1. Introduction

Recurrent aphthous stomatitis (RAS), the most common oral mucosal illness, affects 5 to 25% of the general population [1]. Recurrent painful ulcers that are typically restricted to the oral mucosa without keratin are what RAS is known for. There are three clinical manifestations of RAS: minor, major and herpetiform. The most common type of RAS is the mild variant, which makes up about 85% of all cases. Clinically, it appears as a painful round or oval ulcer with a diameter of less than 1 cm and a gray-white pseudo-membrane with an erythematous halo [2, 3]. Normally, the lesions go away on their own in 7–10 days without leaving any scars. Despite the prevalence of the condition, no definite cause has been identified; thus, a number of risk factors have been proposed, including genetic predisposition, hematologic issues, immunological defects, vitamin deficiencies, mental stress, and local trauma [4, 5]. It has been demonstrated that the pathophysiology of RAS should be linked to bodily immunological issues. Multifunctional cytokines that mediate the immune response and are involved in the inflammatory response may contribute to the development of RAS ulcers [6]. The gene polymorphisms most likely increase a person's susceptibility to RAS by interfering with the metabolism of cytokines like interleukins (IL-1beta, IL-6 and IL-10) [7], tumor necrosis factor-alpha (TNF-alpha) [8] and interferon-gamma (IFN-gamma) [7]. Because of C and T transpositions at positions -511(C T) and +3954(C T) from the transcriptional site, in base pairs (bp), IL-1beta possesses two significant polymorphisms [9]. Numerous gene polymorphisms encoding proinflammatory cytokines, including ILs, TNF-alpha, and 5-HTTLPR, have been shown to contribute to the development of RAS in previous reviews [10].

In addition to environmental factors affecting vitamin D levels, genetic influence has also been questioned in various studies, with some examining whether single nucleotide polymorphisms (SNPs) in vitamin D-related genes [11]. Additional data indicates that several innate immunity-related cytokines may be directly regulated by vitamin D. In co-cultures of infected macrophages, vitamin D promotes the expression of IL-1-beta and IL-8, IL-1, tumor necrosis factor, and interferon (IFN) are examples of other proinflammatory cytokines that are downregulated in infected human peripheral blood mononuclear cells. These findings demonstrate the importance of vitamin D in boosting a rapid response to infection and modifying the acute inflammatory response [12]. Many single-nucleotide polymorphisms (SNPs) in different genes interact with the environment to induce a changed B12 status, according
to current genetic research on vitamin B12 status. The majority of SNPs associated with vitamin B12 status have been evaluated using a candidate gene strategy [13]. Zinc’s capacity to boost the production of IL-1, IL-6, and TNF-in isolated monocytes and peripheral blood mononuclear cells may help to explain how zinc deficiency can act as a modulator of RAS development. In people with zinc deficiency, the synthesis of cytokines is disrupted; low serum zinc levels are associated with a reduction in the production of Th1-type cytokines. Local tissue damage occurs early in the development of aphtha due to an improperly triggered cascade of cytokines [14].

The objective of the current study was to look into any potential connections between RAS patients and IL-1β 542 T>A genetic variants.

2. Materials and Methods

2.1. Study sample

In total, 90 consecutive samples—60 patients with mild RAS and 30 controls—were gathered for the investigation. Patients were enrolled in the oral diagnosis department at Hawler Medical University/College of Dentistry with the same topographical region. Volunteers in the control group had no history of RAS or other systemic illnesses. The population of Erbil City, 16 to 50 years old, with a clinical diagnosis of RAS, were the inclusion criteria. No family history of RAS or suspicion of RAS, as well as the population of Erbil city, were inclusion criteria for controls. Systemic diseases (including HIV infection, Behcet’s disease, systemic lupus erythematosus, Celiac disease, Sweet’s syndrome, inflammatory bowel disease, Reiter syndrome, and PFAPA syndrome), exposure to radiation and drug use, oral cavity tumors, and pregnancy were the exclusion criteria for both cases and controls. The study was authorized by the University Ethics Committee and consent was acquired from all the patients. Five milliliters (ml) of venous blood were collected in (EDTA) and gel tubes and stored at -20°C in the refrigerator for biochemical tests and molecular analysis.

2.2. Biochemical Tests

An automated analyzer HITACHI Cobas C311 and E411 were used to ascertain (Vitamin D, Vitamin B12 and Zinc).

2.3. Molecular analysis

2.3.1. Genomic DNA extraction

Using the ADDBIO DNA blood kit and the supplied instructions, DNA was extracted from the blood. The concentration and purity of the extracted DNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Also, A260/A280 and A260/A230 ratios were recorded. Agarose gel electrophoresis was done to assess DNA intactness and average size for each sample.

2.3.2. Single nucleotide polymorphism (SNP)

Based on earlier genome-wide association studies (GWAS) and meta-analysis studies linked to RAS, the genotyping SNP employed in the study was chosen. TaqMan SNP Genotyping assays were used to genotype a subset of SNPs. Polymerase chain reaction (PCR) primers were created, and 96-well microplate reactions were carried out using an Applied Biosystems SeqStudio™ Genetic Analyzer System sequencing machine. RAS genotyping uses the SNP ID number and assay number.

2.3.3. Polymerase chain reaction (PCR) for genotyping

PCR was carried out in a total volume of 25 µL which contains 2 µL DNA (50 ng), buffer (50 mM KCl, 10 mM Tris–HCl pH 8.4, 0.1% Triton X-100), 1.5 mM MgCl₂, 200 µM dNTPs, Taq DNA polymerase (Promega, USA) and primers (10 pmol/µL). Amplification was completed using primers Forward Primer: (GAGGGCTGGCTTC-TGATT), Reverse primer: (TATCTGTCCCTGGAG-GTGG), Product size: 205 bp Amplicon Tm: 85.3°C, Exon 5: Exon length = 165bp, Product start: 51bp before the start of the exon. Exon excluded: 12bp at the end of exon. Amplification conditions were 94°C for 3 min (Initial denaturation) followed by 35 cycles at 94°C for 30 sec (Denaturation), 57°C for 30 sec (Annealing) and 72°C for 30 sec (Extension). The run was terminated by a final extension at 72°C for 5 min. 2% agarose gel with ethidium bromide DNA Gel Stain was used to resolve the final product. A total of 5 µL PCR product, positive control, negative control and 100 bp ladder were added to 3 µl loading dye (Bromophenol blue). Bands might be seen with the Gel Doc XR + method (Bio-Rad, USA).

2.3.4. Cycle sequencing

Cycle sequencing was carried out in accordance with the manufacturer’s instructions using Thermo Fischer’s brilliant Dye direct cycle sequencing kit. For each 10 µl forward or reverse reaction, 1 µL DNA (4 ng/µL), 1.5 µL of PCR primer mix (0.8 µM each primer), 5.0 µL Bright Dye direct PCR master mix 2.5 µL and deionized water was added. A further cycle sequencing step was completed. Agar gel electrophoresis indicated that the minimum amount of PCR product needed for sequencing was 20 ng. For each 3 µL total reaction volume, 2 µL Bright Dye direct sequencing master mix and 1 µL Bright Dye with forward or reverse primer were used. 3 µL of the sequencing reaction mix was added to the appropriate well in the corresponding forward or reverse reaction plate after the reaction mixture was produced on ice. The reaction plate was briefly spun after being sealed with adhesive film or caps. The reactions were run in a thermal cycler. The final sequencing product was purified using the Bright Dye purification kit according to the manufacturer’s instructions. The final reaction plate was loaded into a 96-standard well in sequencer Applied Biosystems SeqStudio™ Genetic Analyzer System sequencing machine (Applied Biosystems). The acquired sequences were put through a BLAST search to find related genes in GenBank.

2.4. Statistical analysis

The chi-squared test, Kruskal-Wallis and One-Way ANOVA tests were used to assess the statistical significance of differences in allele and genotype distributions between case and control groups in order to determine the influence of certain numerical parameters on gene polymorphisms in case and control groups. The results were considered statistically significant at least at p-value ≤ 0.05. The observed genotype frequencies were compared with those calculated from Hardy–Weinberg equilibrium. Data were entered into a computer and analyzed using the IBM® SPSS® statistics software package version 26.0 (International Business Machines Corp., Armonk, N.Y., USA).
3. Results
A total of 90 cases, including 40 men (44.44%) and 50 women (55.56%), were included in the present study. PCR methods were utilized to amplify the target amplicon with specified primers by using genomic DNA that had been isolated from blood samples taken from study participants. After being resolved in a 2% agarose gel, the amplicons revealed a distinct band with a size of 205 base pairs (Figure 1). In preparation for further use, amplicons were sliced and cleaned. These PCR results were subjected to direct DNA sequencing so that additional confirmation could be obtained. The BLAST search program was used to conduct an analysis of the sequenced segments that were then uploaded to the GenBank database (http://blast.ncbi.nlm.nih.gov).

The participants’ average age was 30.83±1.466 years (range 16–49 years). Among the participants, 60 (66.67%) people have been detected with the RAS diseases. Of the participants who had RAS disease, 27 were men (45% of the male participants), and 33 were women (55% of the female participants). Other demographic information is shown in Table 1. Multiple genetic models, including codominant, dominant, and recessive inheritance patterns, were used in the genotype analysis. The analyses of genotypes showed no significant difference in the target SNPs between groups in the analysis. No significant correlation was found for any inheritance model of SNP IL-1β 542T>A polymorphism with RAS diseases when compared with the control in both parameters such as sex (p-value=0.0023**).

Table 1 shows the genotype distribution of IL-1β 542T>A polymorphism in patients with RAS compared with the control (p-value=0.0113). The genotype distribution of IL-1β in the case group (TT: TA: AA, 8:9:1) differed statistically from those (31: 25: 5) expected from the Hardy–Weinberg equilibrium (p-value=0.002). Also, there was a significant difference in the occurrence of both A and T alleles between RAS patients and controls (p-value=0.0058**), as revealed in Table 2.

The distribution of IL-1β polymorphism genotype frequencies in patients with RAS and controls is displayed in Table 2. There was a higher frequency of the TA genotype in the RAS group than in the control (p-value=0.007**). Also, there was a significant difference in the changing of genotype in IL-1β 542T>A polymorphism.

Table 3. Impact of some variables on IL-1β 542T>A gene polymorphism in patients with recurrent aphthous stomatitis (RAS n = 18) and Healthy controls (n = 10).

Table 2. Genotype distribution of IL-1β 542T>A polymorphism in patients with Recurrent Aphthous Stomatitis (RAS n = 18) and control subjects (n = 10).

Table 3. Impact of some variables on IL-1β 542T>A gene polymorphism in patients with recurrent aphthous stomatitis (RAS n = 18) and Healthy controls (n = 10).
a moderately significant difference occurred when compared to the control (p-value=0.0461). The highest age was recorded in the TA genotype with a mean of 35.44±4.011 and the mean age of the control group was 26.5±2.934 (Figure 2A).

Another factor is vitamin D and B12, which reduce the concentration of both vitamins when compared between the RAS case group and the control group. As can be seen, the mean vitamin D concentration in the control group is 15.29±1.063, but in both genotypes TA and TT the average of vitamin D decreased significantly (9.236±1.312 and 12.94±0.705, respectively) (p-value=0.007) but in genotype AA there was no significant difference (15.6±0.00). On the other hand, vitamin B12 concentrations were similarly decreased, with average concentrations decreasing from (307.9±35.20) in the control group to (247.8±54.09, 243.5±31.5, and 230.0±0.00) in each of the genotypes (TA, TT and AA) respectively (Figure 2B and Figure 2C). This formed a meaningful difference in the genotypes of the interleukin gene (p-value=0.0258) (Table 3).

Decreased plasma zinc concentration is considered to be one of the factors affecting genotype polymorphisms in the IL-1β gene. As shown in Table 3, the average zinc concentration in the control group is (91.9±5.128) which is compared with the average in the plasma of the patients group whose average is (74.1±3.336, 70.0±0.00, and 65.75±1.532) respectively, a clear difference is observed between them (p-value=0.0031) (Figure 2D).

4. Discussion

Recurrent aphthous stomatitis can affect up to 7 out of 10 persons at some point in their lives. The most typical ulcerative lesions of the oral mucosa are recurrent cases of aphthous stomatitis [15]. Although the specific cause of recurrent aphthous stomatitis is unknown, it is thought that a number of potential variables may contribute to its occurrence. Local elements including trauma, oral microbiota dysbiosis, hematric deficiencies (iron, folic acid, or vitamin B12), immunological elements, and psychosocial stress are among them [16]. Additionally, ulcers could exist due to a hereditary susceptibility. Up to 40% of patients have a positive family history. Previous studies have suggested that a number of gene polymorphisms encoding proinflammatory cytokines, such as ILs and TNF-alpha, are responsible for the development of RAS [10]. The meta-analysis of [17] was the earliest quantitative assessment of the correlation between IL polymorphism and RAS vulnerability due to heredity. The overall investigation showed blatant heterogeneity. In order to reduce statistical heterogeneity, researchers conducted a stratified subgroup analysis based on the regional distribution, specifically reducing the value of $F$ to zero.

In our study, the SNP IL-1β 542T>A polymorphism and RAS were not significantly associated when compared to the control group in both categories, including gender (p-value = 0.495) and age groups (p-value = 0.6253) (Table 1). In the study of [18], it was not possible to pinpoint a genetic link between the RAS risk variables and the IL-1β polymorphism. In RAS patients compared to controls, the frequency of the AA and TA genotypes was not noticeably higher (p-value > 0.05). Yet, the allele distribution in this study differed from the results given by other researchers. [8] found that IL-1 heterozygotes were more common in RAS patients than in controls. Additionally, neither the healthy controls nor the patients had a higher prevalence of the CC genotype, according to the researchers. These findings led to the hypothesis that the CT genotype and RAS might be connected. No significant differences were found by [7] in IL-1β C/T at position -511 between patients and controls; nevertheless, the C allele was the most common allele among patients (51.7%) and controls (55.4%), and the T -511 C genotype was the most common genotype in both groups (50% and 59%). The IL-1β polymorphism was not found to have a significant connection with RAS risk, including gender, age and drug use [19].

The age of the volunteers in the RAS patients and control groups, according to this study, had very little bearing on the development of polymorphisms in IL-1β (p-value=0.0461), as indicated in Table (3). When the data were evaluated using a multivariate model that took into account age and gender, the same IL-1β and TNF- genotypes that were previously shown were associated with an increased risk of RAS development (OR 2.40 and 3.07, respectively) [8]. However, because several studies investigated the association between gene polymorphism of cytokines and RAS without taking into account characteristics like age, sex, or the classification of RAS in cases and control groups, the successful achievement did not account for potential confounding factors. As a result, any confounding factors are not taken into consideration in the final result [17].

According to our findings, vitamin D is more associated with polymorphisms in the IL-1β gene (p-value=0.007) than with vitamin B12, which is less linked in sick people than in normal people (p-value=0.0258). According to the findings of a number of research, vitamin D may be able to directly modify the expression of several cytokines that are involved in innate immunity. Vitamin D causes an increase in the expression of IL-1β and IL-8 in macrophage co-cultures that have been infected [12]. [20] also evaluated the connection between each SNP and the impact of vitamin D supplemental intake (i.e., R/NR Respondents and nonrespondents) as well as the pretreatment classification of vitamin D status (S/D/I; adequate, deficient, insufficient). The two SNPs that exhibited the strongest significant connection with S/D/I classification were rs731236
Water-soluble vitamin B12 has been linked to oral mucosal illnesses, including recurrent aphthous stomatitis (RAS), and is essential for the development of hematopoietic stem cells [21]. Some RAS patients have been shown to have deficiencies in zinc, hematinics (iron, vitamin B12, and folic acid), and vitamin D. However, the causes of how hematinic deficits could affect how the immune response develops in RAS have not been sufficiently clarified [22]. [23] discovered that 4 percent of the 25 healthy control subjects and 28% of the 25 RAS patients had zinc deficits. The mean serum zinc level in 25 RAS patients and 25 healthy controls differ in a statistically significant way.

After iron, zinc (Zn) is the trace metal found in the body in the second-highest concentration. But unlike iron, there isn't a specialized zinc store. The roles of zinc can be divided into three categories: catalytic, structural, and regulatory. Inadequate zinc in the body can cause an immune system imbalance, as seen by the greater susceptibility to infections in cases of severe deficiency [24]. In the current study, we found that there is an association between zinc concentration and IL-1β gene polymorphism among patients with Recurrent aphthous stomatitis when compared to control groups (Table 3). The IL-1β network map highlighted the high level of regulation that is responsible for managing the expression of IL-1β. The majority of this control came in the form of negative feedback, which played an important role. It was also intriguing to detect a position for zinc in this network, which was not initially clear while the map was being constructed, but after additional investigation, a role for zinc regulation of ubiquitination was identified. This was a finding that was not immediately obvious. Building the network map is therefore just the beginning of employing a systematic strategy to investigate IL-1β expression in this way [25]. Similarly, according to studies [26], a zinc deficiency that is mild to moderate can have an effect on the production of a number of different cytokines in humans. These cytokines include IL-1, IL-2, IL-6, and TNF-α. It was discovered that LPS-stimulated peripheral blood mononuclear cells (PBMC) from zinc-deficient adults (as induced by experimental diet) produced more IL-1 than their counterparts who had sufficient zinc in their diets. On the other hand, [27] performed an experiment to clarify the association between zinc concentration deficiency and some cytokines parameters, and they found that there was no discernible change in the serum concentrations of IL-1α (p-value=0.888), IL-1β (p-value=0.171), IL-6 (p-value=0.508), TNF-α (p-value=0.940), or CRP (p-value=0.580) as a result of the zinc intervention.

5. Conclusion
In conclusion, the findings of our current investigation indicate that there is a specific IL-1β 542 T>A gene variation that can be associated with the pathogenesis of RAS. The A allele was related to the risk of RAS in the Erbil city population. The development of RAS lesions may be due to the cumulative effect of more than one gene mutation working together. In addition, more research on a more extensive scale is required in order to validate our findings and enable the application of those findings as genetic markers for RAS vulnerability in the study population.

Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The authors read and approved the final manuscript for publication.

Ethics approval and consent to participate
The study was authorized by the University Ethics Committee.

Informed consent
Consent was acquired from all the patients.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

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References
9. Huang W, He BY, Shao J, Jia XW, Yuan Y Di (2017) Interleukin-