https://doi.org/10.33472/AFJBS.6.8.2024.1753-1767

African Journal of Biological Sciences

Open Access Research Paper

MOLECULAR ROLE OF ADAPTOR PROTEIN CARD 9 IN SEVERE DERMATOPHYTE INFECTION

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Article History

Volume 6,Issue 8, 2024
Received:15 Mar 2024
Accepted : 06 May 2024
doi: 10.33472/AFJBS.6.8.2024.1753-1767

Abstract

Background: Dermatophytosis is a common contagious disease caused by fungi known as dermatophytes.

Aim of study: This study was conducted to know the effects of CARD9 genes with severe dermatophytes infections.

Methodology: The study was carried out in the period beginning of December 2022 to end of June 2023.This study has been done at the Al-Hilla Teaching Hospital, Dermatology Unit, Babylon Government; ages range between 1 to ≥ 50 years in male and female. During the study a total 90 clinical specimens (Skin scraping and 5ml of blood) were obtained from all participants (50 dermatophytosis cases and 50 controls). Blood samples (whole blood) introduced for RNA extraction for calculation of gene expression of CARD9 by RT-PCR.

Results: The results for gene expression of CARD9 by RT-PCR revealed that the ΔCt mean of Card 9 mRNA in blood samples of patients group was (10.99) compared to the corresponding ΔCt mean in control group (8.79), significant increasing was observed in ΔCt mean of patient groups compared to the corresponding ΔCt means in control group.

The relative expression (2-ΔΔCt) of Card 9 mRNA was decreased in patient group, significant difference was observed in means of (2¯ΔΔCt) (expression folds) within groups. When the dermatophyte patients were distributed patient group, Asthenozoospermia (AS), Oligoasthenozoospermia (OAS). The t mean of CARD 9 in blood samples of patient groups were [p(0.217)] compared to the corresponding t mean in control group (1.0000),significant p=0.001 decreasing was observed in t mean of CARD9 gene.

Conclusion: Decreased expression level of CARD9 gene in blood samples of dematophyrosis patients groups compared to control group.

Key words: Dermatophyte infections, CARD9 gene, RT-PCR.

1. Introduction

A dermatophyte infection, also known as dermatophytosis or tinea, refers to a group of fungal infections that can affect the skin, hair, and nails. Tinea is caused by the dermatophytes, which include the fungus of the genus Trichophyton, Microsporum, and Epidermatophyton [1].

Many pathogens are known to contribute toward abnormal immune responses in genetically susceptible individuals through molecular mimicry, epitope spreading, bystander activation, or other mechanisms [2].

CARD9-related C-type lectin receptor (CLRs present on pathogens) includes mainly Dectin-1 (best-characterized CLR recognizing β-glucan on pathogens such as fungi), Dectin-2, Dectin-3 and Mincle. Upon recognition of carbohydrate agonists, CLRs recruits tyrosine kinases Syk under Src kinase-mediated tyrosine phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM)-like motif (hem-ITAM) or ITAM in their cytoplasmic tail. Syk is a pivotal mediator involved in coupling activated immunoreceptors in immune cells. Upon recruitment, Syk is phosphorylated and subsequently activates protein kinase Cδ (PKCδ), which leads to the recruitment and phosphorylation of CARD9 [3].

Dectin-1 is the most representative CARD9-dependent pathway, which activates inflammasomes and production of proinflammatory cytokines and chemokines, including TNF-α, GM-CSF, CXCL2, IFN-γ, IL-1β, IL-2, IL-6, IL-10, and IL-23 under the management of CARD9, to control the infection and suppress fungal pathogens [4].

At least 15 CARD9 gene mutations have been identified in people with familial fungus infection, an inherited tendency to develop infections caused by the fungus infections [5]. Most people with familial fungal infection have chronic infections of the skin, nails, and mucous membranes. This pattern of signs and symptoms, which is called chronic mucocutaneous infection, typically begins in early childhood [6]. People with familial fungal infection caused by CARD9 gene mutations can also develop systemic infection, Infections caused by several types of fungi have also been identified in some people with this form of the disorder, which is sometimes called CARD9 deficiency [7].

Rare cases of deep dermatophytosis were associated with genetic defects in the innate signaling adaptor CARD9. CARD9 deficiency can also lead to other uncommon invasive forms of fungal diseases, such as Candida encephalitis, extrapulmonary aspergillosis, and phaeohyphomycosis [8].

Human CARD9 mutation is associated with specific families of pathogenic fungi. c.759dup (p. Lys254fs) mutation in the exon 5 of CARD9 was associated with E. dermatitidis [9].

The fungal infectious diseases were associated with 24 CARD9 mutations, further evaluated the frequency and geographic distribution of CARD9 mutations. Three CARD9 genetic mutations, p.Q289X (c.865C > T), p.Q295X (c.883C > T) and p.D274fsX60 (c.819-820insG), were identified most frequently, which accounted for 25.8%, 17.7%, and 8.1% of the patients, respectively. CARD9 p.Q289X (c.865C > T) and p.Q295X (c.883C > T) mutations were associated with a high risk of candidiasis

and dermatophytosis infection. CARD9 p.Q289X (c.865C > T) and p.Q295X (c.865C > T) accounted for 75% and 37.9% of the African and Asian cases, indicating an obviously different geographical distribution [10].

These CARD9-dependent signaling pathways are regulated by Rubicon, a protein best known for its functions in autophagy. Rubicon competitively binds to CARD9 which results in the disassembly of the CBM complex, thus switching off signaling and preventing excessive inflammatory responses. However, modulation of Rubicon expression levels using lentiviral vectors in mice demonstrated that a reduction in Rubicon expression could help promote fungal clearance and survival by enhancing CARD9-dependent antifungal immune responses, at least in acute infection models [11].

In 2013, Lanternier et al. first demonstrated that 17 non-consanguineous patients with deep dermatophytosis by T. rubrum or T. violaceum, had autosomal recessive CARD9 deficiency without other associated infectious conditions, except oral candidiasis in six of them. These authors described two CARD9 mutations: a homozygous premature stop codon mutation (Q289), identified in 15 patients from seven unrelated Algerian and Tunisian families, and a homozygous missense mutation (R101C) in two Moroccan siblings. The functional consequence of CARD9 mutations was a markedly low level of IL-6 production after stimulation of whole-blood leukocytes with heat-killed C. albicans or S. cerevisiae, but not with TLR agonists. Furthermore, peripheral Th17 cells from CARD9-deficient patients were significantly less frequent than healthy controls. CARD9 Q289 mutation was later described in Egyptian patients with widespread superficial T. rubrum infection of the skin and nails without significant visceral involvement. Recently, this mutation was also reported in an Algerian woman who suffered from cutaneous chronic dermatophytosis by T. rubrum from her childhood and developed an invasive brain infection in her adulthood [12].

The phenotypic variability of dermatophytic infection observed in patients with CARD9 deficiency ranges from extensive skin and nail lesions to potentially lethal lymph node and central nervous system infection [13].

A successful allogeneic hematopoietic stem cell transplantation (HSCT) in two patients with inherited CARD9 deficiency and deep dermatophytosis. More than 3 years after HSCT, both patients have achieved complete clinical remission and stopped antifungal therapy. This evidence points toward deep dermatophytosis pathogenesis in CARD9-deficiency settings might be largely due to the disruption of myeloid cell antifungal response [14].

CARD9 signaling in antigen-presenting cells links innate sensing of fungi to the activation of adaptive immunity and provides a cytokine milieu that induces the development and subsequent of interleukin 17-producingThelper (Th17) cells [15].

2. Materials and Methods

2.1 Materials:

2.1.1 Total RNA Extraction and Amplification Kits.

The chemical materials that are used for total RNA extraction and, after that, amplification by qPCR to perform this study with their companies and countries of origin are listed in table (1).

Table (1): Total RNA extraction and amplification kits for qPCR with their remarks.

No.	Kits	Components	Volume or concentration	Company	origin
1	TransZol Up EasyScript® First-Strand cDNA Synthesis Super Mix	Trizol reagent	100 ml	TRANS	China
		RNA dissolving solution	15 ml		
	GoTaq® qPCR Master Mix	EasyScript® RT-RI Enzyme Mix	20X		
2	TransZol Up EasyScript® First-Strand cDNA Synthesis Super Mix	2x ES reaction Mix	2X	TRANS	
		Anchored Oligo(dT)$_{18}$ primer	0.5 µg/µl		
		RNase-free Water	10 ml		
3	GoTaq® qPCR Master Mix TransZol Up	GoTaq® qPCR Master Mix	2X	Promega	
		Supplemental CXR Reference Dye	300nM		
		Nuclease Free Water	1 ml		
4	EasyScript® First-Strand cDNA Synthesis Super Mix	GoTaq® qPCR Master Mix	2X	Promega	USA
		GoScript™ RT Mix for 1-Step RT-qPCR	50X		
		MgCl2	25mM		
		CXR Reference Dye	30µM		
		Nuclease Free Water	1 ml		

2.1.2 The Primers Used

The primers (table 2), which are used in PCR for the detection of CARD9 and β-actin are provided from Macrogen company (Korea).

2.1.2.1 The specific primer of genes CARD9 and β-actin used in the interaction

Table (2): The specific primer of genes CARD9 and β-actin

Primer		Sequence	T m (C°)	CC (%)	Product size
CARD9	Forward	5'-GCAGGTGTTCCAGTGTGAGG-3'			
	Reverse	5'-GTGAGCCATCTTCCAGGTCG-3			
β-actin	Forward	5'-GGATGCAGAAGGAGATCACTG-3'			
	Reverse	5'-CGATCCACACGGAGTACTTG-3'			

2.2 Methods

2.2.1 Patients and Control Groups

A case control study has been conducted from beginning of December 2022 to end of June 2023. The blood samples have been taken from 100 participants (50 cases with dermatophytosis and 50 controls): 50 Iraqi Patients experiencing dermatophytosis who attended hospitals with consultations for dermatology diseases in Al-Hilla Teaching Hospital, dermatology Unit, Babylon province, and some of outpatients clinics. The laboratory findings as well as clinical examinations have indicated that the patients are experiencing dermatophytosis. Furthermore, information regarding all cases were recorded. Additionally, 50 control group was selected from the same place, but they suffer from diseases other than skin who have no history of dermatophytosis.

2.2.2 Blood Samples Collection

Five ml of venous blood were drawn from all participants, collected in gel tubes and EDTA tubes. The withdrawal of the blood sample was slow via the needle of syringe to prevent hemolysis.

These samples were centrifuged at 15,000 rpm for 10 min to precipitate cell debris and the supernatants were stored at (-80 C) until RNA extraction. RNA was extracted from EDTA tubes for detection of CARD9 gene.

Also, scraping samples were taken from all patients with dermatophytosis for microscopic examination and culture.

2.2.3 Molecular method

2.2.3.1 TransZol Up Genomic RNA Extraction Mini Kit

This Kit used for extraction of genomic RNA from frozen blood.

A. Kit Content

Table (3): Kit Content of TransZol Up Genomic RNA Extraction Mini Kit

Compenent	ET111-01

TransZol Up	100 ml
RNA Dissolving Solution	15 ml

B. Procedure

1. Homogenization

a. Adherent cells

1) Wash culture dish once with 1×PBS.
2) Detach cells with cell spatula. Add 1 ml of TransZol Up to per 10 cm^3 culture dish. Pipetting up and down to lysis the cells.
3) Transfer lysate containing cells to a microcentrifuge tube.
4) Incubate at room temperature for 5 minutes.

b. Suspension cells

1) Transfer suspension cells including culture dish to a microcentrifuge tube. Centrifuge the sample at 8,000×g for 2 minutes at 2-8°C, discard the supernatant.
2) Add 1 ml TransZol Up to per 107 cells.
3) Pipetting up and down until no visible precipitates are present in lysate.
4) Incubate at room temperature for 5 minutes.

c. Animal tissue and plant materials

1) After weighing, quickly transfer the frozen sample into mortar with liquid nitrogen. Grind thoroughly to a powder. Additional liquid nitrogen can be used if needed. Incomplete grind can affect RNA yield and quality.
2) Transfer the tissue powder to a microcentrifuge tube. Add 1 ml of TransZol Up to per 50-100 mg tissue. Homogenize tissue samples with a homogenizer and repeatedly pipette up and down.
3) Incubate at room temperature for 5 minutes.

2. Add 0.2 ml of chloroform per ml TransZol Up. Shake the tube vigorously by hand for 30 seconds. Incubate at room temperature for 3 minutes.

3. Centrifuge the sample at 10,000 g for 15 minutes at 2-8°C. The mixture separates into a lower pink organic phase, an interphase, and a colorless upper aqueous phase which contains the RNA. The volume of the aqueous upper phase is around 50% volume of TransZol Up reagent.

4. Transfer the colorless, upper phase containing the RNA to a fresh RNase-free tube. Add 0.5 ml of isopropanol for per ml TransZol Up used. Mix thoroughly by inverting tube. Incubate at room temperature for 10 minutes.

5. Centrifuge the sample at 10,000xg for 10 minutes at 2-8°C. Discard the supernatant. Colloidal precipitate can be seen at the wall and the bottom of the tube.

6. Add 1 ml of 75% ethanol (prepared with DEPC-treated water), vortexing vigorously (add at least 1 ml of 75% ethanol for 1 ml of TransZol Up used).

7. Centrifuge the sample at 7,500×g for 3 minutes at 2-8°C.

8. Discard the supernatant. Air-dry the RNA pellet (for about 5 minutes).

9-RNA pellet is dissolved in 50-100 µl of dissolving solution.

10. Incubate at 55-60°C for 10 minute. For long-term storage, store the purified RNA at -70°C.

2.2.3.2 Quantitative real time PCR methods

2.2.3.2.1 Total RNA extraction from serum.

Serum samples: samples retrieved from liquid nitrogen and melt at room temperature, after complete melting the sampled mixed well by vortex and RNA extraction workflow as follow:

I. A volume of 1ml of Tranzol up reagent was added directly to liquid samples.

II. Pipetting up and down was done by micropipette until no visible precipitate appeared in lysate.

III. Incubation was for 5 minutes at room temperature.

IV. A volume of 200µl of chloroform was added and shaked well by hand for 30 seconds then incubation for 3 minutes at room temperature.

V. Centrifugation at 10000xg done for 15 minutes at 2-8 C. After centrifugation, the mixture was separated into three layers: lower organic pink layer which contain proteins, pale white middle layer contains DNA and colorless upper layer contain total RNA.

VI. The upper colorless layer was transferred to a new fresh tube and 500 µl of isopropanol were added and mixed well by hand then incubated at room temperature for 10 minutes.

VII. Centrifugation at 10000xg done for 10 minutes at 2-8 C. Then the supernatant discarded and the RNA can be seen as colloidal precipitate at the walls and bottom of tube.

VIII. A volume of 1 ml of 75% ethanol was added to the vortexing tube.

IX. Centrifugation at 7500xg was for 5 minutes at 2-8 C.

X. The supernatant was discarded and air-dried RNA pellet was for 5 minutes.

XI. RNA pellet dissolved in 50 µl of RNA dissolving solution, and incubated at 55-60 C for 10 minutes. Then the RNA solution was stored at (-80 C).

2.2.3.2.2 Estimation of Total RNA Concentration and Purity

The purity of samples was measured by UV/Visible spectrophotometer instrument by adding extracted RNA in the instrument. A260/280 ratios of pure RNA would usually be at 2.0.

2.2.3.2.3 Reference Gene Selection

The reference gene or housekeeping gene or endogenous control gene selected by finding the best and the more stable reference gene expressed in the serum samples. The best reference gene depends on three parameters: high expression level; stable and expressed among all samples; and then showing of converge expression level among all samples (Sauer *et al.*, 2014).

2.2.3.2.4 Determination of CARD9 and β-actin reference gene Expression in Samples by one step RT-qPCR

GoTaq 1-Step RT-qPCR System combines GoScrip Reverse Transcriptase and GoTaq qPCR Master Mix in a single-step real-time amplification reaction. The system, optimized for RT-qPCR, contains a proprietary fluorescent DNA binding dye, Sybr Green Dye. The system enables the detection of RNA expression levels using a one-step RT-qPCR method:

• GoTaq® 1-Step RT-qPCR component, total RNA, primers and Nuclease-free water wre all thawed on ice and each solution was mixed well.
• GoTaq® 1-Step RT-qPCR reaction was prepared, as shown in table (4).
• RT-qPCR reactions were performed using the cycling program shown in table (5).

Table (4):GoTaq® 1-Step RT-qPCR Reaction Mix

Component	Volume	Final Concentration
GoTaq® qPCR Master Mix, 2X	10 µl	1X
GoScriptTM RT Mix for 1-Step RT-qPCR (50X)	0.4 µl	1X
Forward Primer (20X)	0.6 µl	300 nM
Reverse Primer (20X)	0.6 µl	300 nM
MgCl2	1.6 µl	25mM
RNA template	5 µl	100ng
Nuclease-Free Water	1.8 µl	-

Table (5): One-step RT-qPCR programs.

Step	Temperature	Duration	Cycles
Reverse transcription	37 C°	15 min	1
RT inactivation/Hot-start activation	95 C°	10 min	1
Denaturation	95 C°	10 sec	50
Annealing	58 C°	30 sec	
Extension and data collection	72 C°	30 sec	

2.2.3.2.5 Determination of CARD9 and β-actin reference gene expression in samples by two-step RT-qPCR.

2.2.3.2.5.1 Complementary DNA (cDNA) Synthesis.

EasyScript® First-Strand cDNA Synthesis SuperMix kit, is a complete system for the efficient synthesis of first strand miRNA from the total RNA templates. Preparing cDNA according to the manufacturer instructions, as follows:

• The template RNA and all reagents were thawed on ice and each solution was mixed by vortexing gently, then centrifuged briefly to collect the residual liquid from the sides of the tubes.
• The mixture was prepared in a tube on ice, mixed gently and centrifuged briefly to collect the contents and added component as table (6).

Table (6): Reaction components of cDNA synthesis mixture.

Component	Volume final	Concentration
2x ES Reaction Mix	10 µl	1X
Anchored oligo(dT)18 primer	1 µl	0.5 µg/µl
EasyScript® RT/RI Enzyme Mix	1 µl	20X
RNA template	5 µl	0.1 ng-5 µ

RNase-free H2O	up to 20 µl	-

• The mixture was incubated at 42 oC for 15 minutes, followed by 85 oC for 5 seconds to inactivate enzymes.

2.2.3.2.5.2 Determination of expression in samples by qPCR

According to the kit manufacturer instructions:

1. GoTaq® qPCR Master Mix, template cDNA, primers and Nuclease-free water all were thawed on ice, and each solution was mixed well.
2. Master-mix reaction was prepared, as in table (7).
3. Cycling program of qPCR reactions was programmed following 3-step cycling program, as shown in table (8).

Table (7): Component's volume of qPCR mixture.

Component	Volume /reaction	Final Concentration
Forward Primer	0.6 µl	300 nM
GoTaqR qPCR Master Mix	10 µl	(2X)
Nuclease-free water	3.8 µl	-
Reverse Primer	0.6 µl	300 nM
Template cDNA	5 µl	-
Total Volume	20 µl	-

Table (8): qPCR programs

Step	Temperature	Duration	Cycles
Denaturation	95 C^{o}	15 sec	50
Annealing , extension and data collection	60 C^{o}	1 min	
Enzyme activation	95 C^{o}	1 min	1

2.2.3.2.5.3 Calculating Gene Expression (Gene Fold)

There are two strategies for analyzing qPCR data: absolute and relative quantification. The absolute quantification identifies the input gene amount based on a standard curve which created by Livak and Schmittgen. In contrast, the relative quantification determines changes in gene expression relative to a reference genes sample which is accomplished by Pfaffl (Pfaffl, 2001).

Errors caused by standard dilutions when creating a standard curve can also be avoided. In addition, sometimes the relative gene amount between two treatment groups is of more interest than exact DNA/RNA molecular numbers. Therefore, the relative quantification is widely performed.

Gene expression or gene fold or RQ (Relative quantification) value were calculated by Pfaffl equation (Pfaffl, 2001):

$RQ = 2^{-(\Delta\Delta CT)}$.

The gene fold was calculated firstly by collecting CT (CT - cycle threshold) average value from real time PC device for each triplicated sample then ΔCT value was calculated for each sample as follows:

Δ CT = CT (gene of interest) – CT (reference gene).

ΔCT is the difference in CT values for the gene of interest and reference gene for a given sample. This is essential to normalize the gene of interest to a gene, which is not affected by experiment.

Calculating ΔΔCT value is found as follows:

ΔΔ CT = Δ CT (treated sample) – Δ CT (untreated sample (control))

After calculating ΔΔ CT for all samples, the final equation is taken to calculate the gene expression (fold change) as follows:

Fold gene expression RQ = 2-(ΔΔCT).

3. Result and Discussion

3.1 Complementary DNA Reverse Transcription

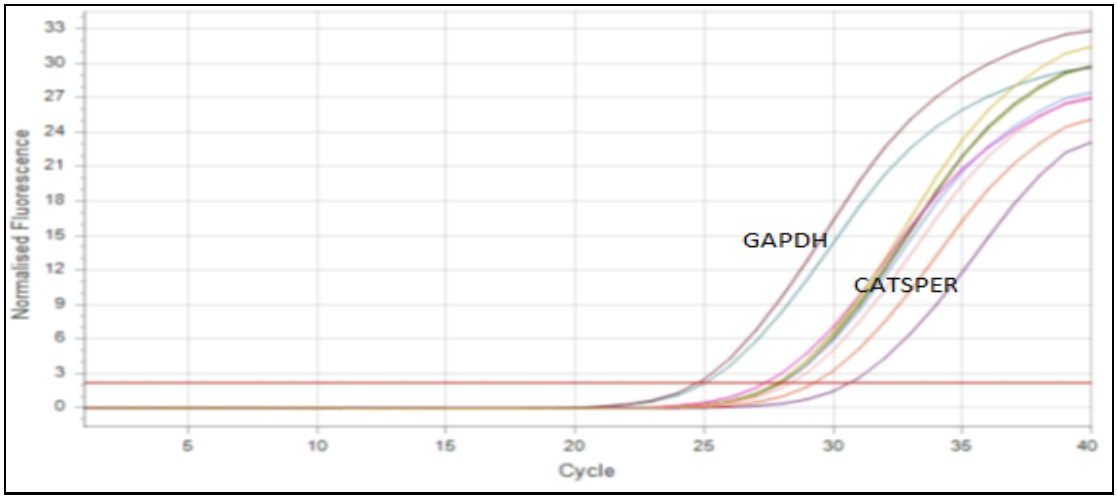
Complementary DNA reverse transcription was conducted on the second day of RNA extraction. A common primer reaction was applied since it was needed to have cDNA for both the gene in the study and housekeeping gene.

The efficiency of cDNA concentration was assessed through the efficiency of qPCR conducted later on All steps were associated with perfect yield reflecting efficient reverse transcription.

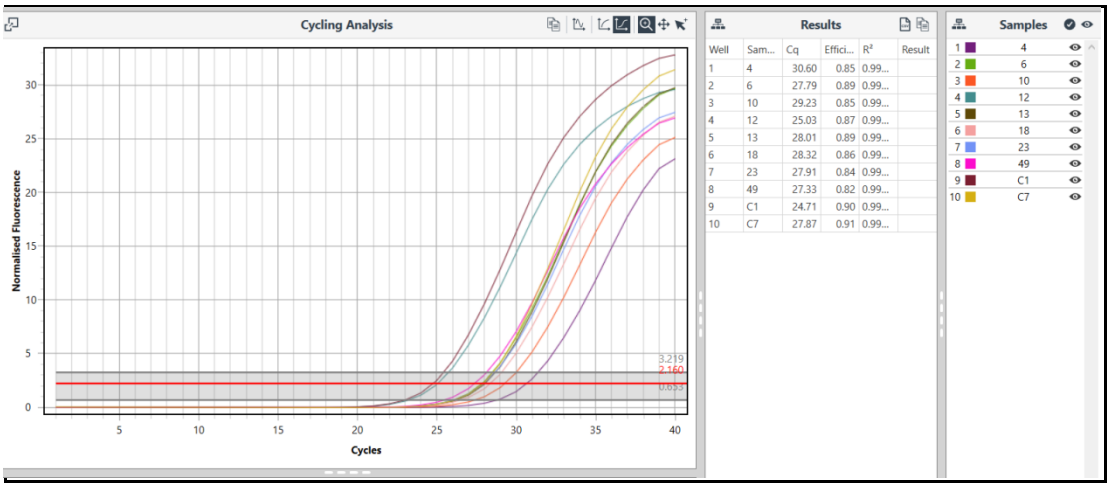
Optimal primers annealing temperature was calculated from the Tm of each primer supplied in the manufactures instructions according to specific equations. The equation calculates the annealing temperature, which requires the sequence of the primer because the amounts of specific nucleotides are needed. The equation is as follow:

- Melting Temperature (Tm) =2 (A+T) + 4 (G+C).
- Annealing Temperature (Ta) = Tm - (2-5) ºC.

Using the equations above, the melting temperatures for the forward primer and reverse primer were calculated. By comparing the annealing temperature for forward and reverse primers, the lowest temperature (ºC) was chosen. Quantitative expression of *CARD 9* gene was determined by Real Time Polymerase Chain Reaction, In which the relative quantitation method was employed. the gene expression was normalized to the level of a housekeeping gene (β actin) and quantified by the ΔCt value and folding ($2^{-\Delta\Delta Ct}$) method. Figures (1 A , B) and (2) show the amplification plots for *CARD 9*.



Figures (1 A) show the amplification plots of CARD 9 gene by RT-PCR. The photograph was taken directly from the monitor of real-time thermo cycler machine.



Figures (1 B) show the amplification plots of CARD 9 gene by RT-PCR. The photograph was taken directly from the monitor of real-time thermo cycler machine.

A representative melt curve *CARD 9* gene for samples analyzed by RT-PCR is given in figure 3-3,in which ,A single peak was observed for the amplicons. Such findings are interpreted that the melt curve represented a pure, single amplicon for each sample, and the specificity of amplification was considered to be great with intercalating dye assay.

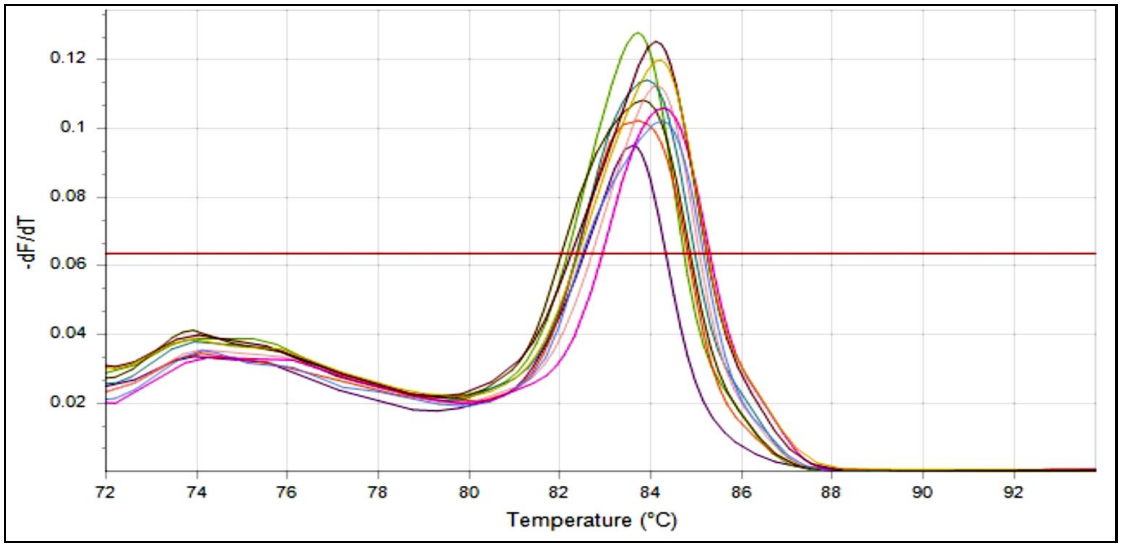


Figure (2): Melt curve of CARD 9 gene amplicons after RT-qPCR analysis showing single peaks.

3.2 Expression level of *CARD 9* Gene in blood samples of studied groups

The ΔCt mean of *Card 9* mRNA in blood samples of patients group was (10.99) compared to the corresponding ΔCt mean in control group (8.79), significant increasing was observed in ΔCt mean of patient groups compared to the corresponding ΔCt means in control group (Table 9 A).

Table (9 A): Expression level of *CARD 9* of the studied groups.

Groups	*P* value‡

ΔCt (mean± SD)		
Control group	Patient group	0.001
8.79	10.99	

‡ **Univariate analysis of variances (ANOVA) significance test;**

The relative expression ($2^{-\Delta\Delta Ct}$) of *Card 9* mRNA was decreased in patient group, significant difference was observed in means of ($2^{-\Delta\Delta Ct}$) (expression folds) within groups. (Table 4-4 B).

Table (9 B): Expression fold ($2^{-\Delta\Delta Ct}$) of *CARD 9* mRNA in blood of the studied groups.

Groups	$2^{-\Delta\Delta Ct}$ (mean ± SD)	*P* value‡
Control group	1.000	0.001
Patient group	0.217	

‡ **Univariate analysis of variances (ANOVA) significance test**

When the dermatophyte patients were distributed patient group, Asthenozoospermia (AS), Oligoasthenozoospermia (OAS). The t mean of *CARD 9* in blood samples of patient groups were [p(0.217)] compared to the corresponding t mean in control group (1.0000),significant p=0.001 decreasing was observed in t mean of CARD9 gene.

In the past two decades, several inherited immune disorders have been discovered, of which mutations in the caspase recruitment protein domain 9 (CARD9) is particularly significant for fungi: dermatophytes are one of the main groups emerging with this disorder [16]. Song *et al.* mentioned that all dermatophyte infections with this type of clinical course and where the host was reported as otherwise appearing healthy, actually had a non-diagnosed CARD9 disorder [17].

In literature before 2010, cases regarding deep dermatophytosis were listed as occurring in otherwise healthy, immunocompetent patients. However, with the advances in genetics and primary immunodeficiencies, the underlying genetic background predisposing to chronic, deep and mutilating dermatophyte infections has been recognized [18].

Song *et al,* reviewed chronic mutilating cases and found solid organ transplant as the prime risk factor, and CARD9 defects as the second. And the most of dermatophytic pseudomycetomata were in patients with a defective signalling pathway with impaired IL-6 and IL-17 production, resulting from CARD9-related immune defects. Cytokine release from innate immune cells is crucial for Th-cell differentiation. Underlying conditions were mostly SOT associated with immunodepression, whereas autosomal recessive CARD9 deficiency was second. In the case presented by song, whole-exome sequencing did not suggest known pathogenic mutations, including CARD9 and genes leading to CD4 lymphocytopenia. However, we still cannot exclude the involvement of other genetic defects in the pathogenesis of the disease, which needs further study [17].

Inherited CARD9 deficiency is mutations may be present in a homozygous or compound heterozygous state. CARD9 is expressed principally in myeloid cells and transduces signals downstream from CLR activation by fungal ligands. Endogenous mutant CARD9 levels differ between alleles (from full-length normal protein to an absence of normal protein). The functional impacts of CARD9 mutations involve impaired cytokine production in response to fungal ligands, impaired neutrophil killing and/or recruitment to infection sites, and defects of Th17 immunity. The key clinical manifestations in patients are fungal infections, including CMC, invasive (in the CNS in particular) Candida infections, extensive/deep dermatophytosis, subcutaneous and invasive phaeohyphomycosis, and extrapulmonary aspergillosis [16].

Cifaldi *et al.* mentioned that the clinical penetrance of CARD9 deficiency is complete, but penetrance is incomplete for each of the fungi concerned. Age at onset is highly heterogeneous, ranging from childhood to adulthood for the same fungal disease [19].

While Alves de Medeiros et al. reported that all patients with dermatophytosis should be tested for CARD9 mutations. Familial screening and genetic counseling should be proposed. Thus our results compatible with this study [20].

Corvilain *et al.* reviewed that he treatment of patients with CARD9 mutations is empirical and based on antifungal therapies and the surgical removal of fungal masses. Patients with persistent/relapsing Candida infections of the CNS could be considered for adjuvant GM-CSF/G-CSF therapy. The potential value of HSCT for CARD9-deficient patients remains unclear [16].

Rare cases of deep dermatophytosis were associated with genetic defects in the innate signaling adaptor CARD9. CARD9 deficiency can also lead to other uncommon invasive forms of fungal diseases, such as Candida encephalitis, extrapulmonary aspergillosis and phaeohyphomycosis [8].

Although reduced Th17 responses were observed in some CARD9-deficient patients, the defect in the IL-17 response may not be the only cause of disease manifestation in these patients. Neutrophil recruitment defects were identified as a leading cause of Candida encephalitis and extrapulmonary aspergillosis as a result of CARD9 deficiency [8]. It remains to be determined if the same applies in deep dermatophytosis, where neutrophils also make part of the dermal lesions.

4. Conclusions

The ΔCt mean of *Card 9* mRNA in blood samples of patients group was (10.99) compared to the corresponding ΔCt mean in control group (8.79), significant increasing was observed in ΔCt mean of patient groups compared to the corresponding ΔCt means in control group.

The relative expression ($2^{-\Delta\Delta Ct}$) of *Card 9* mRNA was decreased in patient group, significant difference was observed in means of ($2^{-\Delta\Delta Ct}$) (expression folds) within groups.

The t mean of *CARD 9* in blood samples of patient groups were [p(0.217)] compared to the corresponding t mean in control group (1.0000),significant p=0.001 decreasing was observed in t mean of CARD9 gene.

5. Ethical Approval

The participants in the study were notified about the study and a verbal agreement has been taken from each participant.

6. Finance

The research was funded by self-efforts.

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