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Alteration in Gene Expression of *STAT4* and *PIAS2* in Individuals with Type 2 Diabetes Mellitus Treated with the Dipeptidyl Peptidase 4 Inhibitor

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ABSTRACT

Objective: Impairment of immune cell signaling molecules is involved in diseases pathogenesis. The evaluation of signal transducer and activator of transcription (*STAT*) 4 and protein inhibitor of activated STAT (PIAS) 2 as well as immunoregulatory role of the dipeptidyl peptidase-4 inhibitor, sitagliptin were investigated in type 2 diabetes mellitus (T2DM).

Materials and Methods: Peripheral blood mononuclear cells (PBMC) were purified from three study groups including healthy controls, T2DM patients with 6 months of sitagliptin treatment, and T2DM patients without sitagliptin. Expressions of *STAT4* and *PIAS2* were assessed with real-time polymerase chain reaction (qPCR).

Results: The expression of *STAT4* in patients without sitagliptin was higher than the healthy controls (p=0.001). Its expression was down-regulated in the sitagliptin treated patient group compared to those without sitagliptin (p=0.005). *PIAS2* expression in patients without sitagliptin was lower than the healthy controls (p=0.009). *PIAS2* was elevated in the sitagliptin treated group versus patients without sitagliptin (p=0.003). A negative correlation between *STAT4* and *PIAS2* was found in individuals without sitagliptin (p=0.01). In patients without sitagliptin, fasting plasma glucose was positively and negatively correlated with *STAT4* and *PIAS2*, respectively (p=0.004 and p=0.001).

Conclusion: Aberrant expression of *STAT4* and reduced expression of *PIAS2* were found in the T2DM patients. Sitagliptin may regulate the cell signaling pathways by elevating *PIAS2* and reducing *STAT4*.

Keywords: Type 2 diabetes mellitus, STAT, PIAS, dipeptidyl peptidase 4 inhibitor

INTRODUCTION

Type 2 diabetes mellitus (T2DM), as a prevalent and intricate metabolic disorder, is primarily defined by hyperglycemia and inflammation (1). Disruption in cytokine secretion and signal transducer and activator of transcription (STAT), and protein inhibitor of activated STAT (PIAS) signaling components play a fundamental role in the inflammation (2, 3).

Several pro-inflammatory cytokines exert their functions through janus kinase (JAK)-STAT (consist of STAT 1-6) signaling molecules (4). STAT4, the most specific member of this family, has a pro-inflammatory impact on the immune system. STAT4 is activated by various inflammatory mediators such as Interleukin (IL)-12, IL-2, and IL-23 in the peripheral blood mononuclear cells (PBMCs) (2). STAT4 can influence a wide range of intracellular processes including T helper (Th) 1 cell differentiation and Interferon (IFN)-γ secretion (5). PIAS family (composed of PIAS 1-4) negatively regulate the intracellular cytokine pathways by acting on STATs molecules. PIAS2 binds to activated STAT4 and suppresses the pro-inflammatory process and signal transduction (3). Previous studies have reported that both STAT4 and PIAS2 take part in various diseases progression. For example, a recent study demonstrated that

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the expression of *PIAS2* was diminished in Parkinson patients, and its levels were involved in disease severity (6). Moreover, *PIAS2* expression was down-regulated in rheumatoid arthritis (RA) fibroblast-like synovial cells (7). Alteration in *STAT4* expression was reported in systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) patients accompanied by association with disease activity (8, 9).

Dipeptidyl peptidase 4 inhibitor, sitagliptin is an anti-diabetic agent prescribed for diabetes patients (10). Previously, we and others have reported diverse anti-inflammatory effects of sitagliptin. For example, this medication has inhibitory effects on the cell proliferation and differentiation (11). Sitagliptin can diminish the protein levels of JAK2 and STAT3 in diabetic rats (12). A reduction in IL-17 and Th17 transcription factor, RORγt, was reported in diabetes patients (13).

Altogether, the changes in STAT4 and PIAS2 levels were implicated in the pathogenesis of several diseases. However, no scientific reports are available in T2DM. In the current study, mRNA levels of *STAT4* and *PIAS2* were investigated in T2DM patients treated with/without sitagliptin in comparison to healthy subjects. The possible regulatory role of sitagliptin on *STAT4* and *PIAS2* was also explored. The relationship between the genes and diabetes parameters was analyzed.

MATERIALS AND METHODS

Subjects

This investigation was confirmed by the local ethics committee (IR. UMSHA. REC. 1402. 364) and written permission was given by all subjects. Three groups were included in this study: 1. T2DM patients without sitagliptin therapy (n=35, 19 females and 16 males, mean age: 50.97 ± 6.44), 2. T2DM patients with sitagliptin (n=35, 20 females and 15 males, mean age: 52.71 ± 5.8), and 3. healthy subjects without any noticeable illness (n=35, 19 females and 16 males, mean age: 50.54 ± 7.58). Patients received sitagliptin by using a fixed dose of 1000 mg/24h in the last 6 months. All patients had received background metformin medication (50 mg/day) one year before blood sampling. More information of the participants is indicated in supplementary Table 1.

Inclusion criterias: 1. T2DM patients were identified according to the American Diabetes Association 2022 principle, 2. fasting plasma glucose (FPG) for each person was > 125 mg/dL, and 3. hemoglobin A1c (HbA1c) was > 6.5%. Exclusion criterias: 1. people with autoimmune and chronic diseases, 2. people with neoplasia and allergic illness, 3. patients who had been taking insulin, immunosuppressants, and antibiotics, and 4. people with diabetes-related problems such as hyperketonemia, retinopathy, and nephropathy.

Cell Purification

PBMCs were isolated from 5 mL of fresh whole blood sample (in EDTA tube) using ficoll gradient centrifugation method (Sigma, MO, USA) (14). Concisely, each sample was diluted 1:2 with phosphate buffered saline (PBS) and was drawn into a tube containing the ficoll solution. PBMC layer was separated by centrifugation at 2200 rpm for 15 min at 4℃. Cells were washed 3 times prior to RNA isolation.

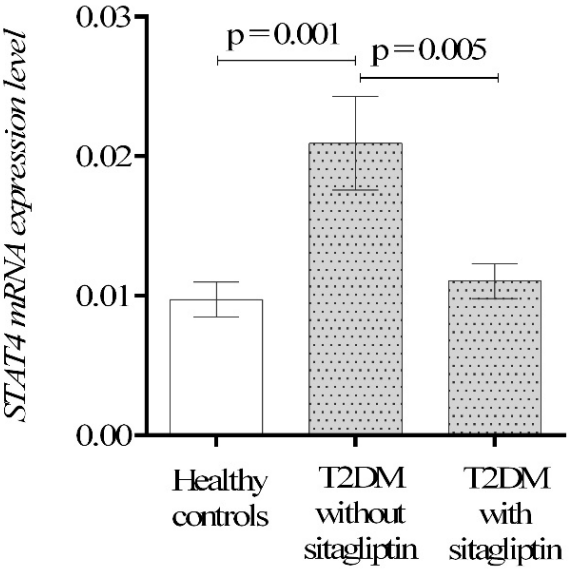

Figure 1. mRNA expression level of *STAT4* in patients and healthy controls. Gene expression level of *STAT4* in PBMCs of T2DM patients with and without sitagliptin, and healthy controls were determined using qPCR. Number of participants for each group is 35.

RNA Preparation and cDNA Generation

Total RNA was prepared from PBMCs. The RNA sensitive kit (Qiagen, USA) with relevant protocol was considered for RNA extraction. A 260/280 nm ratio of ~ 1.8 was accepted for cDNA synthesis. RNA was converted to cDNA using a human kit (Takara, Japan) with recommended protocol.

Real-time polymerase chain reaction (qPCR) (Roche 96 system, Germany) was used to measure gene expression. SYBR green fluorescent reporters (Ampliqon A/S, Denmark) was used to detect the products based on the instructions provided by the manufacturer. Time and temperature for the reactions were along these steps: 1. initial stage (95℃ for 15 min), 2. denaturation (95℃ for 15 s), 3. annealing (60℃ for 30 s), and 4. extension stage (72℃ for 30 s). Housekeeping glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* as an internal control was used for gene normalization. The relative mRNA expression levels of *STAT4* and *PIAS2* were calculated using the $2^{-\Delta Cq}$ method (15).

Specific primers were used as follows: 1) human *STAT4* (NM_003151.4), sense sequence is 5′-*CAGTGAAAGCCATCTCGGAGGA*-3′, and antisense sequence is 5′-*TGTAGTCTCGCAGGATGTCAGC*-3′, 2) human *PIAS2* (NM_173206.4),

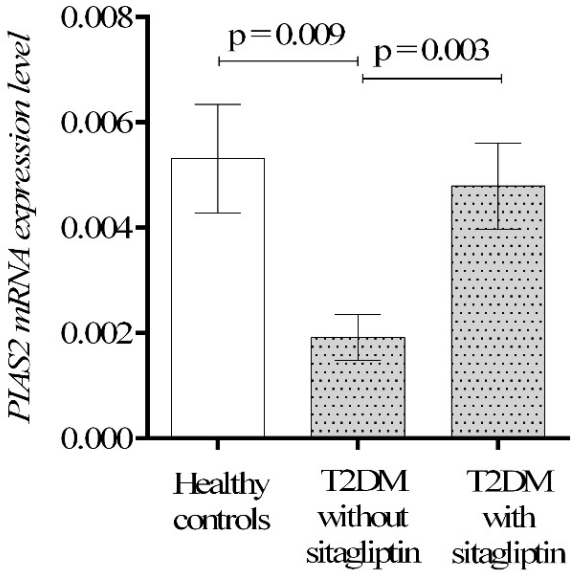

Figure 2. mRNA expression level of *PIAS2* in patients and healthy controls. *PIAS2* expression in PBMCs of patients with and without sitagliptin, and healthy controls were assessed using qPCR. Number of participants for each group is 35.

sense is 5′- *ATCCACGAACTCTTGAAGGACT* -3′, and antisense is 5′- *TGTGGGCTTAGTATCTTGAAGCA* -3′, and 3) human *GAPDH* (NM_002046.7), sense is 5′- *GGAG CGAGATCCCTCCAAAAT* -3′, and antisense primer is 5′- *GGCTGTTGTCATACTTCTCATGG* -3′.

Statistical Analyses

SPSS 21.0 and GraphPad Prism 9.0 were employed for statistical evaluation and graph creation. Analysis of variance (ANOVA) followed by Tukey's test as post hoc (to compare three groups), independent t-test (to compare two groups), and Pearson coefficient for correlations, were applied. Data was expressed as mean ± SD or mean ± SEM (for graphs). A $p<0.05$ was considered statistically significant.

RESULTS

The participants were consisted of T2DM patients without sitagliptin, T2DM patients with 6 months of sitagliptin, and healthy controls. The levels of *STAT4* and *PIAS2* genes were compared between the groups. The expression of *STAT4* was appreciably up-regulated in T2DM patients without sitagliptin when compared to healthy controls (p=0.001, Figure 1). *STAT4* expression was diminished in the T2DM patients treated with sitagliptin compared to the sitagliptin negative group (p=0.005, Figure 1). In contrast, *PIAS2* expression was markedly down-regulated in T2DM patients without sitagliptin in comparison with healthy subjects (p=0.009, Figure 2), and its expression was increased in the sitagliptin treated group compared to the sitagliptin negative group (p=0.003, Figure 2). No significant changes in *STAT4* and *PIAS2* levels were observed among the T2DM patients using sitagliptin and the healthy controls (Figure 1 and 2).

Table 1. Correlation between the *STAT4* and *PIAS2* in study groups.

T2DM patients without sitagliptin		
		PIAS2 expression
STAT4 expression	r value	-0.42
	p value	**0.01**
T2DM patients with sitagliptin		
STAT4 expression	r value	0.26
	p value	0.12
Healthy controls		
STAT4 expression	r value	-0.065
	p value	0.71

Results of Pearson correlation test are shown with r and p values.

In T2DM patients without sitagliptin, a significant negative correlation between the *STAT4* and *PIAS2* was found (p=0.01, Table 1). As indicated in Table 2, FPG was positively and negatively related to the *STAT4* and *PIAS2* expression in patients without sitagliptin, (p=0.004 and p=0.001, respectively). No obvious correlations between the HbA1c and *STAT4 / PIAS2* expression were observed in study groups (Table 2). No correlations in *STAT4* and *PIAS2* were observed in the control and the sitagliptin positive groups. The expression of *STAT4* and *PIAS2* was approximately similar between females and males in the different study groups (Table 3).

DISCUSSION

Chronic inflammation besides an excessive immune activation has received increased focus in T2DM pathogenesis (1). In this study, the gene expression of *STAT4* and its regulator, *PIAS2*, was investigated in T2DM with and without sitagliptin compared to healthy controls. The role of *STATs* as a trigger of inflammation has been suggested by immunological studies (2). *STAT4* participates in the pro-inflammatory immune process including Th1 differentiation and cytokine production (5). The inhibitory effect of *PIAS2* is performed by regulating multiple functional genes including *STAT4* (3). We first observed that the expression of *STAT4* in patients without sitagliptin was much higher than in the healthy controls, whereas the *PIAS2* expression was lower in the patients. *STAT4* expression was reversely related to *PIAS2*. In keeping with this finding, the alterations in *STATs/ PIASs* have previously been published in a diverse range of diseases. A higher mRNA level of *STAT4* in the synovial tissue of RA patients accompanied

Table 2. Correlation between the *STAT4/ PIAS2* expression and diabetes criteria in study groups.

		STAT4 expression	*PIAS2 expression*
T2DM patients without sitagliptin			
Fasting plasma glucose	r	0.56	-0.52
	p	**0.004**	**0.001**
HbA1c	r	0.29	-0.21
	p	0.08	0.20
T2DM patients with sitagliptin			
Fasting plasma glucose	r	-0.008	0.23
	p	0.96	0.18
HbA1c	r	0.13	0.21
	p	0.44	0.22
Healthy controls			
Fasting plasma glucose	r	-0.08	0.023
	p	0.64	0.87
HbA1c	r	-0.80	0.02
	p	0.64	0.87

Results of Pearson correlation test are shown with r and p values.

by correlation with RA progression and rheumatoid factor has been published (16). Activation of *STAT4* by IL-23 stimulation has been reported in multiple sclerosis patients (17). Consistent with our results, the enhanced phosphorylation of JAK2 and STAT3 were noticed in diabetes patients with macrovascular complication *in vitro* (18). Moreover, down-regulated *PIAS2* in RA fibroblast-like synovial cells has been reported (7). It seems that enhanced expression of *STAT4* and impaired expression of *PIAS2* might potentially lead to the amplification of inflammation in T2DM, and it may have an effect on the T2DM pathogenesis.

We next found out that sitagliptin has a regulatory effect on the aberrant expression of *STAT4* and *PIAS2*. The expression of *STAT4* was considerably reduced after 6 months of sitagliptin therapy while *PIAS2* expression was reduced. A few regulatory mechanisms of sitagliptin have been previously reported. For example, sitagliptin treatment reduces Th1 and Th17 cytokines including IFN-γ and IL-17, and increases Treg transcription factor, *FOXP3,* in the patients (13). Sitagliptin can reduce the excessive proliferation of T cells in T2DM (11). Moreover, the modulatory action of sitagliptin on *JAK2*, *STAT3* and suppressors of cytokine signaling (*SOCSs*) were recently demonstrated by our team (19). In a study on diabetic rats focusing on the JAK-STAT pathway, it has been reported that pJAK2/pSTAT3 were significantly diminished following sitagliptin therapy (12). It should be considered that some potential drugs targeting inhibitory genes such as SOCSs were already discussed in diabetes for the protection of pancreatic β-cell function and insulin secretion (20). Our results demonstrate that sitagliptin has a regulatory activity on *PIAS2* and *STAT4*. Further *in vivo* works are needed to evaluate the direct effect of sitagliptin on pancreatic β-cells. Also, the *STAT4* and *PIAS2* targets could be the potential aim for medical intervention.

We realized that increased levels of FPG in T2DM patients without sitagliptin were directly related to *STAT4* and negatively related to *PIAS2*. In line with this finding, a reverse connection among the IL-17 and HbA1c levels was reported in patients with retinopathy by Chen et al (21). Moreover, up-regulation of the *JAK* gene in diabetic mice leads to a deterioration in disease severity and it is linked to an albuminuria complication (22). This finding suggests that *STAT4* and *PIAS2* might have a role in the glucose metabolism by reducing inflammation. However, further works are needed to know the mechanism of action of STAT4 and PIAS2 on diabetes-related clinical parameters.

This work has some limitations. We considered only the mRNA levels of *STAT4* and *PIAS2* genes in PBMCs. Flow cytometric or blotting experiments could be applied to confirm the alteration of these molecules in protein levels. Several cytokines such as IL-12, IL-2, IFNs, and IL-23 could activate or regulate *STAT4* and *PIAS2* signaling molecules, and the contribution of the unique cytokines in relation to *STAT4* and *PIAS2* might require further exploration.

Table 3. Comparison of *STAT4* and *PIAS2* expression in different genders of study groups.

T2DM patients without sitagliptin			
	Female	Male	p value
STAT4 expression	0.018 ± 0.017	0.20 ± 0.02	0.35
PIAS2 expression	0.0019 ± 0.001	0.002 ± 0.003	0.19
T2DM patients with sitagliptin			
STAT4 expression	0.010 ± 0.006	0.012 ± 0.008	0.34
PIAS2 expression	0.0047 ± 0.004	0.0054 ± 0.005	0.87
Healthy controls			
STAT4 expression	0.008 ± 0.006	0.011 ± 0.007	0.29
PIAS2 expression	0.0051 ± 0.0049	0.0056 ± 0.0043	0.79

Mean ± SD values are shown

Following studies could confirm these observations in isolated T cells by using recombinant cytokines to clarify the specific signaling pathways. Moreover, it could be beneficial to investigate the follow-up effects of sitagliptin in the T2DM patients.

CONCLUSION

Impaired gene expression levels of *PIAS2* and elevated mRNA levels of *STAT4* in T2DM patients who did not receive sitagliptin were demonstrated by this study. The beneficial effects of sitagliptin were illustrated by the up-regulation of *PIAS2* and down-regulation of *STAT4* in the T2DM patients. Therefore, sitagliptin might have an immunomodulatory role in the reduction of T2DM-related inflammation through *STAT4* and *PIAS2* molecules. This finding illustrated probable mechanisms underlying the anti-inflammatory action of dipeptidyl peptidase 4 inhibitor, sitagliptin.

Ethics Committee Approval: The study was approved by the ethics committee of Hamadan University of Medical Sciences, Iran (Approval number: IR.UMSHA.REC.1402.364).

Informed Consent: Signed consent was obtained from the participants.

Peer-review: Externally peer-reviewed.

Authors' Contributions: Conception/ design of Study- M.B.; Data acquisition: S.N., F.S., M.B.; Data Analysis/Interpretation: M.B., A.Z.; Drafting Manuscript: S.N., M.B.; Critical Revision of Manuscript: S.N., F.S., A.Z., M.B.; Final Approval and Accountability: S.N., F.S., A.Z., M.B.

Conflict of Interest Statement: All authors declare that they have no conflicts of interest.

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Supplementary Table 1. Information of the study groups.

	T2DM patients without sitagliptin	T2DM patients with sitagliptin	Healthy controls
FPG level (mg/dL)*	139.69 ± 39.81	129.37 ± 27.01	89.25 ± 11.32
HbA1c level (%)*	7.35 ± 0.76	7.21 ± 1.13	4.69 ± 0.54
Duration of diabetes (year)	1.85 ± 0.74	1.95 ± 1.10	-
Body mass index (kg/m^2)	25.39 ± 4.17	24.86 ± 2.04	24.42 ± 2.77

FPG: Fasting plasma glucose, Mean ± SD values are shown and $p<0.05$ was considered significant using ANOVA.

*$p<0.001$; control group compared to each patient group.