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Patients affected by endemic pemphigus foliaceus in Colombia, South America exhibit autoantibodies to optic nerve sheath envelope cell junctions

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ABSTRACT

Background: The majority of the patients affected by a new variant of endemic pemphigus foliaceus in El Bagre, Colombia (El Bagre EPF or pemphigus Abreu-Manu), have experienced vision problems; we have previously reported several ocular abnormalities.

Methods: Here, we aimed to investigate reactivity to optic nerves in these patients. We utilized bovine, rat and mouse optic nerves, and performed immunofluorescence and confocal microscopy to test for optical nerve autoreactivity. We tested 45 patients affected by this disease and 45 controls from the endemic area matched by age, sex and work activity.

Results: Overall, 37 of the 45 patient sera reacted to the optic nerve envelope that is composed of leptomeninges; the reactivity was polyclonal and present mostly at the cell junctions ($P < 0.001$). The immune response was directed against optic nerve sheath cell junctions and the vessels inside it, as well as other molecules inside the nerve. No control cases were positive. Of interest, all the patient autoantibodies co-localized with commercial antibodies to desmoplakins I–II, myocardium-enriched zonula occludens-1- associated protein (MYZAP), armadillo repeat gene deleted in velo-cardio-facial syndrome (ARVCF), and plakophilin-4 (p0071) from Progen Biotechnik ($P < 0.001$).

Conclusion: We conclude that the majority of the patients affected by pemphigus Abreu-Manu have autoantibodies to optic nerve sheath envelope cell junctions. These antibodies also co-localize with armadillo repeat gene deleted in velo-cardio-facial syndrome, p0071 and desmoplakins I–II. The clinical significance of our findings remains unknown.

Introduction

Endemic pemphigus foliaceus (EPF) represents an autoimmune blistering disease presenting in an endemic fashion in South America, Central America, and Tunisia, Africa [1–4]. We have previously described a new variant of EPF in El Bagre, Colombia (El Bagre EPF, or pemphigus Abreu-Manu) [5–7] and also reported ocular problems in these patients including entropion and/or ectropion, trichiasis, blepharophimosis, thinned eyebrows, refractory defects, Meibominitis, and corticonuclear cataracts, among others [8]. In the presteroid era, patients affected by Brazilian fogo selvagem also presented with ocular problems [9]. Here, we aim to continue characterizing the El Bagre EPF autoimmune response by studying optic nerve reactivity in these patients.

Methods

We tested 30 patients affected by El Bagre EPF, and 30 controls from the endemic area matched by age, sex and work activities. A human quality assurance review board approved the studies at the Hospital Nuestra Señora del Carmen in El Bagre. The participants signed informed consent forms, and the patients were evaluated clinically, by hematoxylin–eosin (H & E) histology, direct and indirect immunofluorescence (DIF and IIF), immunohistochemistry, confocal microscopy (CFM), enzyme linked immunoassay (ELISA), immunoblotting (IB), and immunoprecipitation (IP), as previously described [5–12]. Only patients who fulfilled the full diagnostic criteria for El Bagre EPF were included, as follows: (i) patients displayed clinical and epidemiological features described for this disease; (ii) patients lived in the endemic area; (iii) the patient serum displayed intercellular staining between epidermal keratinocytes using DIF, and to the basement membrane zone of the skin by either DIF or IIF, using fluorescein isothiocyanate-conjugated monoclonal antibodies to human total immunoglobulin (IgG), or to IgG4, as previously described [5–12]; (iv) the patient serum was positive by IB for reactivity against desmoglein (Dsg)1, as well as for plakin molecules as previously described [6,7]; (v) the patient serum immunoprecipitated a concanavalin A affinity-purified antigen bovine tryptic 45-kDa fragment of desmoglein 1 (Dsg1) [10]; and (vi) the patient serum yielded a positive result using an ELISA when screening for autoantibodies to pemphigus foliaceus antigens [12].

DIF

Our studies were performed as previously described [5–8]. The slides were counterstained with 4,6-diamidino-2-phenylindole (Pierce, Rockford, IL, USA). We also used antibodies to desmoplakins (DP)-I–II (mouse monoclonal multi-epitope cocktail; Progen Biotechnik [Heidelberg, Germany], cat no. 65146). We utilized antibodies to armadillo repeat gene deleted in velo-cardio-facial syndrome (ARVCF) (polyclonal antibody, source guinea pig, tested in human and bovine; Progen Biotechnik, cat no. GP155); for its secondary, we used Alexa Fluor- 555 goat-anti-guinea pig from Molecular Probes/ Life Technologies/Thermo Fisher Scientific (Waltham, MA, USA). We also utilized an antibody to plakophilin-4 (p0071); Progen Biotechnik, cat no. 651166) and a mouse monoclonal antibody for myocardium-enriched zonula occludens-1- associated protein (MYZAP) [Progen Biotechnik, cat no. 651169]. As a secondary antibody for DP-I–II, the p0071 and the MYZAP, we utilized Texas red-conjugated goat anti-mouse IgG from Thermo Fisher. The samples were consistently run with positive and negative controls. We classified our findings as negative (-), weakly positive (+/-), positive (++) and strongly positive (+++).

IIF

In brief, for IIF we incubated 4-micron thick optic nerve sections from animal sources (cow, rat and mouse) on glass microscopic slides with secondary antibodies as previously described, but with some modifications as follows [5–8]. The rest of the procedure was done as it was for DIF.

Confocal microscopy

Confocal microscopy was performed as previously described [12].

Statistical analysis

We used Fisher's exact test to compare two nominal variables (e.g., positive and negative) of antibody response. We also compared the differences when evaluating: (i) positivity of the El Bagre EPF autoantibodies between patient cases and controls and (ii) patient antibody results versus the commercial antibodies to MYZAP, p0071, DP-I–II and ARVCF. $P < 0.01$ with a 98% of confidence or more was considered statistically significant. We used the software GraphPad QuickCalcs from GraphPad Software (La Jolla, CA, USA).

Results

Using IIF and CFM, 35 of the 45 patients affected by El Bagre EPF displayed autoantibodies to the optic nerve envelope ($P < 0.001$). The predominant autoantibodies were to IgG, IgM, complement/C1q, complement/C3, fibrinogen and albumin; in some cases, we also observed reactivity to IgA, IgD and IgE (see Table 1, that includes the daily dose of prednisone taken by each patient). As mentioned, the reactivity against the optic nerve sheath cells junctions and their vessels was polyclonal and distributed as follows: IgG (n = 37), IgM (28/45), albumin (n = 27), complement/C1q (n = 25), complement/C3c (n = 26),

TABLE 1. Positive staining ratio of patient autoantibodies (Ab) to optic nerve envelope/sheath cell junctions using IIF and CFM with daily doses of prednisone and staining strengths. "Daily dosage of oral prednisone" and "strength of staining" were given with the average of 45 cases.

Percentage of Patients with Ab Positive	Daily Dosage of Oral Prednisone	Number of Positive Cases	Strength of Staining
IgG	10 mg	37/45	(++++)
Fibrinogen	15 mg	37/45	(++++)
Albumin	20 mg	27/45	(+++)
IgM	25 mg	28/45	(+++)
Complement/C3c	30 mg	26/45	(+++)
Complement/C1q	30 mg	25/45	(++)
IgA	30 mg	10/45	(++)
IgD	15 mg	10/45	(++)
IgE	20 mg	10/45	(+)
Kappa	15 mg	36/45	(+++)
Lambda	15 mg	36/45	(+++)

fibrinogen (n = 37), IgE (n = 10), IgA (n = 10) and IgD (n = 10) (see Table 1). The patient auto autoantibodies against the optic nerve sheath cell junctions co-localized with commercial antibodies to DP-I–II, p0071, MYZAP and ARVCF from Progen ($P < 0.001$) using IIF and CFM (see Figures 1, 2). Of interest, the patients with higher autoantibody titers displayed more vision loss and symptoms including blurred, hazy, or "milky" vision, refractory defects and amblyopia ($P < 0.001$). In addition, external examination of the El Bagre-EPF patients displayed entropion and/or ectropion, trichiasis, blepharophimosis, thinned eyebrows, meibominitis, partial obstruction of the Meibomian gland ducts, pinguecula, actinic conjunctivitis, tarsal muscle thickening, edema, and pterygia in higher frequencies relative to the controls ($P < 0.001$).

Discussion

We have previously described ocular clinical alterations in patients affected by El Bagre EPF [8] as well as against nerves and neural mechanoreceptors [8]. We also have previously observed a polyclonal immune response in the patients affected by El Bagre EPF because they are exposed to many xenobiotic agents, as well as other tropical diseases that are putative triggers for this disease. In addition, many patients have a therapeutically induced immunosuppression, taking oral prednisone (usually <30 mg daily) [5,6]. The length of the optic nerve varies widely even between the two eyes of the same person and is on average approximately 35–55 mm from the eyeball to the chiasma (including the intraocular, intraorbital, intracanalicular, and intracranial parts) [13]. Each part can be further subdivided histologically into a fiber layer, a prelaminar region, and a lamina cribrosa region [13]. The optic nerve sheath (dura mater and arachnoid mater) is normally loose near the eyeball, with a much larger subarachnoid space between the optic nerve and its sheath than elsewhere in its course, thus presenting a bulbous appearance just behind the eyeball [13]. Unlike the orbital part of the sheath, the optic nerve in the optic canal is firmly bound to the dura by numerous thick fibrous bands connecting the dura to the pia [13]. These bands not only firmly hold the optic nerve in position in this region, but also hold the dura and the optic nerve close to one another. In this region, the subarachnoid space is reduced to almost a capillary size, which is interrupted by these bands [13]. The arachnoid and pia mater together are sometimes termed the leptomeninges, literally "thin meninges"; these are the two innermost layers of tissue that cover the brain and spinal cord. The outer blood–cerebrospinal fluid barrier is formed by leptomeningeal cells of the arachnoid mater [14]. The structures forming this barrier are tight junctions [14, 15]. Leptomeninges have multiple functions and anatomical relationships. The outer parietal layer of the arachnoid is impermeable to cerebral spinal fluid due to tight intercellular junctions; elsewhere, leptomeningeal cells form desmosomes and gap junctions [16]. In contrast to the tight junctions, specific gap junction cell proteins have also been identified (connexins 26 and 43) [13]. Leptomeningeal cells also form channels in the core and apical cap of arachnoid granulations for the drainage of cerebral spinal fluid into venous sinuses. In the spine, leptomeninges form highly perforated intermediate sheets of arachnoid mater and delicate ligaments that compartmentalize the subarachnoid space; specifically, dentate ligaments anchor subpial collagen to the dura mater and stabilize the spinal cord [12].

The deposits of patient autoantibodies and other inflammatory molecules in the optic nerve sheath envelope cell junc-

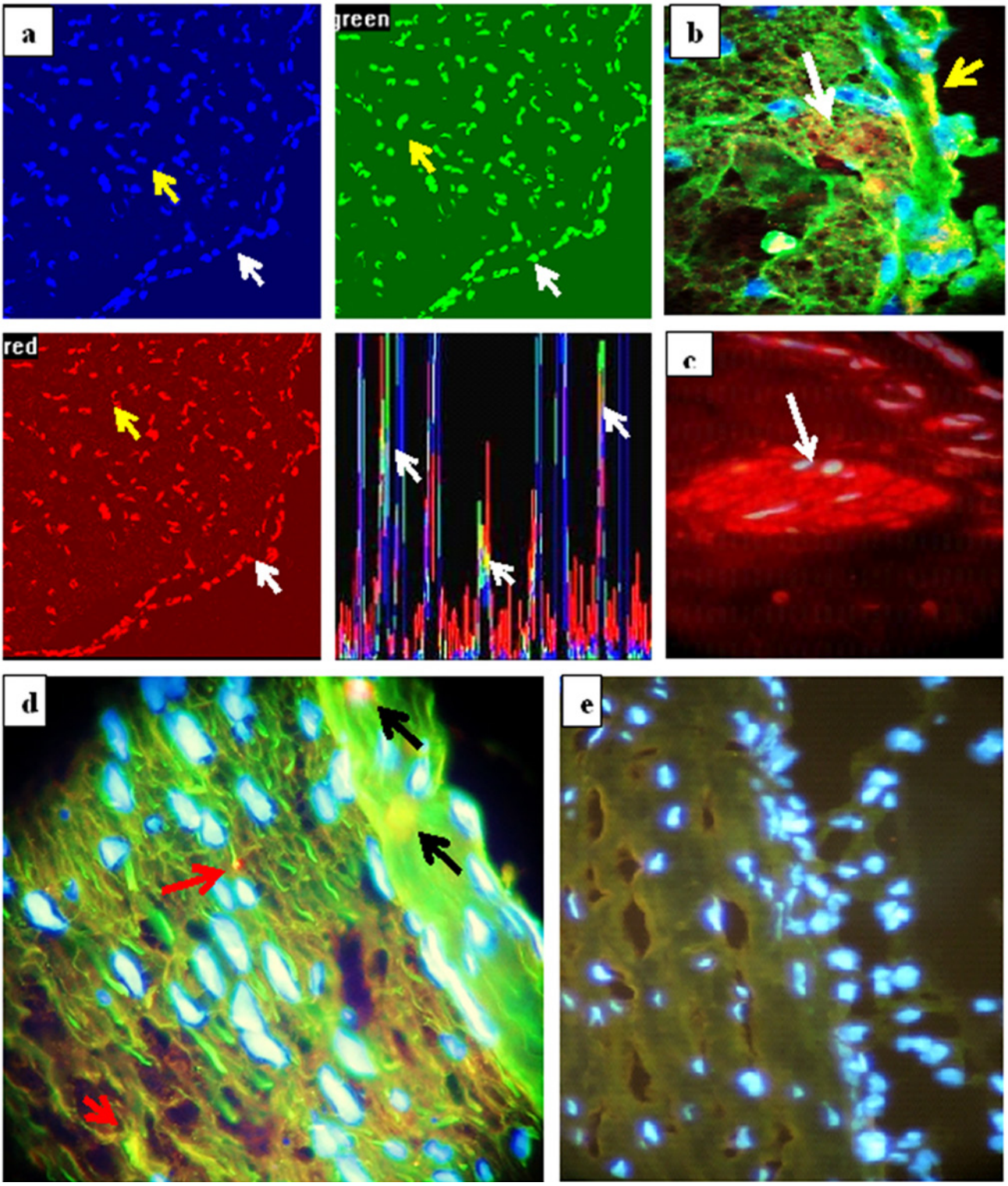

Figure 1. (a) Confocal microscopy using rat optic nerve as an antigen, and showing positive staining with desmoplakin (DP)-I–II (red staining) (+++) around the entire optic nerve envelope (white arrows), as well as inside the nerve (yellow arrows). Note correlating in the panels that the red staining co-localizes with patient autoantibodies labeled with fluorescein isothiocyanate (FITC)-conjugated antihuman immunoglobulin (Ig)G (green staining) (+++) (original magnification 91000). The nuclei of the cells are counterstained in blue with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (+++). Confocal microscopy uses multiple channels of fluorescence. In the presented case, we used a FITC channel (green peaks) (excitation/emission, 495/519 nm), a DAPI channel (blue peaks) (excitation/emission, 360/460 nm), and an Alexa Fluor- 555 channel (red peaks) (excitation/emission, 555/568 nm). The lower right panel shows the co-localization of the peaks of the immunofluorescence of the patient's antibodies (green peaks; white arrows) (+++) with the DP-I–II antibody (red peaks, white arrow) (+++). Both green and red are aligned, demonstrating co-localization. The blue peaks represent our DAPI nuclear counterstaining (+++). Note that yellow peaks are seen because they are showing the overlapping of red and excitation/emission peaks. (b, c) Staining of armadillo repeat gene deleted in velo-cardio-facial syndrome (ARVCF) (red staining, white arrow) (+++) (9200). In (c), note some type of neural receptor (showing the co-localization of both antibodies; white arrow) (+++). (d) IIF. The optic nerve envelope shows positivity with patient autoantibodies (green staining) labeled with FITC-conjugated antihuman IgG (1000X) (+++). Note also the yellow dot positive (a combination of red and

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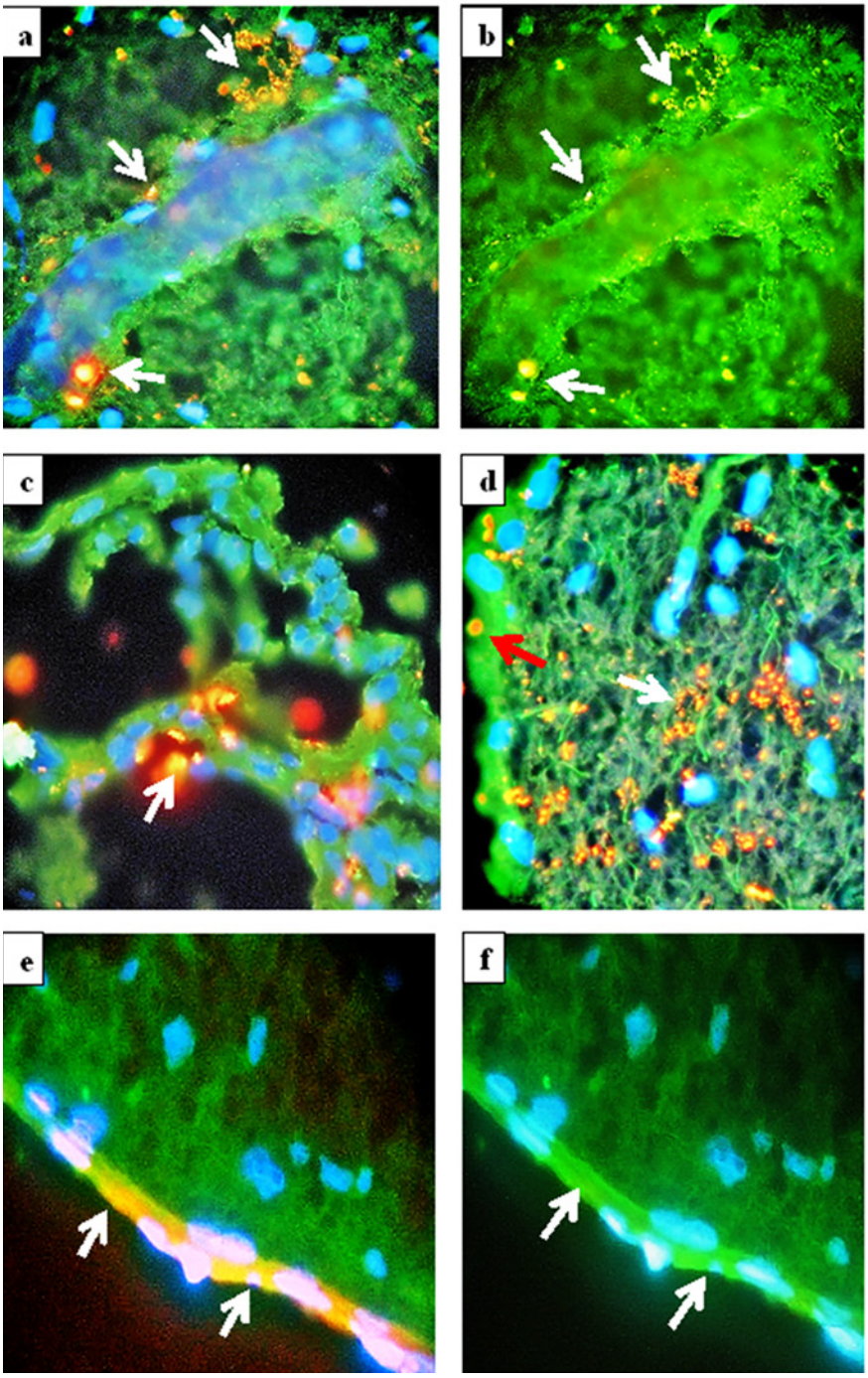

Figure 2. (a) Indirect immunofluorescence (IIF) showing positive staining using patient autoantibodies labeled with FITC-conjugated immunoglobulin (Ig)G (green staining) (+++) and armadillo repeat gene deleted in velo-cardio-facial syndrome (ARVCF) (red staining) (+++) in a folded optic nerve sheath. Note the orange staining (white arrows) as result of co-localization of the green and red staining (original magnification 9100). The nuclei of the cells are counterstained in blue with DAPI (+++). (b) Same as (a), but using FITC IgG (green, excitation/emission, 495/519 nm) (+++) alone. Note that ARVCF is shown in yellowish-orange (white arrows). (c) IIF showing optic nerve sheath detached from the optic nerve and showing positive staining with FITC-conjugated antihuman fibrinogen-labeled patient antibodies (green staining) (++++) co-localizing with MYZAP (orange dots) (++) (white arrow) (9100). (d) IIF showing positive staining for FITC-conjugated antihuman complement/ C1q-labeled patient antibodies (green staining) (+++) co-localizing with ARCVF in the optic nerve sheath (orange staining, red arrow) (++) as well as in some areas inside the nerve (white arrow) (9100). (e) IIF showing positive staining in the optic nerve sheath using FITC-conjugated antihuman IgD-labeled patient antibodies (green staining) (+++) and desmoplakins I and II (red staining) (+++); the combined staining appears orange, white arrows) (9100). (f) Same than (e) but performed with FITC-conjugated IgD alone (green staining, white arrows) (+++). [Copyright: ©2018 Abreu-Velez et al.]

tions likely promote inflammation, and may cause alterations in the shape of the optic nerve including tortuosity. These deposits also could cause edema, which can extend to the meningothelial cells (MECs), the lamina cribrosa and the adjacent extracellular matrix. These changes can, in turn, cause visual anomalies found in these patients. We have already described external anomalies in the eyes of the El Bagre-EPF patients [8].

We, and others, have previously shown that DP-I–II are part of the optic nerve sheet [8, 17]. Here, we show that ARVCF is part of the optic nerve, and co-localizes with autoantibodies from patients affected by pemphigus Abreu-Manu.

Armadillo repeat gene deleted in velo-cardio-facial syndrome (also termed Di George syndrome or 22q11.2 deletion syndrome) is a member of the catenin family [18]. The catenin family plays an important role in the formation of adherens junction (AJ) complexes, which are thought to facilitate communication between the inside and outside environments of cells. The ARVCF gene was isolated in the search for the genetic defect responsible for the autosomal dominant

Figure 1. *(continued)* green) staining in the nerve sheath for p0071 (black arrows) (++). Note also that some parts of the optic nerve are also positive for p0071 (dots stains, red arrows) (++). The cell nuclei are counterstained in blue with DAPI. (e) A negative control, with nuclei counterstained in blue with DAPI (+++). [Copyright: ©2018 Abreu-Velez et al.]

velo-cardio-facial syndrome. The syndrome is a spectrum disorder; features and severity may vary greatly among affected people. Syndromic defects have been noted in the development of the parathyroid glands; "partial" defects with impaired thymic development, and susceptibility to autoimmune disease including pernicious anemia, psoriatic arthritis, Raynaud's phenomenon, myasthenia gravis, warm antibody autoimmune polyendocrine syndrome type 1, rheumatoid arthritis, hemolytic anemia, autoimmune thrombocytopenic purpura, hypoparathyroidism and Hashimoto's thyroiditis, among others (http://www.genecards.org/cgi-bin/carddisp.pl?gene=ARVCF; accessed September 2017) [18]. Based on our data, we suggest that El Bagre EPF may also be associated with ARVCF and, further, that ARVCF may be part of a "predisposing condition for autoimmunity" in patients affected by El Bagre EPF, including the optic nerve envelope [18]. MYZAP represents a component of the cytoplasmic plaques of AJ; AJ connect the endothelial cells of mammalian lymphoid system with desmoplakins-containing complexus adhaerentes of the virgultar cells of lymph node sinuses [19]. MYZAP co-localizes with several cytoplasmic plaque proteins and with AJ-specific transmembrane molecules, including VE-cadherin [19]. Biochemical analyses, including immunoprecipitation, have shown that N-cadherin, DP, desmoglein 2, plakophilin-2, plakoglobin and plectin are very sturdily bound AJ complex partners [19]. These facts support our data findings that MYZAP co-localized with DP-I–II and the El Bagre EPF autoantibodies in the cell junctions of the optic nerve sheath. In regard to the observed antibody co-localization with p0071, MYZAP and DP-I–II and ARVCF, we note that all these molecules are cell junctions; we previously have shown that they co-localized with El Bagre EPF autoantibodies. Our findings may be indicative that they are indeed El Bagre EPF autoantigens [12]. Further research is needed to confirm this possibility.

Conclusion

Patients affected by El Bagre endemic pemphigus foliaceus exhibit autoantibodies to optic nerve sheath envelope cell junctions, and these seem to alter vision in the patients.

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