Evaluation of miR-146a expression level in macrophages exposed to *Candida glabrata*

Arghavan B¹, Sharifi M², Shafiee M³, Mohammadi R¹, ⁴*

¹ Department of Medical Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
² Department of Genetics and Molecular Biology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran
³ Department of Medical Genetics, School of Advanced Medical Technologies, Golestan University of Medical Sciences, Gorgan, Iran
⁴ Infectious Diseases and Tropical Medicine Research Center, Isfahan University of Medical Sciences, Isfahan, Iran

* Corresponding author: Rasoul Mohammadi, Department of Medical Parasitology and Mycology, School of Medicine, Infectious Diseases and Tropical Medicine Research Center, Isfahan University of Medical Sciences, Isfahan, Iran. Email: Dr.rasoul_mohammadi@yahoo.com

(Received: 24 July 2016; Revised: 5 September 2016; Accepted: 17 September 2016)

Abstract

**Background and Purpose:** MicroRNAs are small non-coding RNAs with 192-nt nucleotides in length. Up- or down-regulation of many miRNAs has been shown by stimulation of Toll-like receptors (TLRs) in the innate immune system. Up-regulation of miR-146a has been reported by both TLR and heat-killed *Candida albicans*. In this study, we aimed to evaluate the expression of miR-146a in cultured monocyte-derived macrophages (MDMs) infected by *Candida glabrata* at 12, 24, and 48 hours.

**Materials and Methods:** miR-146a expression was evaluated by qRT-real time polymerase chain reaction (PCR) at three time points in *C. glabrata*-infected MDMs. The data was analyzed using repeated measures ANOVA.

**Results:** miR-146a expression was down-regulated in infected MDMs compared to the control group (P<0.018). The expression of miR-146a was at its highest level at 48 h, as compared to 12 and 24 h (P<0.018). The differences between the experimental group compared to the control group were statistically significant (P<0.018).

**Conclusion:** These results suggest that miR-146a can be involved in regulating macrophage function following TLR stimulation in *C. glabrata*-infected MDMs.

**Keywords:** Candida glabrata, Macrophage, miR-146a

How to cite this paper:
Arghavan B, Sharifi M, Shafiee M, Mohammadi R. Evaluation of miR-146a expression level in macrophages exposed to *Candida glabrata*. Curr Med Mycol. 2016; 2(2): 16-19. DOI: 10.18869/acadpub.cmm.2.2.6

Introduction

*Candida glabrata* has historically been considered as a nonpathogenic normal flora of healthy individuals. Recently, the emerging fungal pathogen, *Candida glabrata*, is the second most common cause of candidiasis after *Candida albicans*, including almost 15-25% of Candida infections [1-3]. The frequency of systemic infections caused by *C. glabrata* is considerably increased due to immunosuppressive diseases and antifungal resistance [4]. There are several inhibitory mechanisms for inflammatory cytokine production including the anti-inflammatory cytokines such as IL-10 [5].

Lately, it has been shown that miRNAs play the role of negative regulators in macrophages [5]. miRNAs are small, non-coding RNAs (containing about 22 nucleotides) that play an important role in the post-transcriptional regulation of gene expression. miRNAs are involved in cell cycle, differentiation, growth and development, metabolism, and immunity in plants, animals, and mammals. They are derived from larger stem-looped hairpin precursors that are transcribed from miRNA genes and are processed by Dicer or Dicer-like proteins [6, 7]. Some of them are expressed through the stimulation of TLRs in the innate immune system such as miR-146a_b, miR-132, and miR-155 in the THP-1 cells stimulated by lipopolysaccharide (LPS).

Increasing miR-146a expression lowers the expression of two key factors (IRAK1 and TRAF6) of the TLR signaling cascade [8]. It has been proposed by Simon et al. [5] that both *Candida albicans* and LPS are able to up-regulate miR-146, miR-155, miR-455, and miR-125a in mouse macrophages. miR-146a, as a negative regulator, can reduce TLR response to prevent further inflammation. This study aimed to evaluate miR-146a-5p expression in human macrophages infected with *Candida glabrata*, compared to non-infected macrophages.

Materials and Methods

**Collection and isolation of mononuclear cells with Lymphodex**

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood using
Lymphodex (Inno-Train, Germany). Briefly, the fresh heparinized blood was placed into 15 ml conical centrifuge tubes. An equal volume of PBS was mixed with the heparinized blood by a sterile pipet. Then, the blood/PBS mixture was gradually layered on the Lymphodex solution by using sterile pipets. The tubes were centrifuged for 30 min at 2000 rpm (900 × g), at 20°C. The cells were counted by hemocytometer protocol and their viability was determined by trypan blue exclusion after re-suspending of mononuclear cells in complete RPMI-1640.

**Monocyte-derived macrophages**

Monocytes were depleted of the PBMCs suspension by adhering to plastic surfaces. In summary, PBMCs were washed and centrifuged three times in RPMI-1640 for 10 min at 1400 rpm (300 × g); then, the supernatant was removed. Monocytes were incubated in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and inactivated fetal bovine serum (Mediatech Inc, Herndon, VA) for 12 h at 37°C in 5% CO₂ humidified incubator. Non-adherent cells were removed after 12 h; tissue culture plates were slowly rinsed with complete RPMI-1640. Monocyte-derived macrophages were cultured on 6-well tissue-culture plate for 12 days (Figure 1) at 37°C in 5% CO₂ in RPMI-1640 medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and inactivated fetal bovine serum.

**Yeast cell culture**

*Candida glabrata* strain (ATCC 90030) was cultured on Sabouraud dextrose agar (SDA) at 37°C for 24 h. Afterwards, it was sub cultured on 1% yeast extract, 2% peptone, and 2% dextrose (YPD) at 37°C in a shaking incubator at 180 rpm. Yeast peptone dextrose overnight cultures were washed twice with PBS and RPMI-1640 for 5 min at 2000 rpm (300 × g). *Candida glabrata* was counted with hemocytometer protocol.

**MDMs infection with C. glabrata**

Monocyte-derived macrophages were exposed to *C. glabrata* suspension. The ratio of MDMs to *C. glabrata* was 1:5. The whole solution in the 6-well tissue-culture plate was scraped and centrifuged; thereafter, the supernatant was removed. Non-infected MDMs were employed as control. Infected and non-infected MDMS were recognized by fluorescent microscope.

**qRT-real time-PCR**

RT-PCR was used to verify the expression of miR-146-a-5p in the MDMs. The total cellular RNA was extracted at 12, 24, and 48 h from infected and control MDMs with the miRCURY RNA Isolation Kit™ (Exiqon, Copenhagen, Denmark), and cDNA was synthesized with the Universal cDNA Synthesis Kit™ (Exiqon, Copenhagen, Denmark). Real-time PCR was performed by using SYBR® Green Master Mix Kit™ (Exiqon, Copenhagen, Denmark) and specific miR-146a-5p primers (Exiqon, Copenhagen, Denmark). The RT-PCR internal controls were synthetic RNA spike-in templates and their primers (Exiqon, Denmark). Real-time PCR was performed by ABI Step One Plus (ABI, USA). 2^{-\Delta\Delta CT} method was used for data analysis.

**Statistical analysis**

Repeated measures ANOVA was performed to evaluate differences between the groups, using SPSS version 21. P-value less than 0.05 was considered statistically significant.

**Results**

The macrophages were infected with *C. glabrata* (ATCC 90030), and after 3 h, 95% of the yeasts were phagocytosed. After 12, 24, and 48 h, the number of phagocytosed yeasts was increased due to replication (Figure 2).

**Down-regulation**

To evaluate the expression of miR-146a, MDMs were infected with *C. glabrata*, and RNA was extracted from both infected and
non-infected MDMs. The results demonstrated down-regulation of miR-146a in infected MDMs compared to the control group (Figure 3). The level of miR-146a expression increased at 12 h after infection of MDMs with *C. glabrata*, and then slowly reduced at 24 h. The expression of miR-146a was at its highest level within 48 h, as compared to the 12 and 24 h time points (Figure 3). The differences between the experimental and the control groups were statistically significant ($P<0.018$).

**Figure 2.** Control (2a), infected monocyte-derived macrophages to *Candida glabrata* after 12 (2b), 24 (2c), and 48 h (2d)

**Figure 3.** miR-146a expression after 12 (25%), 24 (10%) and 48 h (65%); the $2^{-\Delta\Delta Ct}$ method was applied for data analysis and the control group was considered as the reference for 12, 24, and 48 h ($P<0.018$)

**Discussion**

In the innate immunity as the first line of host defense, the primary recognition of pathogens is facilitated via dedicated pattern recognition receptors (PRRs), which are at the surface of phagocytic cells such as monocytes and macrophages. Pattern recognition receptors recognize microbe-specific pathogen-associated molecular patterns (PAMPs) and trigger the activation of some intracellular signaling cascades such as the MAPK and NF-$kB$ pathways.

Subsequently, the production of cytokines including pro-inflammatory cytokines causes inflammation at the site of infection [9-11]. NF-$kB$ is the main transcription factor in the inflammatory signaling pathways and plays an essential role in the regulation of inflammatory cytokines production.

The inflammatory response is essential to combat pathogens, but its inappropriate response can lead to severe problems [12-15]. The miRNAs can control many cellular decision-making networks by targeting the key molecules [16, 17].

miRNAs are also involved in the regulation of the NF-$kB$ pathways (e.g., miR-146a, miR-155, miR-125b, miR-9, and miR-29) [15]. Many of them such as miR-146, miR-155, miR-181b, miR-21, and miR-301a in the NF-$kB$ system are considered negative regulators [18, 19]. Previous studies have shown that the expression of miR-155, miR-146a and miR-146b are induced in response to viral stimuli through TLRs [20, 21]. Monk et al. [5] exhibited that miR-155, miR-146a, miR-125a, and miR-455 can be up-regulated by both TLR and heat-killed *C. albicans*. miR-146a is known as a target key element of NF-$kB$ pathways just as IRAK1 and TRAF6. Generally, the regulatory function of miR-146a leads to the regulation of pro-inflammatory cytokines production. Contrary to the present study, up-regulation of miR-146a in infected macrophages with *C. albicans* has been reported by Monk et al. [5], that is, various species may have different effects on the expression of special microRNA such as miR-146a [22].

**Conclusion**

In the present study, the expression of miR-146a was down-regulated in infected macrophages with *Candida glabrata*. We can conclude that down-regulation of miR-146a can reduce pro-inflammatory cytokines production, which can help *C. glabrata* survive in the macrophages.

**Acknowledgments**

Authors thank central laboratory personnel of Isfahan University of Medical Sciences.
Author’s contribution

B.A. and M S. performed tests, M.S. was a consultant and edited the manuscript, and R.M. supervised the research stages and revised the manuscript.

Conflicts of interest

None declared.

Financial disclosure

The authors gratefully acknowledge the financial support for this article as a part of the thesis No. 394309 that was provided by Isfahan University of Medical Sciences, Isfahan, Iran.

References

1. Pfaffer MA, Messer SA, Moet GJ, Jones RN, Castanheira M. *Candida* bloodstream infections: comparison of species distribution and resistance to echinocandins and azole antifungal agents in Intensive Care Unit (ICU) and non-ICU settings in the SENTRY Antimicrobial Surveillance Program (2008–2009). Intern J Antimicrob Agents. 2011; 38(1):65-9.

2. Pfaffer MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev. 2007; 20(1):133-63.

3. Ruan SY, Chu CC, Hsu PR. In vitro susceptibilities of invasive isolates of *Candida* species: rapid increase in rates of fluconazole susceptible-dose dependent *Candida glabrata* isolates. Antimicrob Agents Chemother. 2008; 52(8):2919-22.

4. Fidel PL Jr, Vazquez JA, Sobel JD. Candida glabrata: review of epidemiology, pathogenesis, and clinical disease with comparison to *C. albicans*. Clin Microbiol Rev. 1999; 12(1):80-96.

5. Monk CE, Huvvagner G, Arthur JS. Regulation of miRNA transcription in macrophages in response to *Candida albicans*. PloS One. 2010; 5(10):e13669.

6. Denti AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the Microprocessor complex. Nature. 2004; 432(7014):2313-5.

7. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, et al. The Microprocessor complex mediates the genesis of microRNAs. Nature. 2004; 432(7014):235-40.

8. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-κB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci U S A. 2006; 103(33):12481-6.

9. Reed DM, Gow NA, Brown GD. Pattern recognition: recent insights from Dectin-1. Curr Opin Immunol. 2009; 21(1):30-7.

10. Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006; 124(4):783-801.

11. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010; 140(6):805-20.

12. Li Q, Verma IM. NF-κB regulation in the immune system. Nat Rev Immunol. 2002; 2(10):725-34.

13. Gilmore TD. Introduction to NF-κB: players, pathways, perspectives. Oncogene. 2006; 25(51):6680-4.

14. Hayden MS, Ghosh S. Shared principles in NF-κB signaling. Cell. 2008; 132(3):344-62.

15. Vaz C, Mer AS, Bhattacharya A, Ramaswamy R. MicroRNAs modulate the dynamics of the NF-κB signaling pathway. PloS One. 2011; 6(11):e27774.

16. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116(2):281-97.

17. Bartel DP, Chen CZ. Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat Rev Genet. 2004; 5(5):396-400.

18. Lindsay MA. microRNAs and the immune response. Trends Immunol. 2008; 29(7):343-51.

19. Kasinski AL, Slack FJ. Potential microRNA therapies targeting Ras, NFκB and p53 signaling. Curr Opin Molecul Therap. 2010; 12(2):147-57.

20. Motsch N, Pfuhl T, Mrazek J, Barth S, Grässer FA. Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) induces the expression of the cellular microRNA miR-146a. RNA Biol. 2007; 4(3):131-7.

21. Cameron JE, Yin Q, Fewell C, Lacey M, McBride J, Wang X, et al. Epstein-Barr virus latent membrane protein 1 induces cellular MicroRNA miR-146a, a modulator of lymphocyte signaling pathways. J Virol. 2008; 82(4):1946-58.

22. Seider K, Brunke S, Schild L, Jablonowski N, Wilson D, Majer O, et al. The facultative intracellular pathogen *Candida glabrata* subverts macrophage cytokine production and phagolysosome maturation. J Immunol. 2011; 187(6):3072-86.