmune blistering disease characterized by the presence of autoantibodies (primarily to desmoglein 1 [Dsg1]). On direct immunofluorescence (DIF) and indirect immunofluorescence (IIF), the EPF autoantibodies display intercellular staining (ICS) between epidermal keratinocytes [1-3]. In Brazil, EPF is titled *fogo selvagem* (FS) and principally affects children and young adults. EPF has also been documented in rural areas of South and Central America (Argentina, Bolivia, El Salvador, Paraguay, Peru and Venezuela) [1-3]. One additional variant has been described in Tunisia, Africa [4].

We previously described a new variant of EPF in El Bagre, Colombia, South America (El Bagre-EPF, or pemphigus Abreu-Manu). El Bagre-EPF differs from other types of EPF because it affects predominantly 30- to70-year-old or older males, and a few peri/postmenopausal females [5-7]. The patients are primarily miners, who also work in agricultural activities. The patients have autoantibodies to dermal eccrine, sebaceous and Meibomian glands, and their neurovascular bundles; to cardiac molecules; to Pacinian receptors and other cutaneous neural receptors; and to tarsal muscles and to other structures [8-10]. For over two decades, we have followed the El Bagre-EPF patients [5-7,8-10]. The disease was originally thought to be a new focus of Brazilian EPF with some differences [19]; however, we later documented it as a new and unique disease, resembling Senear-Usher syndrome. We documented El Bagre EPF to have a unique autoimmune response due to environmental and genetic factors [10-14]. We also previously reported that one-third of the El Bagre-EPF patients present with a systemic form, as observed in systemic lupus erythematosus [10-15].

We previously reported the colocalization of El Bagre-EPF patient autoantibodies with armadillo repeat gene deleted in velo-cardio-facial syndrome (ARVCF) in cardiac tissue, as well as within pilosebaceous units along their neurovascular supply routes [9,11]. ARVCF is an adhesion molecule of the catenin-like protein family that is one of the mutated molecules in the disease called velo-cardio-facial syndrome (VCFS). VCFS, along with DiGeorge syndrome (DGS), and conotruncal anomaly face syndrome are part of the disease complex termed chromosomal microdeletions dissecting (CMD): del22q11 syndrome [11-13]. CMD is known to be associated with multiple autoimmune diseases, as well as some alterations in the immune system [11-13]. ARVCF also is related to the protein p120 subfamily of armadillo-related proteins, which includes p0071 and delta-catenin/NPRAP; these are distantly related plakophilins, and are involved in cell-cell adhesion [11-13]. In our current study, we investigated whether ARVCF was expressed in the skin, including the sebaceous glands and their neurovascular supply structures, and whether we could further confirm colocalization of El Bagre-EPF autoantibodies with ARVCF in the skin.

Materials and Methods

A human quality assurance review board approved the studies at the Hospital Nuestra Señora del Carmen in El Bagre, and all participants provided signed consent. We tested 45 patients affected by El Bagre-EPF, and 45 controls from the endemic area matched by age, sex, demographics, comorbidities, and work activities. All of the tests were performed in both cases and controls. The patients and controls were evaluated by hematoxylin and eosin (H&E) histology, DIF, IIF, confocal microscopy (CFM), indirect immunoelectron microscopy (IEM) immunoblotting (IB), by immunoprecipitation (IP) and by ELISA. Only patients meeting diagnostic criteria for El Bagre-EPF were included; specifically, they had to display clinical and epidemiologic features described for this disease; live in the endemic area; and have serum displaying intercellular staining (ICS) between epidermal keratinocytes and to the basement membrane zone (BMZ) of the skin via either DIF or IIF using fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies to human total IgG or IgG4, as described elsewhere [5-9]. Further, each patient had to be positive by IB for reactivity against Dsg1 [6], as well as for plakin molecules; each patient's serum immunoprecipitated a Concanavalin A (Con A) affinity-purified bovine tryptic 45 kDa fragment of Dsg1 [14]; and each patient's serum had to yield a positive result using an ELISA test when screening for autoantibodies to pemphigus foliaceus (PF) antigens [15].

DIF and IIF: We performed our DIF and IIF as previously described [8,9].

DIF: In brief, we incubated a 4 µm thickness frozen skin section using PBS with 0.1% Triton X-100 and 1% normal goat serum for five minutes for partial permeabilization, to detect cytoplasmic, nuclear, and membrane binding putative antigens, and for blocking non-specific staining. The slides were then washed with PBS [8,9]. The nuclei of the cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Pierce; Rockford, IL, USA). We used antibodies to ARVCF, source guinea pig, Cat. no. GP155; Progen Biotechnik (Heidelberg, Germany). For its secondary, we used Alexa Fluor®555 goat-anti-guinea pig (Molecular Probes Life Technologies/ThermoFisher Scientific; Waltham, MA, USA). All samples were run with positive and negative controls. We classified our findings as negative (-), weakly positive (+), moderately positive (++) and strongly positive (+++). For IIF, our substrate tissue included human, bovine, rat and mouse skin. Written consents were obtained from all patients, and Institutional Review Board permission was obtained from the Hospital Nuestra Señora de El Carmen, in El Bagre, Colombia.

IIF: Performed as the DIF, but utilizing skin from plastic surgeries as an antigen source.

Colocalization of the patient's autoantibodies with commercial antibodies utilizing confocal microscopy (CFM): Our CFM studies were performed as previously described [8,9]. In brief, we utilized standard 20X and 40X objective lenses; each photoframe included an area of approximately 440 x 330 µm. Images were obtained using EZ-1 image analysis software (Nikon, Tokyo, Japan). For colocalization experiments with serum autoantibodies, we used the previously described antibodies to ARVCF.

Indirect immunoelectron microscopy (IEM): Our technique was performed as described [8,9]. In brief, postembedding immunogold labeling was performed on samples on the subjects of the study. Mouse skin was used as an antigen; the tissue was fixed in 4% glutaraldehyde with 0.2% paraformaldehyde, and embedded in Lowicryl® resin [8,9]. The tissue was then sectioned at a 70 nm thickness. The samples were blocked with a solution from Aurion™ (Electron Microcopy Sciences/EMS; Hatfield, PA, USA). Grids were then washed with PBS-BSAC (Aurion™, EMS). The primary antibodies were incubated, washed, and a secondary antibody solution, specifically 10 nm Gold-conjugated protein A PBS-BSAC (Aurion, EMS™) was applied [8,9]. The samples were then double-stained with uranyl acetate and lead citrate. The samples were observed under a Hitachi H7500 transmission electron microscope. Immunogold particle images displaying any pattern of positivity were then converted to TIF format.

Immunoadsorption of skin Dsg1 1 autoantibodies: Patients with El Bagre-EPF have circulating autoantibodies directed against Dsg1 [5,6]. Based on the fact that the purified tryptic fraction of Dsg1 induces loss of adhesion in organ culture and in a neonatal mouse model, it has been proposed that these anti-Dsg1 antibodies play a pathogenic role in blister formation. To investigate if the ARVCF putative autoantibodies reactivity was the same as shown by Dsg1, we performed immunoadsorption experiments as previously described by incubating the tryptic ectodomain of Dsg1 with samples that tested positive for DIF and IIF [14]. We used affinity tryptic-purified antigen, obtained as described using the Con A column [14]. In brief, protein samples were released from bovine snout epidermis by means of trypsin digestion, and purified with the use of a Con A column. The eluted Dsg1 antigen was then incubated with El Bagre-EPF DIF and IIF slides that were positive previously in colocalization of ARVCF with the El Bagre-EPF serum.

Statistical analysis: We used Fisher exact test to compare two nominal variables (e.g., positive and negative) of antibody response. $P < 0.05$ with a 95% of confidence or more was considered statistically significant. We used the software GraphPad QuickCalcs (GraphPad Sofware Inc., La Jolla, CA, USA).

Results

A clinical picture of an exfoliative lesion of El Bagre-EPF is shown in Figure 1a. In Figure 1b, we show the corresponding histologic pattern. Our testing demonstrated positive staining for ARVCF in all layers of the epidermis, on the basement membrane zone and in upper areas of the subjacent dermis (including cell junctions and neurovascular bundles) by DIF, IIF, CFM, IB, and IEM. See Figure 1c, demonstrating one example of DIF staining with colocalization of the El Bagre-EPF autoantibodies and ARVCF.

Overall, 40/45 patients affected by El Bagre-EPF demonstrated autoantibodies that colocalized perfectly with the ARVCF antibody via DIF. In the remaining five patients affected by El Bagre-EPF we observed the expression of ARVCF, but the autoantibodies did not colocalize with the ARVCF antibody. In Table 1 are shown the results of the polyclonal immune response using DIF and IIF, the strength of the stains and the colocalization with the El Bagre-EPF autoantibodies and the ARVCF reactivity.

The CFM studies also demonstrated 100% colocalization of the patient autoantibodies and the commercial antibody to ARVCF ($p < 0.01$) (Figure 1c, d). None of the controls from the endemic area showed autoantibodies colocalizing to ARVCF via DIF, IIF, CMF, or IEM. However, the ARVCF molecule is ubiquitous in the skin.

After the immunoadsorption with the pre-incubated Dsg1 antigen with the patient samples testing positive for colocalization with ARVCF, the immunoreactivity to ARCVF persisted.

Our IEM data (Figure 1e, f) demonstrated positive staining for patient autoantibodies in skin cell junctions. The IB studies confirmed multiple antigens detected by patient sera, that also perfectly colocalized with the Progen antibody to ARVCF (data not shown).

Discussion

To our knowledge, we present evidence for the first time in the medical literature that ARVCF is expressed in the skin via diverse methods. We also demonstrated that El Bagre-EPF autoantibodies colocalized with the commercial antibody to ARVCF from Progen in the skin of 40/45 patients. We further demonstrated that after immunoadsorption with an ectodomain of Dsg1 the reactivity remained, demonstrating that the El Bagre-EPF patient's autoantibodies are directed to a non-Dsg1 antigen.

We noted that 5/45 El Bagre-EPF patients did not display autoantibodies against ARVCF. El Bagre EPF patients present a diverse spectrum of clinical lesions (hyperpigmented, prurigoid, generalized, blistering, etc.) [1,3,5]. The patients may also present in an acute, relapsing or chronic stage of

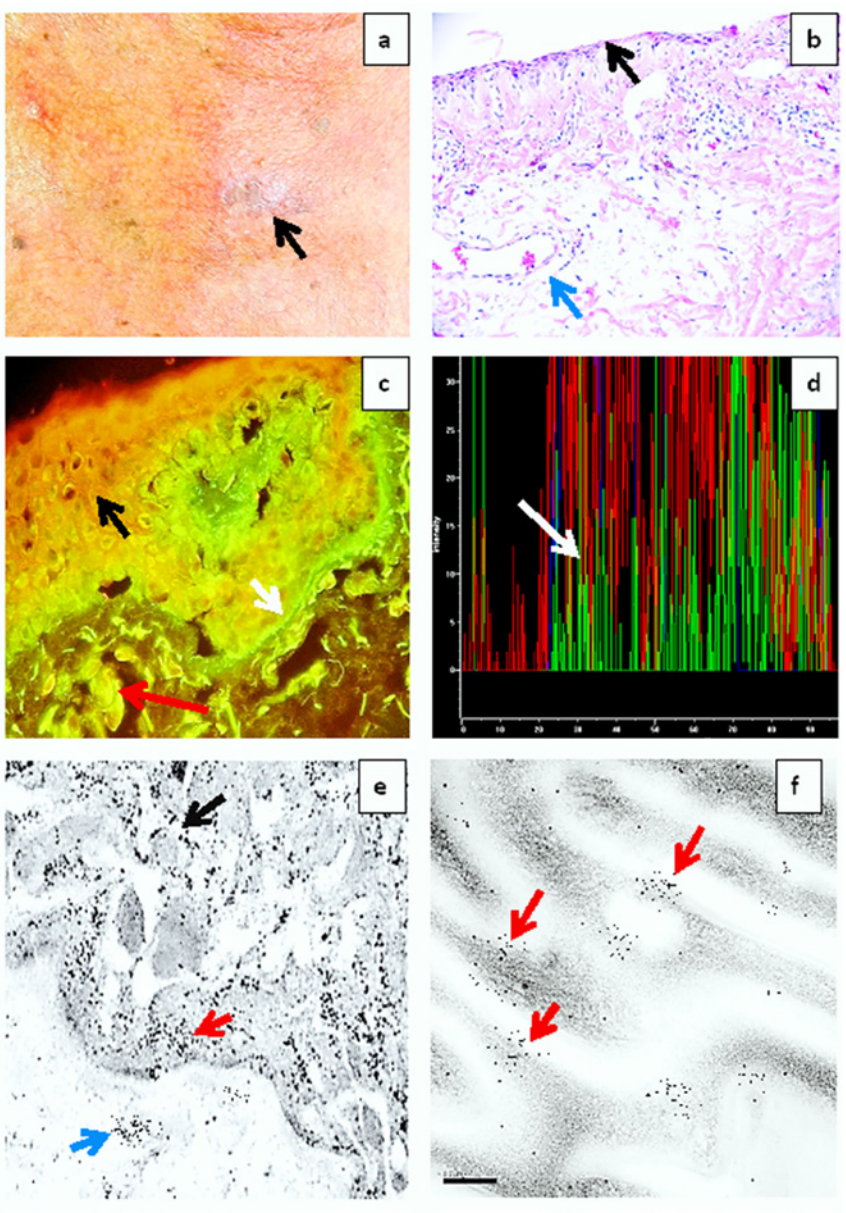

Figure 1. (a) An El Bagre EPF clinical blister (black arrow). (b) H&E staining of one of multiple patterns seen in El Bagre EPF. In this case, note the loss of the upper layers of the epidermis (black arrow), and a dilated dermal blood vessel (blue arrow) with dermal edema and inflammation. (c) DIF of the skin from a patient affected by El Bagre-EPF, showing positive double staining in the epidermis (in orange stain, as result of the patient's autoantibodies in green and the ARVCF in red) (+++) (black arrow), basement membrane zone (greenish stain) (+++) (white arrow) and a dermal neurovascular bundle (yellowish stain) (+++) (blue arrow) using FITC conjugated anti-human IgG (green staining) and Alexa 555 conjugated ARVCF (red staining). The staining observed represents overlap staining of different strength from yellowish-orange to green (400X). (d) Confocal microscopy, with multiple channels of fluorescence utilized. In the presented case we used a FITC channel (green peaks) (Excitation/Emission (nm):495/519), a DAPI channel (blue peaks) (Excitation/Emission (nm):360/460), and an Alexa Fluor®555 channel (red peaks) (Excitation /Emission (nm):555/568). The graphic shows the colocalization of the peaks of the immunofluorescence of the patient's antibodies (green peaks; white arrow) with the ARVCF antibody (red peaks; white arrow). Both green and red are aligned, demonstrating colocalization. The blue peaks represent DAPI (nuclear counterstaining). (e) and (f) IEM photographs of patient skin, showing ARVCF antibodies labelled with 10 nm Gold-conjugated protein A antibodies (tiny black dots). In (e), the antibodies are located in the epidermal cells junctions (black arrow), at the cutaneous basement membrane zone (black dots; red arrow) and in the upper papillary dermis (tiny black dots; blue arrow) (100kV). (f) Positive IEM staining in the upper epidermal keratinocytes cells junction showing ARVCF antibody gold labeled grouped in some areas (tiny black dots; red arrows) (100kV). [Copyright: ©2017 Abreu Velez et al.]

disease. Thus, each case may present a range of autoantigens and immune responses, depending of the clinical phase of the disease. We also showed that the immune response is polyclonal in patients affected by El Bagre-EPF, as previously described [8,9].

We speculate that disruption of epidermal cells in El Bagre-EPF could induce epitope spreading, or expose new molecules that could then become antigenic since like Dsg1, ARVCF represents a cell junction protein. ARVCF is a protein with three cellular locations: in the plasma membrane, the cytoplasm and the nucleus [13-16].

We also hypothesize that ARVC becomes antigenic because this molecule is calcium (Ca^{++}) dependent (similar to DSg1); further, that the El Bagre soils are polluted by mercury, metals and other metalloids [5] that can compete with calcium. El Bagre soils are extremely acidic, contain very scarce organic matter and are thus scarce in calcium, phosphates, magnesium (Mg^{++}), and potassium. Phosphates are constituents of ATP, DNA, RNA, and the phospholipids, which form all cell membranes where the cells junctions are situated. The existence or absence of a given metal/metalloid is crucial to the conformation or activity of most proteins. Thus, the metal/metalloid ions in the environment could compete with physiologic ions (e.g., Ca^{++}), altering the conformation of relevant molecules and generating antigenicity.

ARVCF (A.K.A. catenin delta 1) binds sulfate (S04) as well as magnesium (Mg^{++} are their ligands. DP also has an S04 ligand and to phosphoserine is classified as UniProtKB/

TABLE 1. DIF and IIF autoantibody staining in the skin and colocalization with ARVCF

DIF				IIF		
El Bagre-EPF autoantibodies	Number of positive cases	Strength of staining	Colocalization with ARVCF	Number of positive cases	Strength of staining	Colocalization with ARVCF
IgG	40/45	(+++)	100%	39/45	(+++)	100%
Fibrinogen	39/45	(+++)	100%	38/45	(++)	100%
IgM	38/45	(+++)	100%	38/45	(+++)	100%
Albumin	38/45	(+++)	100%	38/45	(++)	100%
Complement/C3c	35/45	(+++)	100%	35/45	(++)	100%
Complement/C1q	35/45	(++)	100%	35/45	(++)	100%
IgA	15/45	(++)	100%	15/45	(++)	100%
IgD	16/45	(++)	100%	16/45	(++)	100%
IgE	7/45	(++)	100%	7/45	(++)	100%

Swiss-Prot (O00192), and is a member of the catenin family, which contain an armadillo/beta-catenin-like repeat sequence [11-13]. ARVCF is a Ca^{++} dependent cell-cell adhesion molecule, acting via plasma membranes. The catenin family plays an important role in the formation of adherens junction complexes, which are thought to facilitate communication between the inner and outer environments of cells [11-13]. The data suggest that ARVCF is a putative El Bagre-EPF antigen. Our data indicates that ARVCF is a putative non-Dsg1 antigen in El Bagre-EPF patients.

The p120 protein family includes p120-catenin, δ-catenin, p0071, and ARVCF [10]. ARVCF associates with p68, hnRNP H2, SFF1, zonula occludens ZO-1 and ZO-2 (in the last two, binding to PDZ-domain proteins) [11-13].

All three diseases belonging to the CMD syndrome are associated with impaired function of T cells due to abnormal development of the thymus gland, abnormalities of T-cell clonality, and/or in B lymphocytic function [11-13,16]. We believe there is an abnormality in the immune response in El Bagre-EPF patients, which we are currently investigating.

Pemphigus Abreu-Manu differs from previously described forms of EPF. Indeed, El Bagre-EPF is a chronic inflammatory disease that has protean manifestations, including a form fruste (localized to the skin and resembling Senear-Usher syndrome, and with a unique autoimmune response due to environmental and genetic factors) with photosensitivity; and a systemic form [8,9]. Patients affected by this disease may present with relapsing episodes; the systemic form affects multiple organs with a less favorable prognosis [5,6,12].

In conclusion, we demonstrate the expression of the protein ARCVF in multiple areas of the skin. We prove colocalizing or this protein with the majority of the El-Bagre-EPF autoantibodies, and that the reactivity is directed against a non-Dsg1 antigen. We aim in our future research to establish the precise pathologic role of ARVCF in El Bagre-EPF patients and in the skin. The ARVCF UniProtKB/Swiss-Prot (000192) respective sequence is available to researchers to investigate if ARVCF may be a putative antigen in other autoimmune blistering diseases. More than 9% of the protein sequences provided by UniProtKB are derived from translation of the coding sequences.

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