

Letter to the Editor

CARD9 mutations linked to subcutaneous phaeohyphomycosis and T_H17 cell deficiencies

To the Editor:

Phaeohyphomycosis is a group of superficial, cutaneous, subcutaneous, or systemic infections caused by more than 100 species of diverse dematiaceous fungi. It is characterized by dematiaceous yeast-like cells, hyphae, and pseudohyphae in tissue, while sclerotic bodies, characteristics of chromoblastomycosis, are absent.¹ *Phialophora verrucosa*, one of the *Phialophora* species, can be isolated from soil, wood, and rotting vegetation worldwide and has sometimes been reported to cause subcutaneous phaeohyphomycosis.²

During the last decade, host defense against fungal infections has been extensively studied. Our understanding of genetic and immune deficiencies that predispose to chronic mucocutaneous candidiasis has substantially advanced.³ However, the genetic and immunologic defects in patients with phaeohyphomycosis have not been investigated. Here, we report 4 Chinese patients with subcutaneous phaeohyphomycosis caused by *P. verrucosa* with *CARD9* mutations and T_H17 cell deficiencies.

Four patients presenting with persistent red plaques and nodules on their cheeks and faces were recruited in our study (Fig 1, A-C). The clinical manifestations and the outcomes of the 4 patients are summarized in Table E1 in this article's Online Repository at www.jacionline.org. Histological examination revealed intense inflammatory infiltrations with dematiaceous hyphae (Fig 1, D). From tissue cultures, *P. verrucosa* was isolated and identified by the morphological characteristics of black colonies in macroscopic appearance and septate hyphae with typical *Phialophora* type of sporulation in microscopic appearance (Fig 1, E and F), which was confirmed by internal transcribed spacer region nucleotide sequencing (Genbank Accession No. KF360974, KF471126, KF360975, and JX233482). There were no other chronic or opportunistic infections detected or reported in all the patients before their presentation, and they could not recall any trauma. A history of consanguineous marriage was denied. The diagnosis of phaeohyphomycosis caused by *P. verrucosa* was made on the basis of the above clinical, histopathological, and mycological findings.

As is known, both chromoblastomycosis and phaeohyphomycosis can be caused by dematiaceous fungi, but the clinical manifestations and prognosis are different.¹ Patients with *P. verrucosa*-associated subcutaneous phaeohyphomycosis, as demonstrated in our cases, have a relatively early onset, no trauma history, and no known immunocompromised conditions and are resistant to various systemic antifungal agents with remittent aggravation. These clinical features highly suggest that some underlying genetic defects and the subsequent immunodeficiencies should be considered, and thus we carried out whole-exome sequencing in 2 of the sporadic cases (patients 1 and 2).

Written informed consent was obtained from all the patients and 6 ethnically matched healthy volunteers. Ethical approval for molecular genetic studies was obtained from the Clinical Research Ethics Committee of Peking University First Hospital. Genomic DNA was extracted from the peripheral blood leukocytes. Exome

capture was carried out with SureSelect Human All Exon Kit (Agilent, Santa Clara, Calif), and then sequenced by using HiSeq2000 platform. To eliminate the nonpathogenic variants, we filtered out the single nucleotide variants and short insertions and deletions (indels) existing in in-house database and 3 public variation databases (dbSNP134, 1000 Genomes Project, and HapMap8). As a result, including indels, 1333 variants involving 1000 different genes and 1220 variants involving 943 different genes were detected in patient 1 and patient 2, respectively. When we compared these variants with the genes previously reported to be implicated in fungal infection,⁴ only 1 gene (*CARD9*) shared by both patients was found. Sanger sequencing was subsequently performed to validate these 3 variants involving *CARD9* and to screen mutations of *CARD9* in the other 2 patients. Briefly, 3 mutations of *CARD9* were detected: 2 compound heterozygous mutations in patient 1 (c.191-192insTGCT and c.472C>T, p.L64fsX59 and p.Q158X) and 1 homozygous frameshift mutation (c.819-820insG, p.D274fsX60) in patients 2, 3, and 4 (see Fig E1, A, in this article's Online Repository at www.jacionline.org). No relationship or common relatives were reported in patients 2, 3, and 4, who were from different provinces and did not know each other before. We subsequently performed haplotype analysis in these 3 patients to determine whether mutation c.819-820insG was from one common ancestor. Twelve single nucleotide polymorphisms highly polymorphic in Asian populations nearby the mutation were chosen and genotyped. Unexpectedly, all 3 patients shared the same homozygous alleles in all 12 SNPs, suggesting a founder effect (see Table E2 in this article's Online Repository at www.jacionline.org). Parents and siblings of the patients were all healthy and heterozygous carriers of 1 corresponding mutation (the deceased parents of patients 3 and 4 were not enrolled; Fig E1, B). All these detected mutations were not found in 220 ethnically matched control subjects. Thus, *CARD9* was selected as the most likely causative gene underlying phaeohyphomycosis in these patients.

To verify the expression of *CARD9* in our cases, we detected it in PBMCs of patients 1, 2, and 3 by using quantitative PCR and western blot. (Patient 4 was not enrolled in the following study because of traffic inconvenience.) Patients exhibited sufficient level of *CARD9* mRNA transcripts compared with 5 healthy donors, thereby escaping from nonsense-mediated RNA decay (Fig E1, C). Contrarily, patients lacked the expression of the wild-type mature *CARD9* protein, which further confirmed the consequences of premature termination codons in frameshift and nonsense mutations (Fig 2, A).

CARD9 is a critical adaptor that can mediate Dectin-1, Dectin-2, and Mincle-induced NF- κ B activation by forming the *CARD9*-BCL10-MALT1 complex in response to fungal infection.^{5,6} *CARD9* deficiencies may impair pivotal cytokine production in innate immune cells and the differentiation of T_H17 cells. It was reported that *CARD9*-deficient mice were highly susceptible to *Candida albicans* infections.⁷ And homozygous loss-of-function nonsense mutation in *CARD9* (Q295X) has been found to cause chronic mucocutaneous candidiasis in a large consanguineous family, with reduced T_H17 cells and impaired immune response to Dectin-1 agonist.⁸ We then assessed the abundance of T_H17 cells and the related cytokines in patients 1, 2, and 3. It was noteworthy in our observation that the percentages

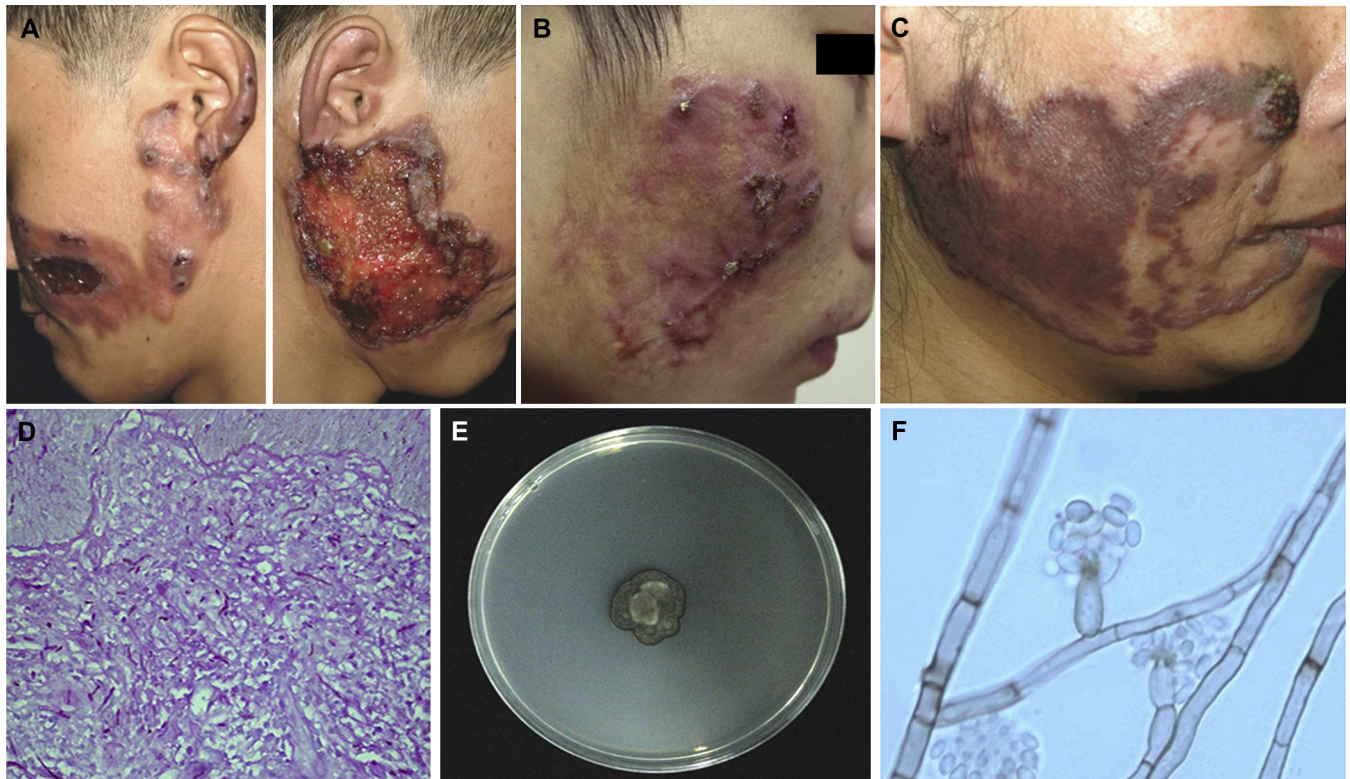


FIG 1. A-D, Clinical manifestations of 4 patients: they have dark red plaques and nodules on their faces, with ulcerations and eschars. Patient 4 has severe skin lesions involving his eyes. E, Skin biopsy specimen of patient 1 showed intense inflammatory infiltration, with slender, strongly septate, dematiaceous hyphae (Periodic Acid-Schiff; original magnification $\times 400$). F, Microscopic appearance of *Phialophora verrucosa*: slide culture showed brown, septate hyphae with typical *Phialophora* type of sporulation (Corn Meal Agar, 14 days at 28°C; original magnification $\times 1000$).

of T_H17 cells (CD4⁺/IL-17⁺) in patients' PBLs were strikingly decreased, and so was the representative effector cytokines IL-17 and IL-22 in serum (Fig 2, B and C). The profound deficiencies of T_H17 responses, as downstream consequences, might lead to the patients' suppressed antifungal immunity.

As the key players in initiating and regulating adaptive immune responses, macrophages and dendritic cells (DCs) secrete cytokines to trigger the differentiation of T_H17 cells.⁹ Therefore, we isolated monocytes from 3 healthy donors and patients 1 and 3, and induced them toward macrophages and immature DCs. Responses to *P verrucosa* spores were detected by the production of IL-6, TNF- α , IL-1 β , and IL-23p19 in supernatants, which were essential for the differentiation, activation, and expansion of T_H17 cells. When challenged with *P verrucosa* spores, both macrophages and DCs from patients secreted much lower cytokines than did those from control subjects (Fig 2, D and E). It is reasonable to speculate that CARD9 deficiencies cause the decreased production of cytokines from innate immune cells, which leads to the deficiencies of T_H17 cells and consequently predisposes patients to recalcitrant subcutaneous *P verrucosa* infections.

An intriguing feature of these patients is that distinct mutations in *CARD9* did not give rise to *Candida* infections in our cases, despite its ubiquity in human skin. Whether *CARD9* is indispensable in different fungal infections, as well as the association of genotype with phenotype, still needs further investigation.

Taken together, our study showed *CARD9* mutations in 4 patients with pheohyphomycosis with marked decreased

proportions and cytokines of T_H17 cells and impaired immune responses against *P verrucosa*, which further confirmed the pivotal role of *CARD9* in antifungal defense. Our preliminary findings of *P verrucosa* linked, for the first time, *CARD9* deficiencies with the susceptibility to opportunistic filamentous fungi, other than yeast or yeast-like fungi. What we have identified in this study will enrich the phenotypic spectrum of diseases underlying *CARD9* deficiencies.

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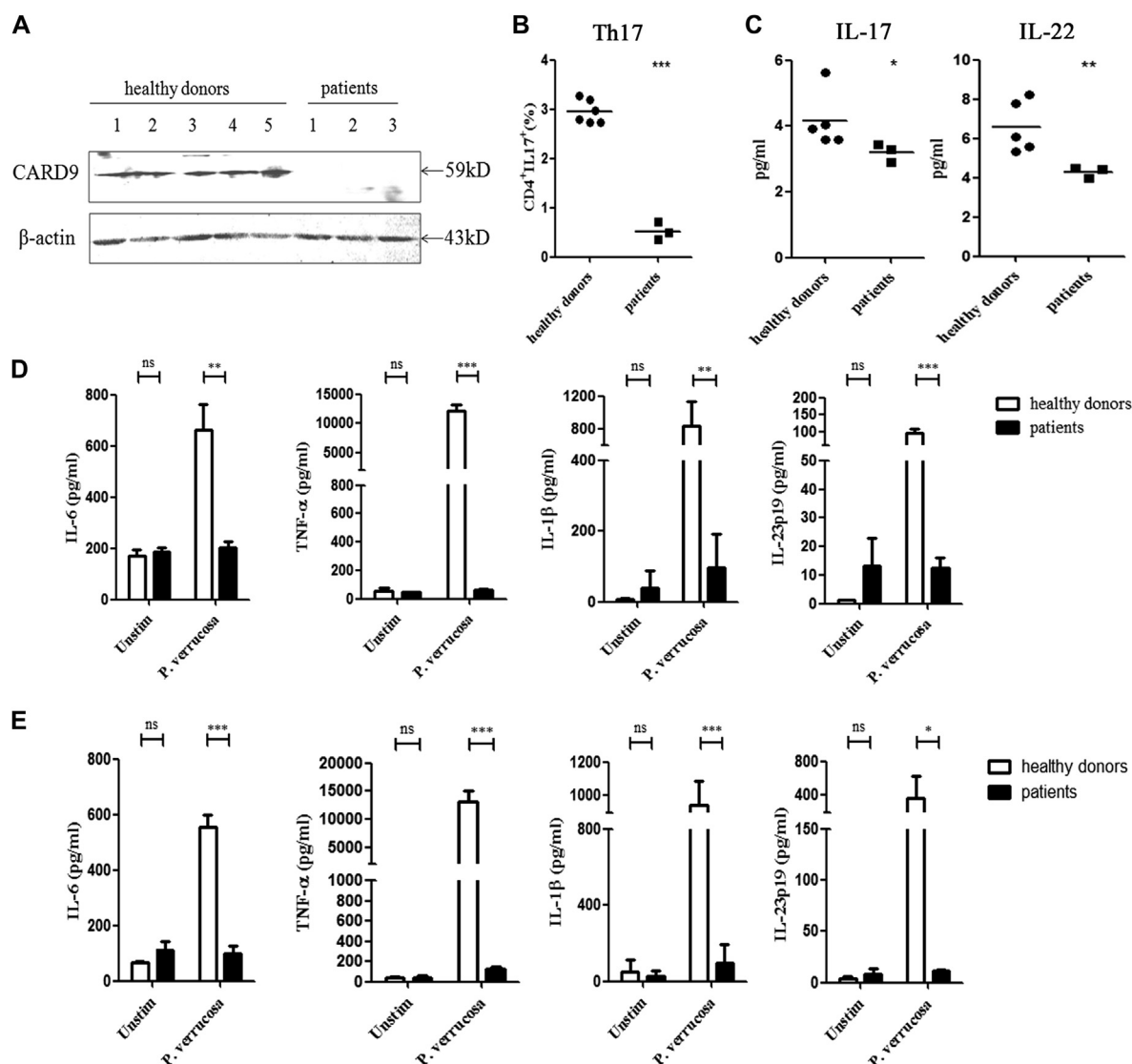


FIG 2. Detection of CARD9 protein and T_H17 cell deficiencies: **A**, Extracted proteins of PBMCs from 5 healthy donors and 3 patients were detected by using Western blot. **B**, Proportions of T_H17 cells ($CD4^+/IL-17^+$) in peripheral lymphocytes were analyzed by using fluorescence-activated cell sorting. **C**, Representative cytokines of T_H17 cells were detected in serum by using ELISA. Macrophages (**D**) and DCs (**E**) derived from peripheral monocytes were stimulated with *Phialophora verrucosa* (10^8 /mL) for 48 hours. Cytokines for the differentiation, activation, and expansion of T_H17 cells (IL-6, TNF- α , IL-1 β , and IL-23p19) were detected in supernatants by using ELISA. Results are depicted as average of 4 healthy donors $\pm$ SD. $P < .05$ was considered statistically significant. * $P < .05$, ** $P < .01$, and *** $P < .001$.

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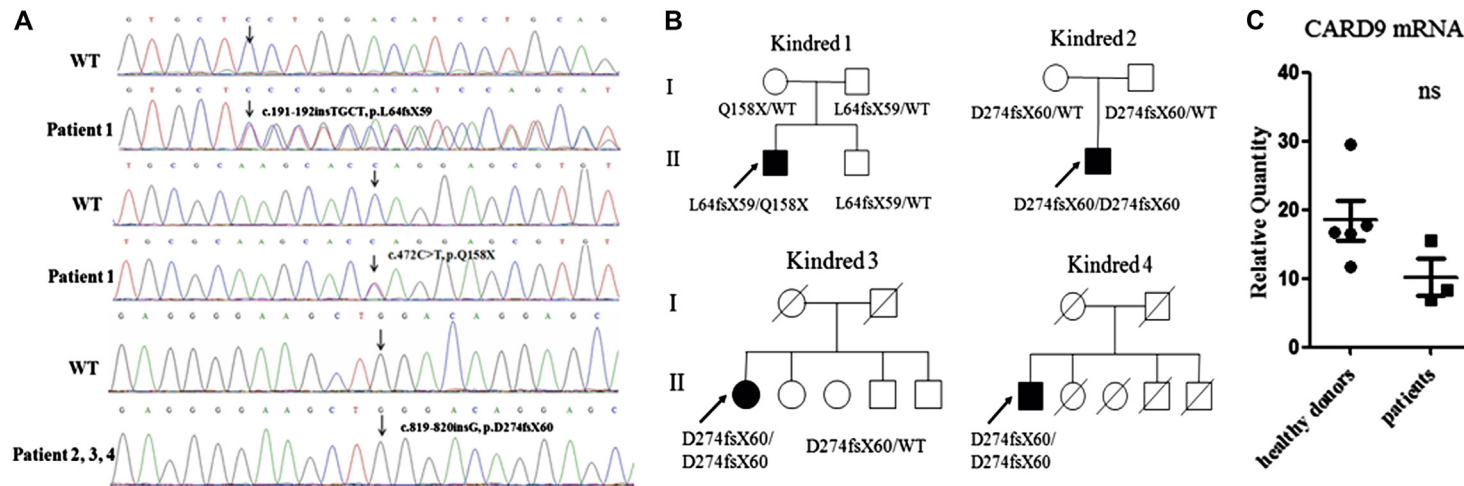


FIG E1. **A**, Chromatograms of Sanger sequencing of the *CARD9* gene in the 4 patients: arrows indicate the mutation sites. **B**, Pedigrees of the 4 patients: arrows indicate the probands. The genotypes of *CARD9* for each individual were listed below. **C**, Identification of *CARD9* transcription: *CARD9* mRNA in PBMCs was detected by using real-time PCR. One of the patients was analyzed as relative control (ns = none of significant, $P > .05$). WT, Wild-type.

TABLE E1. Summary of the clinical manifestations for the patients

Patient	Sex	Age (y)	Age at presentation (y)	Clinical features	Outcome
1	Male	21	13	Progressive dark red plaques and nodules on his bilateral cheeks and ears	Oral itraconazole and intravenous amphotericin B once showed limited effect, but there was no complete healing. Now the lesions aggravated with subcutaneous disseminations.
2	Male	17	6	Gradually enlarged red plaques on his right cheek	Intravenous amphotericin B followed by oral itraconazole for 2 y, with recurrence whenever withdrew the drug.
3	Female	43	20	Slowly enlarged plaques and nodule on her right cheek	Surgical excision of the nodule combined with oral itraconazole therapy for 1 y. It seemed effective, and she was still in treatment.
4	Male	64	48	Severe violet plaque on his face and scalp, which involved his eyes and caused blindness 4 y ago	Oral itraconazole combined with terbinafine for half a year. The lesions improved slightly without satisfactory response.

TABLE E2. Haplotype analysis in patients 2, 3, and 4

SNP	Location (hg19)	Reference allele	Alternative allele	Allele frequency for samples from Asian	Patient 2	Patient 3	Patient 4
rs3812553	Chr 9: 139256495	G	T	0.10	T	T	T
rs10870201	Chr 9: 139257147	G	C	0.31	C	C	C
rs10870202	Chr 9: 139257411	T	C	0.32	C	C	C
rs45592338	Chr 9: 139257422	C	G	0.24	G	G	G
rs4073153	Chr 9: 139259349	A	G	0.31	G	G	G
rs3812555	Chr 9: 139261933	C	T	0.17	T	T	T
rs3812556	Chr 9: 139261991	A	G	0.24	G	G	G
rs59902911	Chr 9: 139265088	C	T	0.09	T	T	T
rs11145769	Chr 9: 139265870	G	A	0.10	A	A	A
rs10781499	Chr 9: 139266405	G	A	0.30	A	A	A
rs4077515	Chr 9: 139266496	C	T	0.31	T	T	T
rs4075760	Chr 9: 139266697	A	G	0.31	G	G	G