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Salivary irisin: potential inflammatory biomarker in recurrent apthous stomatitis patients

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Abstract. – OBJECTIVE: Recurrent Aphthous Stomatitis (RAS) is the most common inflammatory condition of the oral mucosa characterised by recurrent onset of single or multiple painful ulcers mainly affecting the nonkeratinized oral mucosa. RAS mostly occurs in healthy individuals with no associated systemic diseases. Irisin is a newly identified adipomyokine and research has revealed that it has anti-inflammatory effects. The aim of this study was to investigate the significance of salivary irisin levels in patients with recurrent apthous stomatitis (RAS).

PATIENTS AND METHODS: In this investigation, 80 individuals were evaluated. The patient group included 30 patients diagnosed with RAS and each control group consisted of 25 smoker and non-smoker healthy individuals. Saliva samples were collected and salivary irisin, interleukin-2 (IL-2) and interferon-ɣ (IF-ɣ) levels were determined using enzyme-linked immunosorbent assay (ELISA).

RESULTS: IL-2 and IF-ɣ levels in RAS patients were significantly higher than control smoker and non-smoker groups (p=0.0001, p=0.0001, respectively). Irisin level was higher in RAS patients than smoker controls and non-smoker controls. The level of irisin was found as sensitive and specific as IL-2 and more sensitive and specific than IF-ɣ. The salivary levels of pro-inflammatory cytokines IL-2, IF-ɣ and irisin were higher in RAS group compared to controls.

CONCLUSIONS: This is the first report evaluating the irisin an adipo-myokine as an inflammatory biomarker in RAS.

Key Words:
Inflammation, Irisin, RAS.

Introduction

Adipose tissue and muscle that were previously considered metabolically passive have been shown to play significant role in metabolic regulation through secretion of a number of hormones and hormone-like peptides, such as leptin, ghrelin, nesfatin, preptin, adropin and irisin. These peptides are described to communicate with cells in an autocrine (the cells that produce them)/paracrine (nearby cells), or in an endocrine manner (distant tissues). Myokines are mainly secreted in skeletal muscle and adipokines are in adipose tissue. The myokines described in the literature that are additionally known to be secreted by adipocytes are termed as adipo-myokines[1]. There are two types of adipose tissue in mammals: brown and white, which is mainly in humans. The white adipose tissue (WAT) stores excess energy in the form of triglycerides. Conversely, the brown adipose tissue (BAT) is specialized in energy expenditure and responsible for non-shivering thermogenesis called adaptive thermogenesis[2]. WAT is the main source of adipokines with pro- and antiinflammatory properties, including leptin, adiponectin, interleukin-6 (IL-6), interleukin-10 (IL-10), monocyte chemoattractant protein-1 (Mcp-1) and tumor necrosis factor-α (Tnf-α)[3]. Adipokines are secreted both from adipocytes and other cells of the adipose tissue, such as endothelial cells and macrophages[4]. Obesity, especially visceral adiposity, is characterized by a state of low-grade systemic inflammation. Proinflammatory adipokines and molecules secreted from adipose tissue are implicated as the cause of increased cardiovascular disease risk, development of insulin resistance and so-called metabolic syndrome associated with obesity.

Since its first report in 2012 by Boström et al[5] in Harvard University, irisin, a novel myokine, has been one of the most investigated peptides in recent years. It is a 112 amino acid cleavage

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product of fibronectin type III domain-containing protein 5 (FDNC5), which is, in turn, stimulated by peroxisome proliferator-activated receptor-ɣ co-activator 1-α (PGC-1α). Originally it was described as a myokine secreted in response to physical excercise from skeletal muscle, but later it was found to be also secreted from adipose tissue[6]. Therefore, irisin has been included in the adipomyokine family. Although secreted primarily by muscle, especially cardiac muscle and adipose tissue, pancreas, sebaceous glands, liver, lung, testes, kidney, salivary glands, rectum, stomach, tongue, neuronal cells and sweat glands have shown strong immunoreactivity to irisin and its precursor FNDC5 mRNA indicating irisin synthesis in these tissues[7,8]. Irisin promotes "browning" of mature white adipocytes in response to exercise, increases energy expenditure, decreases insulin resistance and adipogenesis. It has been proposed to mediate beneficial effects of exercise on metabolism[8]. It was found that addition of recombinant irisin decreased the expression of inflammatory markers and stimulated the phenotypic switching of adipose tissue macrophages from M1 (pro-inflammatory) to M2 (anti-inflammatory) state in adipose tissue[9]. Serum irisin levels have previously been investigated in obesity, chronic kidney disease, type 2 diabetes mellitus, metabolic syndrome, inflammation and various types of cancer[10-20].

Recurrent Aphthous Stomatitis (RAS) is the most common inflammatory condition of the oral mucosa characterised by recurrent onset of single or multiple painful ulcers mainly affecting the nonkeratinized oral mucosa[21]. RAS mostly occurs in healthy individuals with no associated systemic diseases. The prevalence of RAS shows great variation between 2% and 50% in the general population, depending on the population studied, environmental factors and diagnostic criteria[22]. RAS is more prevalent in adult women, school children, non-smokers, and those with high socioeconomic status[23,24]. Onset of the disease mostly occurs in childhood and usually the severity and frequency of episodes decreases with age[25]. Despite marked research and clinical attention, the etiology and pathogenesis of RAS remain unclear. Several factors such as genetic predisposition, viral and bacterial infections, psychological stress, food allergies, local trauma contribute to development of the aphtous ulcers. Deficiencies of hemoglobin, iron, vitamin B12, Vitamin D and folic acid and abnormally high blood homocysteine level were found to be associated with RAS[26,27].

It is suggested that in genetically predisposed subjects, triggering factors initiates a disrupted immunologic respose and inflammation with subsequent apthous ulcer formation in RAS. Enhanced immunologic reaction occurs through inappropriately iniated cascade of cytokines. In many reports, cytokine profile in RAS patients have been studied. Increased production of Type 1 pro-inflammatory cytokines IL-2, IFN-γ and TNF-α, as well as IL-5, IL-6, and IL-8 and decreased production of anti-inflammatory cytokines IL-10 and TGF-β were found in peripheral blood of RAS patients compared to healthy individuals[28]. Elevated levels of IL-2, IFN-γ, and TNF-α mRNAs and lower resting levels of IL-10 mRNA were detected in oral mucosa of RAS patients compared to healthy controls[29]. There are epidemiologic studies[30] indicating lower prevalence of RAS in smokers compared to non-smokers with an increase in the incidence of RAS following smoking cessation. In a research[31] conducted on RAS patients, although smoking was not shown to be an independent risk factor, a negative association between smoking and the frequency of RAS recurrences has been reported. Since the etiology of RAS cannot be determined precisely, there is no effective treatment and protection. The main goals in treatment are avoidance of local traumatic factors, suppressing local immune response, controlling pain, shortening ulcer time/accelerating healing and preventing relapses and secondary infection. Treatments are usually palliative, but none provide permanent remission[32].

Saliva is one of the best-known easy collect and non-invasive biological materials. It comprises 99.5% water and the remaining 0.5% contains antibacterial compounds such as secretory IgA and lysozyme, electrolytes, mucus, glycoproteins, enzymes and various peptide hormones. Saliva generally reflects blood peptid concentration saliva plays a key role in the oral health so that it can preserve the integrity of the oral mucosal membrane through liquefaction and amelioration of the soft tissue[33]. Patient acceptance of saliva sample collection is much higher when compared to obtaining serum or biopsy samples[34].

Based on the objective of investigating inflammatory biomarkers in RAS we investigated the irisin, an adipomyokine that has not been previously studied in RAS. We compared irisin level with IL-2 and IF-γ levels which have been previously investigated in RAS patients. In addition, we also aimed to determine the irisin levels in

both smoking and non-smoking controls to elucidate the relationship between smoking and salivary irisin, which is not previously investigated. As mention above, RAS is diagnosed from a history of recurrent ulcers together with an oral examination. Salivary molecules (such as EGF, IL-2, etc.) play a pivotal role in maintaining the oral health and ameliorating the oral ulcers that irisin secretion from salivary glands and relation with inflammation, caused to wonder it can be a marker in RAS patient. This research aimed to gain new information to the literature by determining whether irisin can be used as an inflammatory biomarker of RAS.

Patients and Methods

Study Population

The study protocol was approved by Ordu University (ODU) Clinical Research Ethics Committee (Decision No: 2018/16). This investigation involved 30 patients previously diagnosed with RAS, 25 healthy non-smokers and 25 healthy smokers. The patients were recruited from Otolaryngology Clinics of University Research Hospital. All subjects provided written consent. Patients over the age of 18, having a diagnosis of RAS (at least 3 spontaneous aphtous ulcer occurrence per year), were included in the study. Exclusion criteria included presence of autoimmune disease, diagnosis of cancer and pregnancy. Exclusion criteria was also applied for age and sex-matched healhty control subjects. All of the RAS patients were free of active aphtous ulcer at the time of saliva collection.

Our study included the following steps: collection of saliva samples, determination of Irisin, IL-2, IF-γ levels in saliva samples by ELISA method, and statistical analysis. The methods and procedures used in these steps are detailed below.

Collection of Saliva Samples

Saliva has a circadian periodicity such that all saliva samples of patients and controls were taken at 10:00 am in the morning; thus, the collection was carried out at the same time of the day.

All subjects were seated on the examination chair and cotton swabs were given to the subjects (Salivette, Sarstedt AG&Co, Nümbrecht, Germany). Subjects placed the swabs in the mouth and chewed them for about 60 seconds to stimulate salivation. Then swabs that absorbed saliva were placed in the tubes and cover closed, centrifuged for 2 minutes at 1000 x g. The bottom part of the salivette that contained the clear saliva was removed and saliva transfered into 1.5 ml eppendorf tubes and stored in -80°C deep freezer until working time.

Determination of Irisin, IL-2, IF-γ Levels in Saliva Samples by ELISA Method

Human irisin salivary levels were determined using an Enzyme Linked Immunosorbent Assay (ELISA) kit (USCN, Life Science Inc., Catalog No. 201-12-5328, Sunred Biological Technology, Shanghai, China) in line with the manufacturer's instructions.

Absorbance of samples was measured at 450 nm using a BioTek Instrument EL800 Microplate Reader (Winooski, VT, USA). Results were expressed as ng/mL.

Human IL-2 salivary levels were determined using an enzyme linked immunosorbent assay (ELISA) kit (USCN, Life Science Inc., Catalog No: 201-12-0095, Sunred Biological Technology, Shanghai, China) in line with the manufacturer's instructions.

Absorbance of samples was measured at 450 nm using a BioTek Instrument EL800 Microplate Reader. Results were expressed as ng/L.

Human IF-ɣ salivary levels were determined using an enzyme linked immunosorbent assay (ELISA) kit (USCN, Life Science Inc., Catalog No: 201-12-0106, Sunred Biological Technology, Shanghai, China) in line with the manufacturer's instructions. Absorbance of samples was measured at 450 nm using a BioTek Instrument EL800 Microplate Reader. Results were expressed as ng/L.

Statistical Analysis

The test results were analyzed on SPSS 13.0.1 (License No. 9069727) statistical software (Statistical Package for the Social Sciences; SPSS Inc., Chicago, IL, USA). Data were shown as mean±-standard deviation for normal distributed and median (interquartile range) for non-normal distributed variables. The distribution of irisin, IL-2, IF-γ levels in each group was calculated by Kolmogorov-Smirnov test. Comparisons of the groups were done by ANOVA for normal distribution and by Kruskal Wallis test for non-normal distribution. Post hoc evaluation within group of IF-γ was performed using the "Tukey" and "Tamhane T2" tests The Mann Whitney U test was used to compare nonparametric two-way parameters for İrisin and IL-2. Spearman correlation analysis was

Table I. Irisin, IL-2, IF-γ concentrations, age of RAS, smoker and non-smoker control groups.

Parameters	RAS group (n=30)	Control smoker group (n=25)	Control non-smoker group (n=25)	*p*-value
Irisin (ng/mL)	19.3 (15.4-32.9)[a]	12.1 (10.0-14,8)	15.6 (10.9-21.3)	0.0001
IL-2 (ng/L)	151.9 (114.1-166,8)[a,b]	48.9 (35.1-62.3)	38.3 (27.9-54.3)	0.0001
IF-ɣ (ng/L)	136.3±32.8[a,b]	84.3± 25.2	64.6± 27.7	0.0001
Age	27.5 (20.0-44.0)	21.0 (20.0-30.0)	23 (19.0-37.0)	0.172

Data were expressed as: mean ± SD for parametric tests, median (inter quarter range for 25-75%) for nonparametric tests. p; according to one-way ANOVA for IF-ɣ, Kruskall Wallis for irisin and IL-2, age. Post-hoc evaluation within group of IF- ɣ was performed using the 'Tukey' and 'Tamhane T2' tests The Mann Whitney U test was used to compare nonparametric two-way parameters for irisin, age and IL-2 a, significantly different from the control smoker group. b, values differ significantly from the control non-smoker group.

used to assess the relationships among the parameters considering the skewness of data distribution. Receiver operating characteristic (ROC) curves were analyzed on Medcalc software version 11.5.1.0 (MedCalc software BVBA, Ostend, Belgium). Statistical significance was accepted as $p<0.05$.

Results

Thirty RAS patients, 25 control smoker and 25 non-smoker subjects were enrolled in the study. RAS patients with a median age of 27.5 (range 20 to 40). The healthy control smoker and non-smoker group with median age of 21 (range 20 to 30) and 23 (19 to 37), respectively. There was no significant difference between the groups in terms of age (year). None of the RAS patients were smokers. Distribution of biochemical parameters, and age were shown in Table I. Comparison of three groups revealed significantly elevated irisin, IL-2 and IF-γ levels in the patients with RAS (p=0.0001, p=0.0001, p=0.0001, respectively). As expected, the levels of IL-2 and IF-γ were high in RAS patients. The level of irisin was studied for the first time in RAS patients and we found irisin level as high as IL-2 and IF-ɣ. Spearman correlation analysis results of irisin, IL-2, and IF-γ in RAS patient was shown in Figures 1 and 2. There is a positive and strong correlation between irisin and IL-2 (p=0.0001, R=0.780), IF-γ and IL-2 (p=0.0001, R=0.782). However, there is no strong relationship between irisin and IF-γ. Optimum diagnostic Irisin, IL-2, IF-γ cutoff point, AUC according to the receiver operator characteristic (ROC) curve data are shown in Table II. As seen in the ROC analysis (Figure 3), irisin is more specific and sensitive than IF-γ at least as much as IL-2 in the diagnosis of the disease.

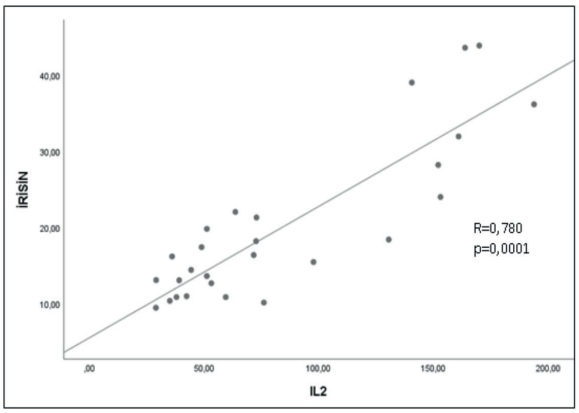

Figure 1. Irisin and IL-2 correlation.

Table II. Optimum diagnostic Irisin, IL-2, IF-ɣ cutoff point, AUC according to the receiver operator characteristic (ROC) curve.

Parameters	AUC	95% CI	Cutoff point	*p*-value
Irisin (ng/mL)	0.929	0.722- 0.995	>16.21	0.0001
IL-2 (ng/L)	0.980	0.867-1.000	>65.08	0.0001
IF-ɣ (ng/L)	0.879	0.656-0.980	>92.14	0.0001

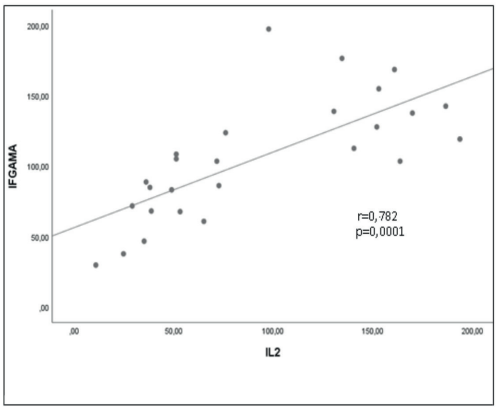

Figure 2. IF-ɣ and IL-2 correlation.

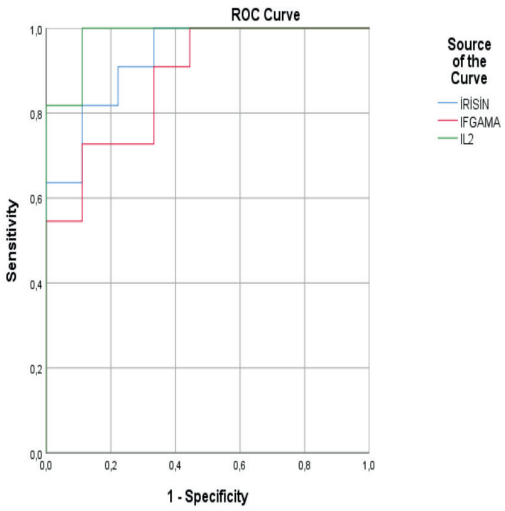

Figure 3. ROC Analysis of Irisin, IL-2 and IF-ɣ.

Discussion

Adipose tissue and muscles are two important tissues that secrete myokines and adipokines, which play an important role in the regulation of metabolism and inflammatory process. Adipose tissue is more important in all inflammatory diseases than it is stated. WAT is the main source of adipokines with pro and antiinflammatory properties including leptin, adiponectin, IL-6, IL-10, TNF-α, and newly identified irisin. Irisin is known as adipo-myokine and until this time serum irisin levels have previously been investigated in obesity, chronic kidney disease, type 2 diabetes mellitus, metabolic syndrome, inflammation, and various types of cancer[10-20]. In patients with myocardial Infarction (MI), the level of irisin in serum and saliva has been shown to be reduced and it has been said that irisin can be considered as a candidate marker for the diagnosis of MI beside CK-MB and cardiac troponin[35]. Again, same researchers reported that salivary and serum irisin levels were correlated and they found significantly higher irisin levels in saliva than the serum levels in both obese and normal weight subjects[6]. It was found that addition of recombinant irisin decreased the expression of inflammatory markers and stimulated the phenotypic switching of adipose tissue macrophages from M1 (pro-inflammatory) to M2 (anti-inflammatory) state in adipose tissue[9]. RAS is the most common inflammatory condition of the oral mucosa characterised by recurrent onset of single or multiple painful ulcers with unknown etiology. Irisin has been associated with inflammation but has never been studied in RAS patients before. We demonstrated elevated irisin levels in RAS patients accompanied by elevated IL-2 and IF-γ levels and stronger correlation of irisin with IL-2. Besides, irisin was more specific and sensitive than IF-γ and as much sensitive and specific as IL-2 in our RAS patients. In an experimental study[36] conducted in colitis induced rats, it has been shown that exercise prior to induction of colitis diminished the severity of colonic damage, associated with marked increase in plasma irisin levels confirming that irisin could be involved in the mechanism linked to the mucosal healing of colitis. We hypothesized that elevated irisin levels in RAS can be explained by anti-inflammatory role of irisin in compensatory process to overcome ongoing inflammation in RAS.

According to the literature, RAS formation is suppressed in smokers. A negative epidemiological association has been reported between aphthous stomatitis and smoking. It is claimed that smoking decreases the risk of developing aphthous ulcers by increasing keratinization in the oral mucosa and/or by decreasing the inflammatory response through immunosuppression[37,38]. We also investigated the relationship between IL-2, IF-γ, and irisin levels and smoking. None of the subjects in RAS group was smoking so RAS patients were not included in statistical analysis. Our study is the first one that investigates the relation of irisin with smoking so far. We compared irisin levels of the smoker and non-smoker control groups. These two groups were similar in terms of age. The salivary IL-2 and IF-γ levels were significantly higher in control smokers compared to non-smokers while decreased irisin

level was observed in control smokers although insignificant. Tymkiw et al[39] compared the healthy and periodontitis sites between smokers and non-smokers and contrary to our results demonstrated no significant differences in levels of IF-γ and IL-2 despite decreased production of several pro-inflammatory cytokines in smokers. Immunosupression and decreased inflammatory state may be the reason for decreased irisin level in smoking subjects. Wung et al[40] reported a significant association between the salivary EGF and improvement of the ulcer induced by RAS.

Conclusions

This study is the first research to show the relationship between inflammation and irisin in saliva samples of RAS patients. We demonstrated elevated level of irisin in RAS patients. When we analyze the results, irisin is more sensitive and specific than IF-γ and as sensitive and specific as IL-2 for RAS. Irisin is decreased and two pro-inflammatory cytokines, IF-γ and IL-2, are increased in salivary samples of smoking healthy subjects.

The major limitation of our investigation is the relatively small number of patients and controls involved. Irisin and other inflammatory parameters were studied only in saliva samples, and the results could not be evaluated in serum/plasma samples due to budget limitation. Also, salivary samples were taken from the RAS patients during their ulcer free period and this can be a drawback. During active ulcer phase in which the inflammation is expected to be more pronounced, differences would be more significant. Therefore, irisin is involved in inflammation and whether it has proanti-inflammatory role needs to be further elucidated.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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Ethics Committee Approval

Ethics Committee Approval was received from the Ordu University Local Ethical Committee under Reference No. 2018-16.

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