The Impact of MicroRNA Gene Polymorphisms on Immune Response to Diphtheria Vaccine in Iraqi Children and Adolescents

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Abstract

Background: Since its invention in the first quarter of the last century, diphtheria toxoid was successfully used for vaccination against diphtheria. However, sporadic case and even an epidemic have been recorded in many parts of the world, suggesting the presence of genetic factors influencing the immune response to this vaccine.

Aim: To assess the role of three single nucleotide polymorphisms(miRNA-146a G> C, miRNA-49 T> G and miRNA-196a2 C> T) in miRNA genes in immune response to diph- theria vaccine.

Subjects and Methods: This study recruited 68 children and adolescents who received three doses of diphtheria toxoid. DNA was isolated from the blood lymphocytes and miRNA- 146a,miRNA-149 and miRNA-196a2 genes were amplified, and then directly sequenced. Serum levels of anti-diphtheria toxoids IgG antibodies were determined by enzyme linked immunosorbent assay, and accordingly the immune response was categorized into good, moderate and low immunity .The association of different genotypes and alleles with immune status was illustrated statistically.

Results: Good immune response to diphtheria vaccine was significantly associated with the C allele of the polymorphism miRNA-146a G> C. Out of 14 carriers of GG geno- type,11(78.57%)showed good immunity compared to only 48.15% of GG carriers who had such immunity (P=0.039).The mutant allele of miR-149 T> G was more prevalent than wild type allele G among subjects with good immunity .However, the difference was insignificant
.Genotypes and allele frequencies of the SNP miR-196a2 C> T were very close in different immunological statuses.

Conclusion: The results of the current study suggest the significant role of miRNA-146a G> C gene polymorphism and the insignificant role of both miRNA-149 T>G and miRNA- 196a2 C>T gene polymorphisms in response to diphtheria toxoids vaccine. More studies are required to find out the associations of different polymorphisms in miRNA genes with immune response for different vaccines.

Keywords: miRNA gene, single nucleotide polymorphism, diphtheria toxoid vaccine

1. Introduction

Diphtheria is a bacterial disease caused by Corynebacterium diphtheria. Toxogenic C. diphtheria produces exotoxin which is absorbed by the mucous membrane and causes destruction of epithelium with a superficial inflammatory response [1]. Before vaccine era, diphtheria was widely spread among children. The invention of diphtheria –tetanus- pertussis(DTP) vaccine significantly reduced and even eliminated the disease in almost all developed and developing countries. However, an epidemic had emerged in the formal Soviet Union at the end of 1980s, followed by many cases of the disease in different parts of Europe [2] . This raises the question whether the immunity induced by the vaccination regime is sufficient to

protect the individual against the disease, or if there are some other factors influence this immunity. In fact, individual variation in immune response after vaccination with DTP has been observed in many studie [3] [4] [5] .

MicroRNAs are groups of very short non-coding RNAs which have many important physiological and pathological activities [6] [7]. Such activities are mainly attributed to post transcriptional modulation exerted by miRNA for multiple gen [8]. In respect to im- mune system, specific miRNAs are involved in the regulation of both innate and adaptive arms through controlling the development of immune cell progenitors and the differentiation and functions of mature cells. Thus, it is not surprising to find certain SNPs in miRNA genes having significant influence on the immune response and antibody titer to human vac- cines [9][10]. Not only do SNPs occur at miRNA genes, but also in pri-miRNA, pre-RNA and mature miRNA sequences [11]. Affected miRNA may exert an alteration in expression and/or maturation with subsequent influence on immune system, depending on the involved miRNA [12].

Although a huge number of studies investigated the role of miRNA gene polymorphisms in different pathologies, very few studies have addressed the association of such polymor- phisms with the response to human vaccines. Thus, this study aimed to assess the role of three polymorphisms in miRNA-146a, miRNA-149 and miRNA-196a2 in response to diphtheria vaccine among a sample of Iraqi children and adolescences. To the best of our knowledge, this is the first published study to evaluate the association of immune response to diphtheria vaccine with miRNA gene polymorphisms.

2. Subjects and Methods

The Study Population

A total of 68 children and adolescents (age range 8-13 years, 37 male and 31 female) who were attending Al-Zahraa' Center for primary health care/ Baghdad during the period from November 2015 to April 2016 were recruited for this study. All subjects have received three doses of diphtheria-tetanus-pertussis (DTP) vaccine according to the center records.

Blood Samples, DNA Extraction, Gene Amplification and Genotyping from each subject, 5 mL of venous blood samples were obtained, 3 mL of which was kept in plain tube where the serum was isolated, while 2 mL in EDTA-tube for DNA isolation. DNA was isolated from lymphocyte using a ready kit (Favor prep DNA extraction mini

kit/ Favor Gene Biotechnologies/ Taiwan) following manufacturer's instructions. Three SNPs (rs2910164 in pre-miRNA146a , rs11614913 in pre-miRNA-196a2 and rs71428440 in pri-miRNA-149) were selected for this study. These SNPs are considered as candidates for miRNAs involved in antigen recognition or immune response activation [13]. The primers and PCR conditions are reported elsewhere [14]. PCR products were directly sequenced and analyzed by Chromas pro software.

Anti-Diphtheria Antibody Titer

Immunoglobulin G antibodies for diphtheria toxoids were measured using enzyme linked immune-sorbent assay (ELISA, Diphtheria IgG ELISA kit, My Biosource/ China) according to manufacturer's instructions. Briefly, serum samples or standards are pipetted into the wells of the microtiter plate. A binding between the IgG antibodies of the serum and the antigen takes place. After incubation at room temperature, the plate was rinsed with washing solution to remove the bound material. Anti-human-IgG peroxidase conjugate was added and incubated for 30 minutes, followed by washing and then addition of TMB solution and incubated for 20 minutes. Stop solution was added, and the resulted color was measured spectrophotometrically at wave length 450 nm. A standard curve was plotted between five standard concentrations and their optical density (figure 1). From this curve, the concentration of each sample was calculated in relation to its optical density.

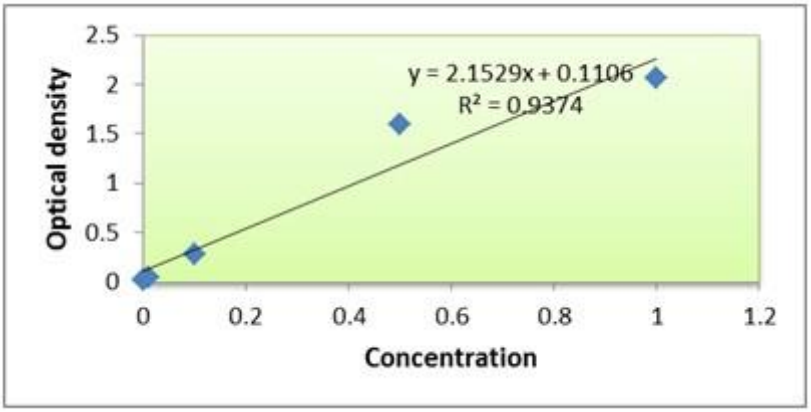

Figure 1: Standard curve foranti-diphtheria toxoid IgG antibodies

The result of each sample was assessed according to the manufacturer's instructions as follows:

<0.01 IU/mL: Low immunity
0.01 – 0.1 IU/mL: Moderate immunity
> 0.1 IU/mL: Good immunity

3. Statistical Analysis

Prism GraphPad software version 4.0 was used for statistical analysis. Genotypes and allele frequencies were directly counted and compared between each two groups by Pearson Chi square. Continuous variables were expressed as mean ± standard deviation (SD). Differences were considered statistically significant at $P<0.05$.

4. Results

Antibody Concentration in Different Ages and Genders

Table 1 shows the immunity against diphtheria vaccine in different ages and genders of the study population. Overall, there were no significant differences neither between age groups nor between genders. Although age group 8-10 year had a higher percentage of subjects with better immunity (65.51%) than the age group 11-13 years (46.15%), the difference was insignificant (P=0.090). Similarly, males showed an insignificantly higher percentage of subjects with better immunity (56.77%) than female (51.61%).

Table 1: The immunity against diphtheria vaccine in different ages and genders of the study population

Categories	Good immunity	Moderate immunity	Low immunity
Age			
8-10 years (29 subjects)	19(65.51%)	7(24.14%)	3(10.34%)
11-13 years (39 subjects)	18(46.15%)	15(38.46%)	6(15.38%)
P- value	0.090	0.162	0.409
Gender			
Male (37 subjects)	21(56.77%)	11(29.73%)	5(13.51%)
Female (31 subjects)	16(51.61%)	11(35.48%)	4(12.9%)
P-value	0.429	0.402	0.614

Association between Different Genotypes and Immunity

Both miRNA-146a G>C (146 bp)and miRNA-149 T>G (250bp) appeared in two geno- types (figures 2 and 3), while miRNA-196a2 C>T (150bp) had three genotypes (figure 4).

Table 3 shows the frequency of different genotypes and alleles of the three SNPs in association with different immune response. The heterozygous GC genotype of the SNP miRNA-146a G>C is more frequent in subjects with better immunity than homozygous GG genotype with significant difference (P=0.039). At allele level, the frequency of C allele is significantly higher than G allele in those subjects (P=0.049).

On the other hand, different genotypes and alleles of both miRNA-149 T>G and miRNA- 196a2 C>T polymorphisms did not show any significant association with immunity to diph- theria toxoids vaccine, although there are many variations in the percentage of these geno- types and alleles among different levels of immune response (table 3).

Table 2: Genotypes and allele frequencies of miRNA-146a G>C, miRNA-149 T>G and miRNA- 196a2 C>T polymorphisms and their associated with different levels ofimmune response to diphtheria toxoids vaccine

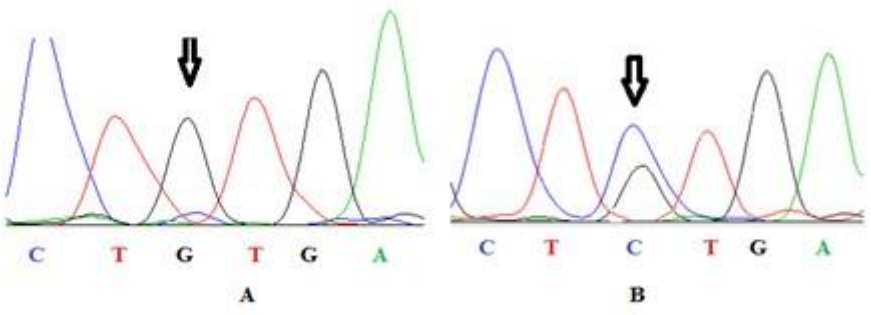

Figure 2: Different genotypesof the SNP rs2910164 (miRNA-146a G>C).A: Homozygous wild type GG, B: heterozygous genotype CG.

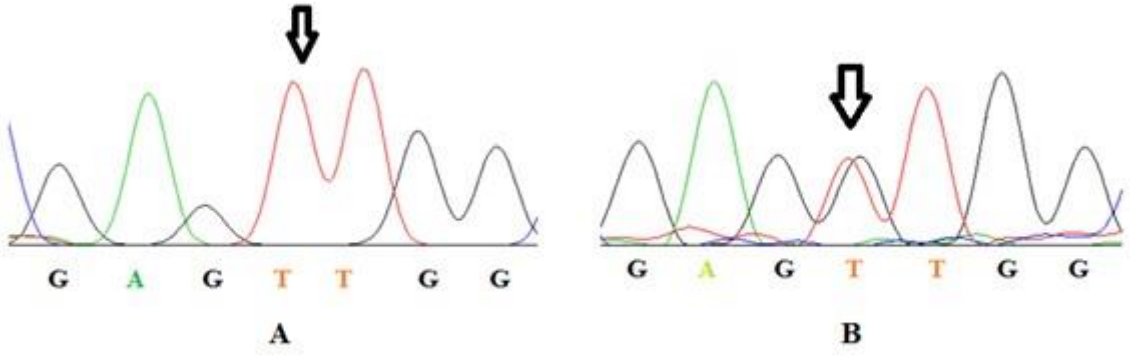

Figure 3: Different genotypesof the SNP rs71428440 (miRNA-149 T>G).A: Homozygous wild type TT, B: heterozygous genotype GT.

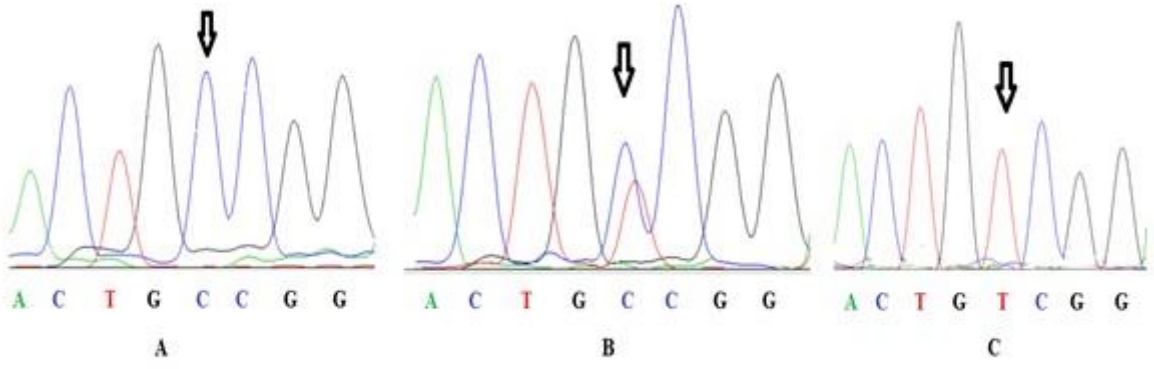

Figure 4: Different genotypesof the SNP rs11614913 (miRNA-196a2C>T). A: Homozygous wild type CC, B: heterozygous genotype CT, C: homozygousmutant genotype TT

Table 2: Genotypes and allele frequencies of miRNA-146a G>C, miRNA-149 T>G and miRNA- 196a2 C>T polymorphisms and their associated with different levels of immune response to diphtheria toxoids vaccine

Polymorphisms	Good immunity (37)	Moderate immunity (22)	Low immunity (9)
MiR-146a G>C			
Genotypes			
GG (54 subjects)	26(48.15%)	20(37.04%)	8(14.81%)
GC (14 subjects)	11(78.57%)	2(14.29%)	1(7.14%)
P-value	0.039	0.093	0.403
Alleles			
G (122 frequency)	63(51.64%)	42(34.43%)	17(13.93%)
C (14 frequency)	11(78.57%)	2(14.29%)	1(7.14%)
P-value	0.049	0.107	0.417
MiR-149 T>G			
Genotypes			
TT (46 subjects)	22(47.83%)	19(41.3%)	5(10.87%)
TG (22 subjects)	15(68.18%)	5(22.73%)	2(9.09%)
P-value	0.93	0.108	0.513
Alleles			
T (114 frequency)	59(51.75%)	43(37.72%)	12(8.33%)
G (22 frequency)	15(68.18%)	5(22.73%)	2(9.09%)
P-value	0.118	0.134	0.598
MiR-196a2 C>T			
Genotypes			
CC (21 subjects)	12(57.14%)	7(33.33%)	2(9.52%)
CT (28 subjects)	15(53.57%)	9(32.14%)	4(14.29%)
TT (19 subjects)	10(52.63%)	6(31.58%)	3(15.79%)
P-value	0.517(CC vs CT)	0.585(CC vs CT)	0.482(CC vs CT)
	0.512(CC vs TT)	0.587(CC vs TT)	0.451(CC vs TT)
	0.592(CT vs TT)	0.612(CT vs TT)	0.600(CT vs TT)
Alleles			
C (70 frequency)	39(55.71%)	23(32.86%)	8(11.43%)
T (66 frequency)	35(53.03%)	21(31.82%)	10(15.15%)
P-value	0.444	0.522	0.349

5. Discussion

he only SNP which was found to have significant association with antibody response to diphtheria vaccine was miRNA-146a G>C (rs2910164). This result is in accordance with that obtained by Xiong et al. [15] who found significant effect of this SNP on the humoral immune response to hepatitis B vaccine in Chinese population. Furthermore, the current result agrees with many previous studies in that the SNP miRNA-146a G>C has a significant effect on the innate and adaptive immune response. For example, [16] showed a significant association of this polymorphism with the susceptibility to severe sepsis in the Chinese population, while [17] recorded similar association with the susceptibility to leprosy among Brazilians.

Among the many significant roles of miRNA-146a is its role as a negative regulator of the immune response [18] . There is almost a general agreement that miRNA-146a acts mainly on two adaptor molecules which are Interleukin-1 receptor-associated kinase 1(IRAK-1) and Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6) through 3 prime un- translated region (3'UTR) causing down-regulation of expression of these molecules [19].

As these molecules are very important as signaling molecules to activate both

innate and adaptive immune response, high levels of miRNA-146a exert a negative regulatory effect on the immune system. Many experimental studies supported this role. Researches on miRNA- 146a-deficient animals revealed an increase in both IRAK1 and TRAF6 accompanied by an increase in the percentage of IFN-γ producing T-cell subset. Moreover, decreased levels of miRNA-146a have resulted in increased number, but an impaired function of T-regulatory cells (Treg) with massive lymphocyte activation [20] [16].

Polymorphisms in miRNA genes were found to have a great influence on biogenesis and maturation as well as on the gain or loss of function of miRNA [21]. The SNP rs2910164 is located in the stem region of pre-miRNA-146a and the presence of mutant allele (allele C) affects the integrity of the stem loop and the processing of precursor miR-146a to mature one [22]. Practically, C allele was found to be associated with a decrease the mature miRNA- 146a production and unmasking the expression of both TRAF6 and IRAK-1 [23] [24] . Thus, robust immune response is expected with the presence of this allele and that explains the association of this polymorphism with several autoimmune diseases [25].

The immune response to diphtheria toxoids is of humoral type represented by IgG an- tibodies. The current results imply that C allele carrier has higher IgG titer than those carrying G allele. As mentioned above, C allele associates with increased gene expression of both IRAK-1 and TRAF6. Although these two adaptor molecules are primarily influenced innate immune response through toll-like receptors (TLRs), there are strong evidences that they have significant roles in antibody production [26] showed that TRAF6 is essential for B cell development and function. Among the most important findings of those authors is that signaling of TRAF6 was very important for the generation and maintenance of B cell pool. Furthermore, TRAF6 was necessary for CD40 function in B cell during T-cell dependent humoral immune response.

Although less prominent, IRAK-1 also indirectly influence antibody production through TLRs carried on B cells. Upon ligand engagement, the transmemebrane and cytoplasmic interleukin receptor (TIR) domain of TLR interact with myeloid differentiation protein 88 (MyD88). This interaction recruits IRAK-4 which stimulates the phosphorylation of IRAK- 1, a step that is necessary for recruitment and activation of TRAF6 [27] .

Contrary to miRNA-146a G>C, both miRNA-149 C>T and miRNA-196a2 T>G showed no association with humoral immune response to diphtheria toxoids vaccine. Despite some evidence for the role of miRNA-149 as a negative regulator for TLR-induced inflammatory response [28] , its role in adaptive immunity is not well documented, and the SNP miRNA- 149 was reported frequently to have no relevance with immune respons [15] [29] .

MiR-196a2 C>T has been reported to have a role in the pathogenesis of different patholo- gies such as colon-rectal cancer [30] and inflammatory bowel disease [31] . However, a large number of studies suggested a null association with many other diseases especially those related to immune response. [15] did not find any significant effect of this SNP in immune response to hepatitis B vaccine. Almost similar result was obtained by [29] in studying pul- monary tuberculosis in Chinese population. More recently, [32] reported a null association of this polymorphism with asthma phenotype, disease duration, and age of onset in Egyptian population.

These data strongly suggested the role of miRNA-146a G>C in the humoral response to diphtheria toxoid vaccine, although more studies with larger sample size are required for the more solid conclusion.

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الخلاصة

منذ ان تم اختراعه في الربع الاول من القرن الماضي استخدم ذيفان الدفتريا المقتول بنجاح في التلقيح ضد مرض الخناق ومع ذلك فقد سجلت حالات فردية بل وحتى اوبئة في اجزاء عديدة من العالم من ما يشير الى وجود عوامل وراثية توثر على الاستجابة المناعية لهذا اللقاح. هدفت الدراسة الحالية الى تقييم دور ثلاث تغايرات جينية (miRNA−146aG>C ، miRNA−149T>G ، miRNA−1962aC>T) في جين MicroRNA في الاستجابة المناعية ضد لقاح الدفتريا. استخدمت هذه الدراسة 68 طفلا ومراهقا ممن تلقوا ثلاث جرع من لقاح الدفتريا . تم عزل من خلايا ومضاعفة جينMicroRNA المناظر للتغايرات الجينية المطلوبة ، ومن ثم اجراء اختبار تتابع القواعد . حددت المستويات المصلية للاجسام المضادة بطريقة الادمصاص المناعي الانزيمي وعلى ضوء ذلك صنفت الاستجابة المناعية الى جيدة , متوسطة او منخفضة ، وتم تحديد علاقة الانماط الجينية والاليلات المختلفة بمستوى المناعة باستخدام الطرائق الاحصائية . ارتبطت المناعة الجيدة ضد لقاح الدفتريا معنويا بالاليل C للتغاير الجيني MicroRNA , ومن بين 14 فردا يحملون النمط الجينيCG اظهر 11 (78.57%) استجابة مناعية جيدة مقارنة بـ 48.15% فقط مما يحملون النمط الجيني GG (P=0.039) وكان الاليل الطافر(G) للتغاير الجيني miRNA−149T>G اكثر اتشارا من الاليل T بين الافراد ذوي المناعة الجيني الا ان الفرق لم يكن معنويا . اما توزيع الانماط الجينية والاليلات للتغاير فقد كان متقاربا جدا في الحالات المناعية المختلفة .
تشير نتائج الدراسة الحالية إلى دور مهم لتغاير جين miRNA−146aG>C ودور غير مهم لكلا من تغايرات جين miRNA−149 T>G و miRNA−196a2 C>T في الاستجابة المناعية للقاح الدفثريا , ويجب إجراء المزيد من الدراسات للكشف عن علاقة التغايرات الجينية المختلفة في جيناتMicroRNA مع الاستجابة المناعية للقاحات المختلفة.

الكلمات المفتاحية : جين MicroRNA, التغايرات الجينية ، ذيفان البكتريا المقتول