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IMMUNOLOGICAL AND MOLECULAR STUDY OF INTERLEUKIN 10 (IL 10) IN PATIENTS WITH RECURRENT APHTHOUS STOMATITIS

Eman Abbas Aboud*[1], Baha Hamdi Hakim Al Amiedi[2], Suha Abdulhussein Hindy[3] and Haydar O. Hashim[4]

[1,2]Department of Microbiology, Faculty of Dentistry, Babylon University, Iraq.
[3]Department of Oral Pathology , Faculty of Dentistry, Babylon University, Iraq.
[4]Laboratory Clinical Sciences, Faculty of Pharmacy, Babylon University, Iraq.
*e-mail : emanabbasaboud@gmail.com

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ABSTRACT : Recurrent aphthous stomatitis is the most common inflammatory ulcerative condition of the oral mucosa. Interleukin-10 *(IL-10)* also known as cytokine synthesis inhibitory factor, *IL-10* was demonstrated to be an important cytokine-suppressing autoimmunity and inflammatory response. The study aimed to investigate the association between the Interleukin-10 (*IL-10)* gene polymorphism and its salivary level with recurrent aphthous stomatitis in Babylon province. The total subjects of the present study is 94, divided into 2 groups; 45subjects with recurrent aphthous stomatitis and 49 subjects healthy controls, Un-stimulated salivary sample was taken from each subject and genotyped for interleukin-10 polymorphism (rs 1518111) by sequencing after SSCP technique. On the other hand, the level of interleukin-10 (IL-10) in saliva was estimated by the ELISA technique. In this study, the result show that the allele T had a significant association with recurrent aphthous stomatitis (OR.1.8, PV.0.3) and inherited as a recessive allele in which TT genotype has OR of 3 compared to other genotypes. On the other hand, the salivary interleukin-10 level did not record any significant difference between the case and control group. The results suggest that Interleukin-10 (*IL-10*) rs1518111 gene polymorphism was a putative risk factor for recurrent aphthous stomatitis.

Key words : Recurrent aphthous stomatitis, interleukin-10 (*IL-10*) rs1518111.

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INTRODUCTION

Recurrent aphthous stomatitis is the most common inflammatory ulcerative condition of the oral mucosa. The lesions are localized, painful, shallow ulcer typically on nonkeratinized or poorly keratinized mucosa, often covered by a gray fibromembranous slough and surrounded by erythematous halo sites of predilection include the ventral surface of the tongue, the floor of the mouth, and buccal, labial, soft palatal and oropharyngeal mucosa (Scully *et al*, 2000). The lesion typically onset in early age with three main clinical types: Minor aphthous stomatitis, Major aphthous stomatitis, and Herpetic form aphthous stomatitis (Scully *et al*, 2000).

The genetic risk factors which may determine the individual susceptibility to recurrent aphthous stomatitis include various DNA polymorphisms distributed in the human genome. Special attention should be paid to the alterations in the metabolism of cytokines, which include: interleukins (IL-1â, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12), interferon ã (IFN-ã) and tumor necrosis factor-á (TNF-á), serotonin transporter gene and endothelial nitric oxide synthase gene (Œlebioda *et al*, 2013).

Cytokines are soluble proteins that bind to specific receptors on target cells and initiate intracellular signaling cascades resulting in phenotypic changes in the cell via altered gene regulation. Cytokines play a major role in various biological activities such as differentiation proliferation, regeneration, development, repair inflammation and homeostasis they play a fundamental role in inflammation including periodontal disease. (Hassan *et al*, 2019; Mohammed *et al*, 2020; AL-Mumin *et al*, 2020). Anti-inflammatory cytokine is produced by T lymphocytes (mainly Th2 subsets), B lymphocytes, NK cells, mast cells, eosinophil's, dendritic cells, monocytes, and macrophages. IL-10 was demonstrated to be important cytokine-suppressing autoimmunity and inflammatory response (Hu *et al*, 2015). Interleukin *(IL-10)* has pleiotropic effects on immune regulation and

inflammation by down-regulating the expression of Th1 cytokines (Moore *et al*, 1993). Therefore, it could be a good candidate for oral tolerance (Najafi *et al*, 2014).

Accordingly, it is maybe possible to investigate the involvement of the genetic polymorphisms of the gene in these complications, taking into account the role of high-frequency SNPs in the onset of many metabolic dysfunctions (Mohammed *et al*, 2020).

This project aimed to study the association of Interleukin-10 (IL-10) rs1518111 gene polymorphism and its salivary level with Recurrent aphthous stomatitis in the Iraqi population.

MATERIALS AND METHODS

Sampling

The project ethics was approved by the ethics and sciences community of Dentistry College, Babylon University. A total of (94) subjects of both genders were enrolled in this study. The age range (10-50) years. Most of the subjects were from attendants to the Department of oral medicine/College of Dentistry, University of Babylon (Hillah city, Iraq) from December 2019 to April 2019. a questionnaire was designed to include: name, age, dental history, family history of recurrent aphthous stomatitis disease and history of systemic and chronic diseases.

Table 1 : Primers for Interleukin 10(*IL-10)* genotyping.

Primers	Sequence (5'-3')	Amplicon size bp
Interleukin-10 (*IL-10*)	F/GGCATCAAAAAGACCGCATT R/TAGCGATCCTCCTTCACCAGA	336 bp

The enrolled individuals were divided into two categories:

1. Control group (C): - included (49) subjects who had not any signs or family history of recurrent aphthous stomatitis.

2. Case group (P): - included (45) subjects who had been diagnosed with recurrent aphthous stomatitis, The determination of disease severity was based on classification on recurrent aphthous stomatitis (Vivek and Nair, 2011).

Exclusion Criteria

Each individual with the following criteria had been excluded from the study: diabetes mellitus, rheumatoid arthritis, cardiovascular diseases, hepatic diseases, kidney diseases, Smoker, pregnant or lactating woman, alcohol drinking, and whose undertaken Systemic antibiotic or anti-inflammatory therapy within the last one weeks.

Collection of salivary samples and DNA extraction

Five mL of participant saliva were collected in a clean sterilized plastic container and divided into two tubes, one for DNA and the other for ELISA measuring. The DNA was extracted from the saliva according (Quinque *et al*, 2006).

Genotyping and PCR Amplification of Interleukin-10 (IL-10)

The Genotyping technique was selected according to Hashim and Al-Shuhaib (2019). A pair of specific primers were designed with the aid of NCBI primer blast online software according to the protocol described by Hashim *et al* (2015). The primer amplifies 336 bp DNA fragment around the rs1518111 polymorphism (Table 1). For each PCR reaction 8 µl of 2.5X master mix (Cyntol, Russia), 1 µl of each primer (Macrogen, Korea), 2 µl of extracted DNA and 8 µl of molecular grade water (Promega, USA). The PCR thermo cycling conditions were optimized by gradient annealing temperature (analytic Jena, Germany), the optimized thermo cycling conditions were: initial denaturation at 95Cº for 5 min flowed by (35 cycles of 95Cº for 30 sec, 60 Cº for 30 sec, 72 Cº for 30 sec) then a final elongation at 72 Cº for 5 min. the PCR product was resolved on 2% agarose gel (Fig. 1).

Single-strand conformation polymorphism (SSCP) and sequencing

SSCP was performed according to Imran *et al* (2020) and the gels were stained by silver nitrate according to the procedure described by Byun *et al* (2009). Three different patterns were detected by using the SSCP technique (Fig. 2). Two random samples from each pattern were subjected to direct sequencing according to provider protocol (Microgene, South Korea).

The sequencing reactions indicated the exact identity of this genetic fragment after performing NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The results of sequence analysis indicate that each SSCP pattern belong to a specific rs1518111 genotype (Fig. 3).

Salivary Interleukin 10(IL-10) concentration measurement

The salivary Interleukin 10 (IL-10) concentration was determined by Human IL-10 ELISA Kit (ab100549) according to the manufacturer's instructions (Abcam, USA).

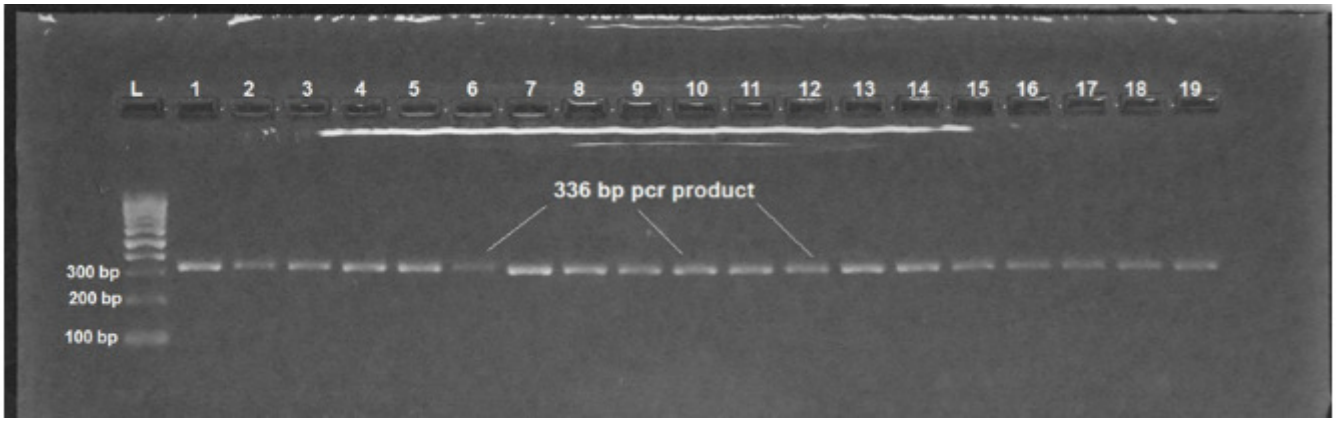

Fig. 1 : Agarose Electrophoresis of PCR amplicon (336 bp).

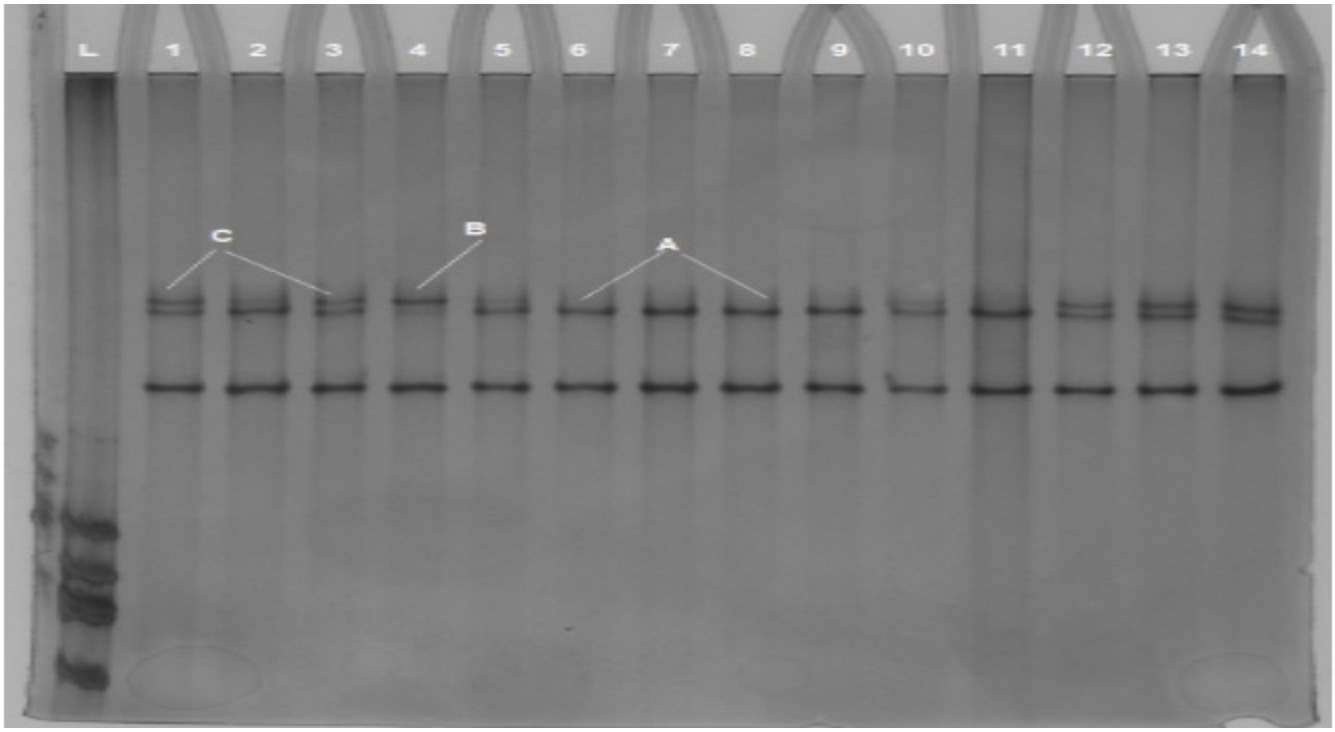

Fig. 2 : show the SSCP pattern of IL-10 amplicon, the corresponding genotype of each pattern as the following (lanes 1,2,3, 12,13 and 14 TC genotype: lanes 4 TT genotype : lanes 5,6,7,8,9,10 and 11 CC genotype).

RESULTS

The association of Interleukin-10 (*IL-10*) rs 1518111 Gene polymorphism with Recurrent Aphthous Stomatitis

An allelic frequency of Interleukin-10 *(IL-10)* rs1518111 gene polymorphism for case and control groups are listed in Table 2. The results showed that there was a significant allelic frequency difference between the case and control groups. The T allele represents a risk allele with an odds ratio of 1.882 (C.I. 1.049-3.376) (p-value 0.033).

Table 3 represent the Hardy-Weinberg equilibrium, the fisher's exact test shows that the control group follows Hardy-Weinberg equilibrium, while the case group has a significant deviation.

The association of each genotype with RAS disease was further tested under different models of inheritance. The result shows that the T allele represents a recessive

Table 2 : show the Allelic association of Interleukin 10 (rs1518111) gene polymorphism with R.A.S.

	Control		**Case**			
Allele	**Count**	**Proportion**	**Count**	**Proportion**		
C	63	0.64	44	0.49	**0.531 (0.296-0.953)**	**0.033**
T	35	0.36	46	0.51	**1.882(1.049-3.376)**	

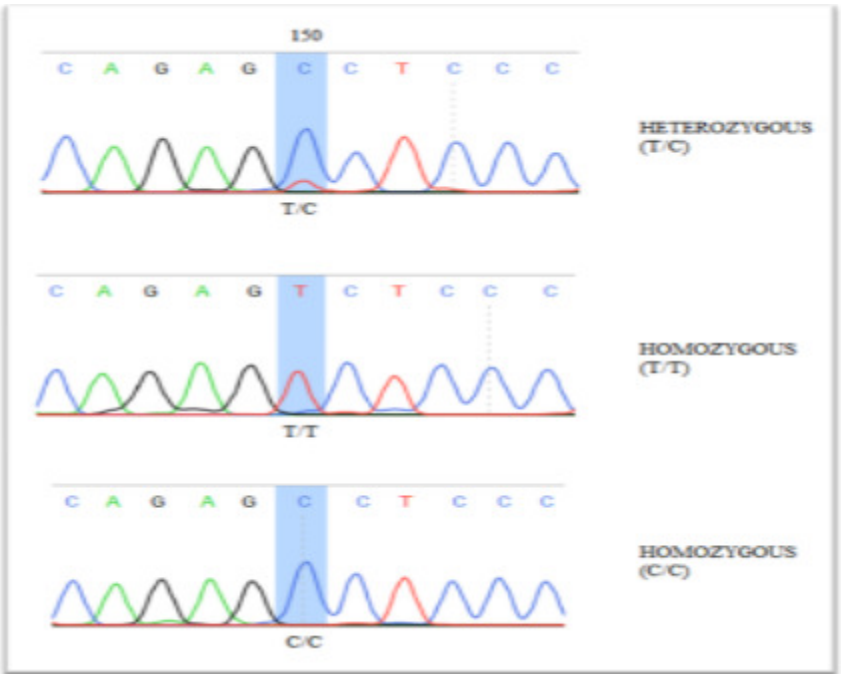

Fig. 3 : Sanger's sequence chromatogram traces of different SSCP patterns.

Table 3 : Hardy-Weinberg equilibrium association of interleukin-10 gene polymorphism (rs 1518111).

	CC	TC	TT	C	T	P-value
All subjects	35	37	22	107	81	0.059
Control	21	21	7	63	35	0.76
Case	14	16	15	44	46	**0.072**

Table 4 : The association of each genotype with RAS.

Model	Genotype	Control	Case	OR (95% CI)	P-value
Codominant	C/C	21 (42.9%)	14 (31.1%)	1.00	0.087
	T/C	21 (42.9%)	16 (35.6%)	1.14 (0.45-2.92)	
	T/T	7 (14.3%)	15 (33.3%)	**3.21 (1.05-9.89)**	
Dominant	C/C	21 (42.9%)	14 (31.1%)	1.00	0.24
	T/C-T/T	28 (57.1%)	31 (68.9%)	1.66 (0.71-3.88)	
Recessive	C/C-T/C	42 (85.7%)	30 (66.7%)	1.00	**0.028**
	T/T	7 (14.3%)	15 (33.3%)	**3.00 (1.09-8.25)**	
Over dominant	C/C-T/T	28 (57.1%)	29 (64.4%)	1.00	0.47
	T/C	21 (42.9%)	16 (35.6%)	0.74 (0.32-1.69)	

Table 5 : Association of Interleukin-10 (*IL-10*) level in saliva with RAS.

	Group	N	Mean	Std. deviation	T value	P-value
IL10	Control	49	46.7342	21.11319	-0.906	0.367
	Case	45	50.0375	12.86993		

risk allele, individuals with TT genotype have an odds ratio of 3 to develop the RAS disease (Table 4).

On the other hand, the salivary level of Interleukin-10 did not record any significant difference between cases and the control group (Table 5).

DISCUSSION

The etiology and pathogenesis of RAS remain unclear. Multiple factors are associated with the development of this disease, including positive family history, food hypersensitivity, smoking, psychological stress and immune disturbance (Rivera, 2019).

A family history of ulcers is found in approximately 40 % of patients and the highest incidence is found in siblings of parents both of whom have RAS, Identical twins show a 90% concordance, implicating a genetic component. Several recent studies have examined polymorphisms of individual genes in factors thought to be associated with the pathogenesis of RAS (Challacombe *et al*, 2015).

Few studies documented the relationship between RAS and interleukin-10 gene polymorphisms. a study conducted by Najafi *et al* (2014) on the Iranian population

Which include (140) healthy individuals and (64) patients with RAS. The study found a significant association of interleukin-10 (IL-10) gene polymorphisms at positions 1082 (C/A),-819(C/T), and -592(C/A) with RAS (Najafi et al., 2014).

The same results were also revealed among the Asian population. A study by Yang *et al* (2017), which studied the association of interleukin-10 (IL-10) gene polymorphism with RAS, the study recruit (779) RAS patients and (1016) healthy control individuals, the study reveals a significant association of RAS with interleukin-10 gene polymorphisms (Yang *et al*, 2017).

Our results also reveal that the salivary concentration of interleukin-10 (IL-10) did not has a significant difference between cases and control. These results agree with several other studies in different populations. A study conducted in the united states concluded that the Interleukin-10 gene expression did not differ between cases and control groups (Huff *et al*, 1998). On the other hand, several other studies indicate the significant association of interleukin-10 concentration with RAS. Furthermore, Farmaki et al found that the interleukin-10 (IL-10) level in saliva can be considered as the main immune response against RAS (Albanidou-Farmaki *et al*, 2007; Avci *et al*, 2014).

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