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ORIGINAL ARTICLE

Early-Onset Invasive Infection Due to *Corynespora cassiicola* Associated with Compound Heterozygous *CARD9* Mutations in a Colombian Patient

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Abstract

Purpose *CARD9* deficiency is an inborn error of immunity that predisposes otherwise healthy humans to mucocutaneous and invasive fungal infections, mostly caused by *Candida*, but also by dermatophytes, *Aspergillus*, and other fungi. Phaeohyphomycosis are an emerging group of fungal infections caused by dematiaceous fungi (phaeohyphomycetes) and are being increasingly identified in patients with *CARD9* deficiency. The *Corynespora* genus belongs to phaeohyphomycetes and only one adult patient with *CARD9* deficiency has been reported to suffer from invasive disease caused by *C. cassiicola*. We identified a Colombian child with an early-onset, deep, and destructive mucocutaneous infection due to *C. cassiicola* and we searched for mutations in *CARD9*.

Methods We reviewed the medical records and immunological findings in the patient. Microbiologic tests and biopsies were performed. Whole-exome sequencing (WES) was made and Sanger sequencing was used to confirm the *CARD9* mutations in the patient and her family. Finally, *CARD9* protein expression was evaluated in peripheral blood mononuclear cells (PBMC) by western blotting.

Results The patient was affected by a large, indurated, foul-smelling, and verrucous ulcerated lesion on the left side of the face with extensive necrosis and crusting, due to a *C. cassiicola* infectious disease. WES led to the identification of compound heterozygous mutations in the patient consisting of the previously reported p.Q289* nonsense (c.865C > T, exon 6) mutation, and a novel deletion (c.23_29del; p.Asp8Alafs10*) leading to a frameshift and a premature stop codon in exon 2. *CARD9* protein expression was absent in peripheral blood mononuclear cells from the patient.

Conclusion We describe here compound heterozygous loss-of-expression mutations in *CARD9* leading to severe deep and destructive mucocutaneous phaeohyphomycosis due to *C. cassiicola* in a Colombian child.

Keywords Phaeohyphomycosis · *Corynespora cassiicola* · compound heterozygous mutations · CARD9 · invasive fungal disease · primary immunodeficiency · inborn errors of immunity

Introduction

Chronic and life-threatening fungal diseases in humans frequently develop from acquired immunodeficiency states and are responsible for substantial morbidity and mortality throughout the world [1, 2]. In addition, fungal infections may also develop in patients with rare inborn errors of immunity and in most cases, as part of a spectrum of diverse infectious susceptibility in which patients commonly also

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exhibit bacterial and/or viral infections [3, 4]. In contrast, fungal infections in otherwise healthy children or young adults with no known underlying risk factors, may occasionally present in the form of organ-specific disease (OSD) or invasive fungal disease (IFD) [5]. Such is the case in which individuals with mutations in genes involved in IL-17 (*IL17F*, *IL17RA*, *IL17RC*, and *ACT1*) [6] or *CARD9* immunity develop mucocutaneous or invasive fungal infections with a narrow range of fungal species [5, 7].

The caspase-associated recruitment domain (CARD)-containing protein 9 (*CARD9*) is a critical adaptor protein primarily expressed in myeloid-lineage cells downstream of several C-type lectin receptors such us Dectin-1, Dectin-2, and the Mincle receptor, which are critical for the recognition of fungal components such as β-glucan, α-mannan, and α-mannose during antifungal immune responses [8, 9, 10]. *CARD9* plays a critical role in innate and adaptive immunity and functions to induce the production of pro-inflammatory cytokines after microbial invasion (reviewed in [9, 7]). Specifically, *CARD9* is centrally positioned in a complex intracellular signaling pathway after fungal pattern recognition by the CLR family of receptors, leading to the induction of IL-1β, TNF, IL-6, and other cytokines for effective antifungal responses [11, 14]. In humans, bi-allelic loss-of-function mutations in *CARD9* result in autosomal recessive (AR) complete *CARD9* deficiency (OMIM *607212), a rare condition characterized by selective predisposition to mucocutaneous as well as invasive fungal infections. The molecular characterization of the antifungal response in mice as well as in *CARD9*-deficient patients, has revealed the critical role of *CARD9* in the induction of pro-inflammatory cytokines and chemokines, neutrophil killing, and neutrophil trafficking to skin and subcutaneous tissues, the central nervous system (CNS), and extrapulmonary sites [7, 12, 13, 15–20].

Most reported cases have been associated with *Candida* spp. (mainly *C. albicans*) [21] and dermatophytes (*Trichophyton* spp.) [7, 23, 24]. More recently, bi-allelic LOF mutations in *CARD9* have been identified in individuals affected with systemic infections due to *Aspergillus* spp. [18] as well as invasive mucocutaneous infections by phaeohyphomycetes or dematiaceous fungi (phaeohyphomycosis), including *Exophiala* spp., *Phialophora verrucosa*, and *Corynespora cassiicola* [20, 24–26,]. Here, we report the first case of *C. cassiicola* infection in a child affected with severe, invasive, and deep subcutaneous facial phaeohyphomycosis, in whom we identified compound heterozygous mutations in *CARD9*.

Materials and Methods

Panfungal PCR for Detection of *C. cassiicola*

Genomic DNA was extracted from tissue samples and cultures grown in Saboraud dextrose agar using the QIAamp DNA mini kit (QIAGEN, Germantown, MD), following the manufacturer's recommendations. The DNA was amplified using site-specific primers for the D1 and D2 domains (~ 600 bp) and the ITS (internal transcribed spacer) region 1 (600–900 bp) of the nuclear large-subunit (28S) ribosomal DNA [27]. The PCR products were bidirectionally sequenced by Sanger (Macrogen, MD, USA). Sequences were edited using the software Sequencher v 5.0 (Gene Code Corporation, Ann Harbor, MI) and aligned for confirmation using BLAST (NCBI, Washington, DC) and CBS-KNAW (Westerdijk Fungal Biodiversity Institute, Ultretch, The Netherlands).

Whole-Exome Sequencing (WES), Bioinformatic Analysis, and Sanger Sequencing

Genomic DNA was extracted from whole blood from the proband using the Puregene DNA Purification Kit (Gentra Systems, Minneapolis, MN). Exome capture was performed with the Agilent Sureselect V4/V5, (Agilent Technologies) and paired-end sequencing was performed on a Hiseq4000 platform (Illumina) generating 100-base reads. The reads were aligned to the reference human genome GRCh37/Hg19 using the Burrows-Wheeler Alignment tool (BWA v 0.7.12-r1039) [28]. Bioinformatic analyses were performed using an in-house software pipeline developed in collaboration with the Centro Nacional de Secuenciación Genómica CNSG (Medellín, Colombia). Variants were confirmed by Sanger sequencing in genomic DNA from the patient, both parents, and the four siblings, using site-specific oligonucleotides flanking the coding and intron regions of exons 2 and 6 of *CARD9*. Amplicons were sequenced using BigDye terminator technology with a Genetic Analyzer Sequencer 3500XL (Applied Biosystems, Foster City, CA). Subsequently, the data was collected and aligned with the reference sequence deposited in the NCBI data bank, using the software analyzer Genius R9 v9.1.3 Snapgene (GSL Biotech LLC) and 4picks (Nucleobytes B.V. Gerberastraat, The Netherlands).

Flow Cytometry and Immunophenotyping

Whole blood was either processed directly or used for the isolation of peripheral blood mononuclear cells (PBMCs) on a Ficoll density gradient. The PBMCs obtained were then either processed directly. We lysed the red blood cells by incubating 100 μl fresh whole blood with fluorophore-conjugated antibodies for 20 min and then with FACS lysing solution (BD) for 10 min at room temperature. The antibodies used for staining were CD45-FITC, CD3-PE-Cy7, CD19- PE-Cy7, CD4–Pacific Blue, CD14-V450, CD8-PE-Cy5, CD8-V450, or CD16-CD56-PE, CD45RA-FITC, CCR7-PE, CD27-FITC, IgD-PE, CD24-FITC, CD38-APC, and CD3-APC. Fluorescence was measured on a FACSCanto II flow cytometer (BD).

Western Blotting

Human PBMC were isolated from whole blood and protein extracts obtained using 10X RIPA cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with protease inhibitors. Lysates were separated by electrophoresis (10% SDS PAGE) and transferred to Immobilon-P PVDF membranes (MerkMillipore, Germany). Proteins were blotted with rabbit anti-human *CARD9* (Protein Tech, Thermo Fisher Scientific, Waltham, MA) or rabbit anti-GAPDH (Santa Cruz Biotechnology, Paso Robles, CA), and later incubated with goat anti-rabbit IgG-HRP conjugate (Bio-Rad, Hercules, CA) or anti-rabbit IgG-peroxidase conjugate (Sigma, St Louis, MO) and revealed with the Clarity™ Western ECL Substrate (Bio-Rad).

Ethics Statement

This study was approved by the local review board of the Universidad de Antioquia (F8790-07-0010) and conducted according to the "Scientific Standards for Technical and Administrative Health Research" established by the Colombian Ministry of Health Resolution 008430 of 1993. Informed consent was obtained from the patient or their family members included in this study.

Results

Invasive Fungal Infection by *C. cassiicola* in a Colombian Child

The patient is a female born at term in 2006 in a rural area of the north of Colombia (South America) from non-consanguineous parents, and she has three healthy younger siblings. She received all the vaccines for her age according to the guidelines of the Colombian Official Immunization Program (PAI), and no adverse effects were reported including those related to the BCG vaccine. At the age of 4 years, she presented spontaneously with recurrent epistaxis associated with an indolent nodule in the nasal dorsum and foul-smelling mucopurulent rhinorrhea that led to a nasocutaneous fistula and subsequently, to the perforation of the nasal palate. At the regional hospital in her hometown, the pediatrician diagnosed her with an invasive nasocutaneous infection of unknown origin. A non-contrast computed tomography (CT) of the head showed an expansive mass affecting the maxillary sinus, and histopathologic examination of a skin sample obtained from the nodule revealed a mycotic granuloma that was suggestive of an infection due to *Mucorales*. By then, the patient was referred to an infectious diseases specialist in the city of Barranquilla, and he considered the diagnosis of fungal vs parasitic infection (possibly rhinocerebral mucormycosis vs mucocutaneous leishmaniasis). She was hospitalized for further microbiologic testing and placed on IV amphotericin B (AmB) for 1 month, and although apparently marginal therapeutic response was attained, she was discharged pending results from the tests. Four months later, the patient was hospitalized again, and surgery was performed to remove the nasal mass, and amputation of the inferior turbinates, anterior and posterior ethmoidectomy, and medial maxillary antrostomy were performed. After this, she was discharged with outpatient treatment, but unfortunately, we could not obtain the medical records describing the therapy and the identification of the microorganism. She did not return for revision and was lost to follow-up.

At the age of 8 years, the patient was hospitalized in the city of Santa Marta. Progressive growth and destruction of the mucocutaneous invasive nasofacial infection was observed, despite multiple treatments. Surgical tissue samples were obtained from the affected soft palate and skin, and these were sent to the National Institute of Health in Bogotá (Colombia). The pathologist reported post-treatment severe and chronic inflammation with granulomas and abscesses with fungal structures positive for Gomori-silver staining in the interior. He suspected and considered these findings to be compatible with *Aspergillus* spp., and remarkably, he suggested the possibility of an underlying primary immunodeficiency (PID), but this was not pursued any further. Based on the suspicious of *Aspergillus* infection, galactomannan was measured twice by ELISA in the patient's serum and both tests were positive with indexes of 1.06 and 0.86 respectively (ref. value, > 0.5). At this time, an ELISA for HIV was negative and quantitation of T cells subsets (Tritest, BD Biosciences, San Jose, CA) was within normal values for age (not shown). Based on these results, the diagnosis of mucocutaneous invasive aspergillosis was established and treatment was initiated with oral voriconazole (10 mg/kg/day) for 10 weeks with moderate improvement, and the patient was discharged for follow-up. Ten weeks later, she came back for revision and marginal response of the affected tissues with periorbital cellulitis of the left eye was noted, and she was hospitalized again. Intravenous AmB (1 mg/kg/day) was initiated for 1 month along with IV vancomycin (40 mg/kg/day) plus clindamycin (30 mg/kg/day) for 14 days to treat a superimposed infection. The bacterial infection cleared, but no significant improvement of the mucocutaneous infection was noted, and although the treating physicians considered switching AmB to liposomal (LAmB), this presentation was not available. Hence, the treatment was adjusted with IV voriconazole (6 mg/kg/day) plus caspofungin (50 mg/m^2/d) for 14 days, followed by oral voriconazole for 12 weeks and the patient was discharged.

Four months later, the infection had extended to adjacent tissues (Fig. 1a, b) and the patient consulted to the hospital in her hometown. A surgical sample of the mucosa from the maxillary

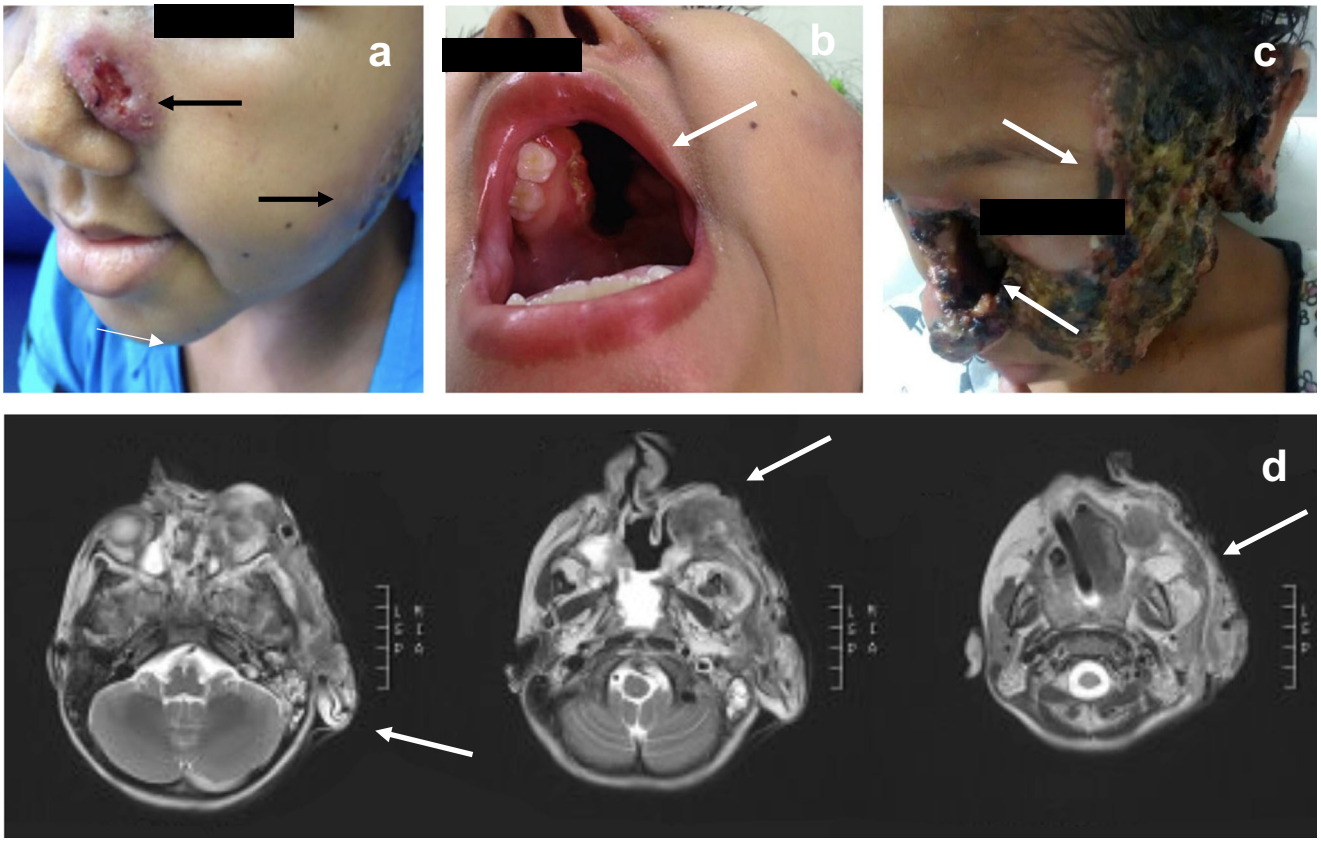

Fig. 1 Clinical findings in the CARD9 deficient patient. **a** Ulceration of the nasal dorsum and extension of the infection to the soft tissues of the left chin. **b** Complete loss of the left soft palate and teeth after debridement. **c** Perforation of the nasal dorsum and bridge and palate and extensive compromise of the left side of the face and external ear with widespread cutaneous necrosis. **d** Contrast-enhanced MRI at of the head taken during her last hospitalization of June of 2015 before the start of the antifungal therapy. The images show severe damage of soft tissues and bone structures but no compromise of the orbit and the base of the skull and brain

sinus was obtained and it was sent to the Clinical Mycology Reference Laboratory Corporacion para Investigaciones Biológicas (CIB, Medellín, Colombia). A fungal culture was negative, however an in-house Panfungal PCR assay was performed to amplify the ITS and D regions of the large 28S subunit of rRNA from genomic DNA of the tissue. The PCR products were sequenced by Sanger and sequence homology analyses confirmed 99% homology to *C. cassiicola*. She was hospitalized again, and therapy was initiated with IV LAmB (5 mg/kg/day) plus liquid oral posaconazole (20 mg/kg/day) for 1 month; following clinical improvement, she was discharged with oral posaconazole plus terbinafine (125 mg/day). However, the treatment was stopped due to issues unknown to the authors, and she was lost to follow-up again. Six months later, she consulted to the local hospital in another city nearby of Sincelejo (South Caribbean area) and due to the depth and extension of the facial infection, she was transferred to a tertiary center in Medellín.

Upon admission, the physical exam revealed a non-febrile but chronically malnourished child. A large, indurated, foul-smelling, and verrucous ulcerated lesion was noted in the left side of her face, extending to the left ear and beneath the mouth with widespread necrosis and crusting, almost complete loss of tissue of the nasal dorsum and complete perforation of the nasal bridge and palate (Fig. 1c). A contrast-enhanced magnetic resonance imaging (CE-MRI) of the head revealed an infiltrative lesion with extensive damage and deep destruction of soft tissues and cranial bones on the left side of the face, the infiltration affected maxillary sinuses, nasal septum, turbinates, palate and oral cavity and nasopharynx, floor of the left orbit and adjacent muscles, and external auditory canal, and destruction included the cranial posterior fossa but excluded any cerebral or ocular involvement (Fig. 1d). Therefore, extensive surgical debridement was performed and new tissue samples were obtained for microbiologic and molecular analyses. In addition, antifungal therapy was initiated with IV LAmB (5 mg/kg/day) plus posaconazole (20 mg/kg/day), as well as IV cefepime (150 mg/kg/day) and clindamycin (40 mg/kg/day) for 10 days to treat a superimposed bacterial infection that eventually cleared.

Scrapings from the affected skin were examined using the potassium hydroxide preparation (KOH prep) test revealing abundant hyaline and hemiacetal hyphae (not shown). Histopathologic examination of a skin biopsy showed extensive acute necrosis and chronic granulomatous inflammation (Fig. 2a), and the methenamine silver-staining revealed numerous mycotic structures with extensive angioinvasion (Fig. 2b).

Although no isolates were recovered from either aerobic or anaerobic bacterial cultures, numerous gray velvety colonies with short aerial mycelia were grown in Saboraud dextrose agar (BBL Becton Dickinson, Sparks, MD) suggestive of a mold (Fig. 2c). These results did not allow the characterization of the fungus. A new panfungal PCR from genomic DNA of the colonies and the skin biopsies were performed, identifying *C. cassiicola* again. The antifungal susceptibility testing demonstrated that the fungus was sensitive to at a low level to posaconazole (MIC 1.0), AmB (MIC 0.125), and voriconazole (MIC 0.75). After 1 month, the clinical response to the antifungal treatment was modest, therefore caspofungin (50 mg/m^2/day) was added to therapy but with minor clinical improvement. Then at this point, the infectious diseases specialists discussed the case with physicians from the national referral center for PID in Medellin and decided to pursue an inborn errors of immunity.

During this hospitalization that lasted 6 months, aggressive wound care and nutrition (via gastrostomy) were continued, and the patient demonstrated significant clinical response to the antifungal therapy with substantial resolution of the mucocutaneous lesions in the face; however, the severe soft tissue and bone deformities persisted as sequelae. The patient was discharged with oral posaconazole plus terbinafine, but 1 week later, she was readmitted due to a cutaneous nodule that developed in the base of the nasal dorsum, leading to suspicion of reactivation of the infection. Serum levels of posaconazole were measured and were considered to be in adequate range (1.65 μg/ml (ref. value, 0.7–1.0). Intravenous LAmB was restarted, and a new MRI of the head was performed revealing significant healing of soft tissues; however, there were signs of reactivation and the damage to the bone structures was considered extreme (not shown). Therefore, a medical staff was called to determine if more surgical debridement might help to control the spread of the fungus, but they concluded that due to the uncontrolled spread of the infection and progressive and extensive damage of the soft tissues and bone structures, a surgical approach was not suitable. Moreover, the risks of attempting a facial reconstruction were high enough to compromise the life of the patient. Therefore, she was discharged and sent home with the same oral antifungal therapy and nutritional support. After this, the patient had multiple readmissions to the hospital in her hometown due the uncontrolled infection for which she continued to receive rescue therapy with IV AmB with minimal clinical response and she has continued to deteriorate.

Immunological Findings in the Patient

Complete white blood cell counts (WBC) during this hospitalization showed normal to mild leukocytosis with normal absolute counts of lymphocytes (TLC) and neutrophils (TNC), but persistently high numbers of monocytes and platelets and in several occasions with mild eosinophilia (Table S1 and Fig. S1). Quantitation of serum Ig revealed normal IgM, IgA, and IgE; high IgG (Table S2); and a dihydrorhodamine (DHR) test after phorbol myristate acetate stimulation of patient's neutrophils was normal, excluding the diagnosis of classic chronic granulomatous disease (data not shown). Immunophenotyping of PBMC by flow cytometry revealed normal absolute counts of total monocytes and lymphocytes as well as normal T, B, and NK cells. Further immunophenotyping of B cell subpopulations revealed increased naïve B cells (IgD$^+$/CD27$^-$) and decreased marginal zone (IgD$^+$/CD27$^+$), switched memory (IgD$^-$/CD27$^+$), and transitional (CD24^{++}/CD38^{++}) B cells (Table S2). In addition, phenotyping of T cell subpopulations revealed increased numbers of terminally differentiated (CD45RA$^+$ CCR7$^-$) and memory (CD45RA$^-$CCR7$^-$) effectors CD4$^+$ T.

Compound Heterozygous Mutations in *CARD9* and Abolition of *CARD9* Expression in PBMC

WES was performed in genomic DNA from the patient revealing compound heterozygous mutations in *CARD9* (Fig. 3a). One mutation consisted of a small mono-allelic deletion of seven nucleotides, c.23_29del in exon 2, resulting in a predicted frame shift leading to a premature stop codon 10 residues later (p.Asp8Alafs*10) not previously reported. This

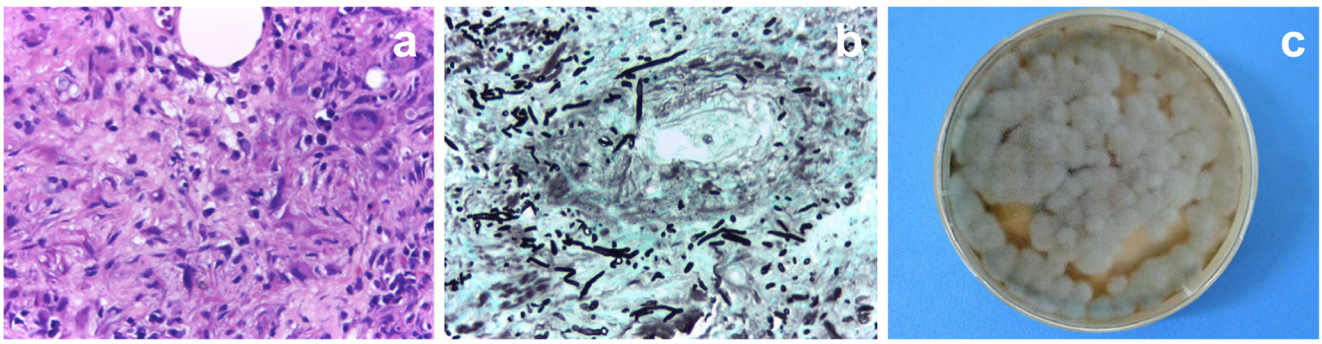

Fig. 2 Histopathology of tissue samples and isolation of the fungus in the CARD9 deficient patient. **a** hematoxylin-eosin stain (× 40) showing necrosis and abundant inflammatory infiltrate composed of lymphocytes, histiocytes, plasma cells, neutrophils, and multinucleated giant cells. **b** Methenamine silver stain (× 40) showing wide septate hyphae and conidia invading the wall of a vessel. **c** Colonies of *C. cassiicola* isolated from Sabouraud dextrose agar medium

variant predicted a mutated protein of a molecular weight of 1.7 kDa that removes the coiled coil domains (Fig. 3b). This variant was not found in gnomAD, and in silico analysis by SIFT and PolyPhen predicted this variant to be deleterious and damaging, respectively [29, 30]. The other mutation was a substitution in exon 6 (c.865C>T) resulting in the nonsense mutation, p.Q289*, and has been previously reported [21, 22, 31]. No mutations in genes involved in other PID as reported by the IUIS were identified. Both mutant alleles were confirmed by Sanger sequencing in the patient. The mother and two out of the three younger brothers were heterozygous for p.Q289*, while the c.23_29del was found only in the patient (Fig. 2c). Finally, expression of CARD9 protein was absent by western blot analysis in total extracts from PBMCs from the patient compared to the normal expression of the protein in two healthy controls (Fig. 3d).

Discussion

We report a Colombian child affected with a severe, invasive, and deep subcutaneous facial infection due to *C. cassiicola* and bi-allelic mutations in *CARD9*. Since its first description in 2009 in a family affected with chronic mucocutaneous candidiasis [21], *CARD9* mutations predisposing to invasive fungal infections in humans are being increasingly reported [7]. In addition, the spectrum of fungal pathogens has grown beyond *Candida* spp. to include now dermatophytes [22], dematiaceous fungi [16], and *Aspergillus* spp. [18]. The mechanisms by which *CARD9* deficiency leads to increased susceptibility to fungal infections are not well understood. In humans, *CARD9* deficiency leads to reduced numbers of $Th17^+$ T cells [16, 21], defective neutrophil killing [15], and trafficking to the central nervous system [17], as well defective pro-inflammatory chemokine and cytokine production [18, 20, 22,

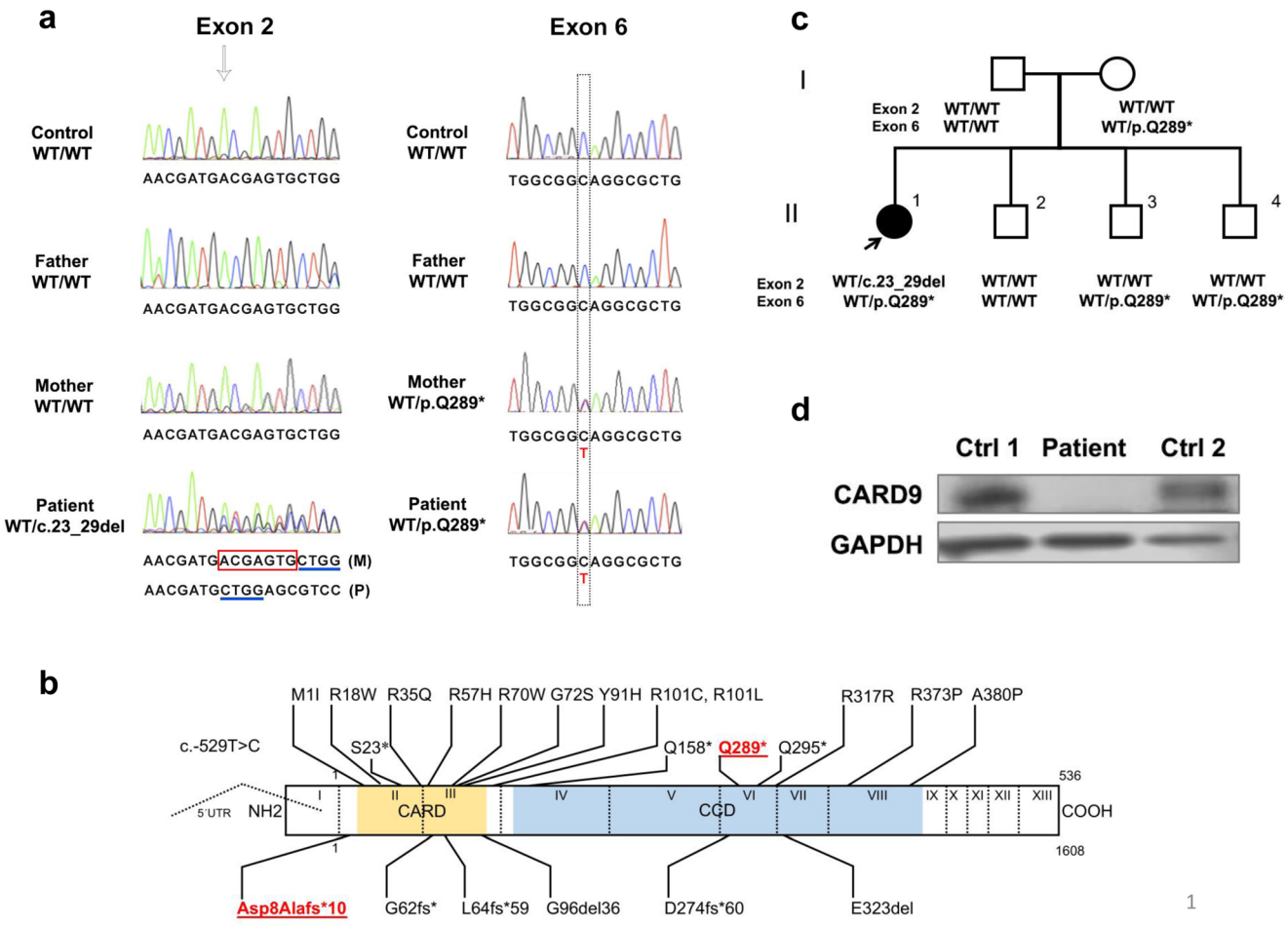

Fig. 3 Genetic and molecular analysis of *CARD9* mutations. **a** Sanger sequencing chromatograms. The heterozygous mutation p.Q289* in exon 6 is present in the patient, the mother, and the two youngest siblings. The heterozygous 7 base pair ACGAGTG (red box) deletion in exon 2 (c.23_29del) is only present in the patient. **b** Schematic representation of human CARD9 protein with its functional CARD domain and coiled-coiled domain (CCD) showing the physical location of previously reported mutations [7]. The 13 exons are indicated by Roman numerals, the first exon is nonprotein-coding. The c.23_29del and cQ289* mutations are shown in underlined red. **c** Pedigree including the *CARD9* genotype with the variants in the cDNA in exons 2 and 6. Each generation has a roman numeral (I-II) (left margin); the arrow indicates the proband. **d** Immunoblotting of CARD9 from protein extracts of peripheral blood mononuclear cells (PBMCS) from the patient (P) and two healthy controls (C1 and C2)

24, 53]. In *CARD9* knockout mice, the inability to control dematiaceous fungi has been associated with lack of Th17 differentiation and reduction of TNF, interleukin IL-1β, IL-6, and IL-17A production in footpad homogenates [12]. Unfortunately, although we demonstrated the lack of CARD9 protein expression in PBMC, we could not perform other tests to identify any of the reported immune abnormalities as no samples were available for these studies. However, the results strongly suggest that the *CARD9* mutations in our patient are responsible for the predisposition to the infection due to *C. cassiicola*.

Phaeohyphomycosis are fungal infections caused by melanin-producing fungi and over 100 species have been associated with localized or systemic infections in humans [33]. Most of these fungi are ubiquitous saprophytes in soil and decaying matter, but some are important phytopathogens. *C. cassiicola* has been rarely reported to cause human disease and up to date, there have been only five confirmed cases of the infection in adults from Africa and Asia [25, 34–37] (Table S3). All patients were farmers except one, and risk factors including comorbidities predisposing to immunosuppression were identified in only one individual; the clinical presentation ranged from maduromycetoma (a form of chronic granulomatous fungal infection affecting the foot) to invasive necrotic subcutaneous infections affecting the hands and legs. To date, only one patient affected with a severe destructive infection of the face due to *C. cassicola* has been reported and associated with a compound heterozygous mutations in *CARD9* [25]. To the best of our knowledge, the patient reported here is the first child to be described with an invasive subcutaneous facial infection due to *C. cassiicola,* and the second human in whom bi-allelic mutations in *CARD9* have been identified associated with a *C. cassiicola* infection.

Our patient's clinical outcome turned out to be very poor. Different circumstances beyond our control led to a delay in the proper identification of the fungus responsible for the infectious disease as well as in the instauration of the most appropriate therapy. First of all, a consistent approach must be in place always to rapidly and unambiguously identify the etiologic agent, both histologically and molecularly. In infected tissues, *Mucorales* typically show characteristic broad, hyaline, ribbon-like, irregular fungal hyphae with wide-angle branching (aseptate hyphae) [38], while *Aspergillus* display the characteristic acute angle branching septate hyphae [39] and phaeohyphomycetes as dark-pigmented hyphae (dematiaceous) [40]. These characteristics must be considered when trying to distinguish between these three fungi structures. In addition, biochemical testing and molecular methods such as panfungal PCR should be used during the first stages of the infection both with negative and positive cultures to precisely define the etiology [41]. In our patient, early attempts to identify the fungal species causing the facial infection was ambiguous. Initially, an infection due to *Mucor* was suspected (though no microbiological records could be obtained) but later on, the diagnosis of aspergillosis was contemplated, based on histological findings and positive galactomannan testing (although *Aspergillus* was not isolated from cultures). Ultimately, we identified *C. cassiicola* as the etiologic agent by molecular testing (panfungal PCR). However, this does not allow us to firmly conclude if our patient at some point had aspergillosis as a co-infection or a super-infection that might have responded to the concomitant therapy, leading to the subsequent unmasking of the infection by *C. cassiicola*. Galactomannan antigenemia has been reported in immunocompromised patients with phaeohyphomycosis as an indicative of invasive aspergillosis [42]. Therefore, caution must be exercised in this interpretation because false-positive serum galactomannan has been reported due to cross-reaction with other existing non-*Aspergillus* fungal infections [43, 44], the use of certain antimicrobials [45, 46], and conditional fluids [47], amongst other causes.

With respect to the treatments, in three patients with *C. cassiicola* infection (P2-P4, Table S3), the use of different antifungals such as voriconazole, AmB, terbinafine, pimaricin, and micafungin, as well as different routes of administration (topical vs oral or parenteral) led to the resolution of the infections in less than 1 year [35, 37]. Furthermore, in the patient reported by Yan et al., a slight improvement of the lesions was noted after 2 weeks of administration of a total dose of 690 mg of AmB, however the treatment was not continued as the patient left the hospital prematurely [25]. In our patient, different regimens of antifungal therapies were used, some of them empirically. Some of these therapies, exhibit potential fungicidal activity against *Corynespora* spp. However, it is not clear for us if those treatments were ever completed and supervised properly as the patient was lost to follow-up in several occasions. It is well known that triazole levels in children are particularly low, especially with voriconazole [48, 49]. Therefore, it is critical to perform therapeutic drug monitoring as a routine adjunct to their use. Lastly, although our patient was finally referred to a specialized center where appropriate antifungal treatment was prescribed (according to the antifungal susceptibility testing), the nature of the invasiveness and destruction of soft tissues and bone might have affected the bioavailability of the antifungals in the affected tissues, leading to lack of therapeutic response. Little is known about the necrotrophic and invasive nature of *C. cassiicola*, although recent genomic studies into the pathogenicity of this fungus suggest that the fungus has significant necrophilic properties that might explain its aggressiveness in susceptible hosts [50].

Peripheral blood eosinophilia (PBE) is defined as an absolute eosinophil count of > 500/μL and classify < 1000/μL as being mild and those > 1500/μL as being marked [51, 52]. PBE that range from mild and marked eosinophilia [7, 18, 22–24, 53–56], as well as cerebral spinal fluid eosinophilia [15, 53, 57] have been reported in patients with CARD9 deficiency with different fungal etiologies. Moreover,

eosinophilia has been reported in patients with pulmonary and disseminated coccidioidomycosis [58, 59], gastrointestinal basidiobolomycosis [60], and sporadically in other fungal diseases affecting different organs [51, 61–63]. In addition, eosinophilia is a common feature due to hyper-reactivity to *Aspergillus* in patients with allergic bronchopulmonary aspergillosis (ABPA) that develops with asthma or cystic fibrosis [64, 65], and rarely in patients with spontaneous *Aspergillus* empyema without ABPA [66]. In our patient, during the last hospitalization lasting over 6 months, PBE was documented on several occasions towards the middle of her stay, but never as high (marked eosinophilia) as some other patients with CARD9 deficiency (Fig. S1) [22–24]. On the other hand, high-serum IgE levels have been reported in association with CARD9 deficiency and fungal infections affecting the skin, the digestive tract, and the joints [67]. Serum IgE levels were measured in our patient only once and they were normal. Therefore, it is tempting to speculate both, eosinophilia and high levels of IgE, could be features of this inborn errors of immunity, as it has been demonstrated for several disorders of immune deficiencies or immune dysregulation [68]. However, eosinophilia has to be interpreted with caution, as other non-infectious disease-related factors have been documented to induce eosinophilia as well [52].

In conclusion, we advise considering mutations in CARD9 deficiency in rare and unusual fungal etiologies associated with mucocutaneous and/or invasive fungal infections. In addition, it is necessary to develop more studies that further explore potential adjuvant therapies based on the use of recombinant GM-CSF [69], as well as the use of hematopoietic stem cell transplantation (Grumach A, manuscript in preparation) [7, 70] that might lead to the resolution of the disease.

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Author's Contributions JLF and AAA are the principal investigators and conceived the study. CAAF, SDO, and LC performed the experiments and took samples from the patient. JLC, AP, and JB contributed to the design of the study. JLF analyzed the flow cytometry data. MMV, JFA, AAA, and FC were responsible for genetic analysis. CPB, CM, AR, MT, LVG, AMM, VM, DYC, ACR, CG, IB, and JCO contributed to the collection of samples from the patients and provided a clinical oversight. IB contributed with the fungus identification. All authors revised the manuscript and approved the final manuscript. CAAF, AAA, JB, and JLF analyzed the results and wrote the manuscript.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the local review board of the Universidad de Antioquia (F8790-07-0010) and conducted according to the "Scientific Standards for Technical and Administrative Health Research" established by the Colombian Ministry of Health Resolution 008430 of 1993. Informed consent was obtained from the patient or their family members included in this study.

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