Accepted Manuscript

Title: Evaluation of SOCS1 Methylation in patients with Behcet's disease

Authors: Ali abdi, Alireza Khabazi, Ebrahim Sakhinia, Shahriar Alipour, Mahsa Talei, Zohreh Babaloo

PII: S0165-2478(18)30167-6
DOI: https://doi.org/10.1016/j.imlet.2018.07.001
Reference: IMLET 6220

To appear in: *Immunology Letters*

Received date: 30-3-2018
Revised date: 26-6-2018
Accepted date: 6-7-2018

Please cite this article as: abdi A, Khabazi A, Sakhinia E, Alipour S, Talei M, Babaloo Z, Evaluation of SOCS1 Methylation in patients with Behcet's disease, *Immunology Letters* (2018), https://doi.org/10.1016/j.imlet.2018.07.001

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Evaluation of SOCS1 Methylation in patients with Behcet's disease.

Ali abdi[1,2], Alireza Khabazi[3], Ebrahim Sakhinia[4], Shahriar Alipour[4,5], Mahsa Talei[1,2], Zohreh Babaloo[1,2*]

[1]. *Immunology Research Center, Tabriz University of Medical Sciences, Iran.*

[2]. *Department of Immunology Medicine faculty, Tabriz University of Medical Sciences, Iran.*

[3]. *Connective Tissue Disease Research Center, Tabriz University of medical science, Iran.*

[4]. *Dept. of Genetics, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.*

[5]. *Molecular Medicine, Faculty of Advanced Medical Sciences, Tabriz university of Medical Sciences, Iran.*

[*]***Corresponding author Emails***: Zbabaloo20@gmail.com

Abstract:

Introduction: Epigenetic discusses to inherited changes in mitosis and meiosis in the gene expression pattern which is independent of primary DNA sequence. Since, SOCS1 hyper-methylation can activate JAK / STAT signaling pathway and activation of this pathway can directly affect the impact of different cytokines on cell function and subsequently lead to pathophysiology of diseases, in particular autoimmune diseases that interact directly with the amount of cytokines and due to the fact that the cause and pathology of Behcet's disease (BD) have not ever been completely determined. So, the purpose of this study was to evaluate the methylation pattern of SOCS1 gene in patients with BD and compare them with healthy group.

Methodology: This study was a case-control study in which 50 patients with BD and 60 subjects as healthy group participated. Blood samples were collected from all participants and then Peripheral Blood Mononuclear Cells (PBMCs) were isolated through Ficoll method. After extraction of DNA by Salting out method and its analysis with Nano-drop, the methylation level of SOCS1 was examined using qMS-PCR technique.

Results: Findings about methylation and gene expression in SOCS1 gene showed that the level of SOCS1 methylation was increased in patient groups compared with healthy subjects (control group) which the increase was statistically significant ($p\text{-value}<0.05$). Also, the results of gene expression revealed that the fold change of SOCS1 gene expression was decreased in patient group compared with healthy subjects which the decrease was statistically significant ($p\text{-value}<0.05$).

Discussion and conclusion: According to the results of this study, it can be suggested that the DNA methylation of SOCS1 gene is likely to affect the gene expression and thereby contribute to the pathogenesis of Behcet's disease.

ACCEPTED MANUSCRIPT

Keywords: DNA methylation, Behcet's disease, SOCS1 gene, qMS-PCR

Introduction:

BD is characterized by recurrent oral pseudoephedrine, uveitis, aphthous stomatitis and genitalia ulcers and skin lesions. Since vascular manifestations are common in this disease, it is considered as vasculitis (1, 2). Although small vessels are specific in the wall without microscopic changes, thrombophilia or clotting is common in small and large vessels. However, arthritis is rare in this disease. In this regard, BD is unlikely to be comparable to other vasculitis (3). The disease is more prevalent in Silk Road countries. Silk Road is an ancient road between Mediterranean and East Asia, where has been known as the main place of this disease prevalent (4). The exact etiopathology of BD has not yet been clarified. However, most studies have shown that the disease is caused by environmental factors such as infectious agents or contamination in patients with a history of genetic predisposition (5). Genetic and epigenetic alterations that involved in chronic disease suggests new pathways for research and will prepare new goals for treatment of these diseases such as BD (6).

Recent studies have shown that there are strong evidences on the role of epigenetic in pathogenesis of autoimmune diseases in addition to genetics (7, 8). DNA methylation is one of the most important epigenetic mechanisms involved in gene expression regulation process. Abnormal methylation of CpG islands in association with a promoter can lead to loss of gene expression, which is an alternative mechanism of gene deactivation in functional reduction mutations (9). Regulation of inflammatory cytokines network through epigenetic mechanisms has been proven in many studies. On the other hand, the methylation amount of CpG increases in aging, which suggests the spread of chronic diseases and can be attributed to the progression of essential genes' inactivation (10).

Various cytokines such as interferons, interleukins, and colony stimulating factors are attached to a large family of type I / II cytokine receptors on the membrane and lead to their phosphorylation through their JAK3 proximal membrane (11).

SOCS1 hyper-methylation can activate JAK / STAT signaling pathway and activation of this pathway can directly affect the impact of various cytokines such as IL-6[1] on cell function and subsequently lead to pathophysiology diseases, especially diseases such as autoimmune diseases, which are directly related to the amount of involved cytokines (12). Interestingly, SOCS1 function is not limited only to regulation of JAK / STAT signaling pathway, since SOCS1 also inhibits signaling transmission of cytokines and factors such as insulin and toll-like receptors, which each of them activates JAK / STAT pathway. SOCS1 inhibit the cytokine production through an interaction with Jak protein family. The SH2 domain of SOCS1 joins

[1] IL-6 refers to Interleukin 6 in humans. Interleukin 6 is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory cytokine

ACCEPTED MANUSCRIPT

to a JH1 domain of Jak2 and prevents its phosphorylation, thus downregulating the Jak/STAT pathway (13, 14). Existing evidence indicates the inaccurate expression of SOCS1 in human disease, especially in some malignancies (15). The results obtained from some studies suggest that SOCS1 plays an important role in function and accuracy of Treg cells through maintaining expression of Foxp3 and inhibiting production of IFNγ and IL-17a which are driven by STAT1 and STAT3, respectively (16).

Since rheumatologic diseases are among diseases associated with environmental factors such as race in addition to genetics and due to the fact that epigenetic processes affect expression of genes through environmental factors (17, 18), the main purpose of present study was to investigate the effect of methylation and expression of a number of genes on this disease in Azeri race. Due to proven role of JAK1 and STAT3 genes in rheumatologic and BD and lack of study on the amount of methylation and expression of genes involved in this pathway, the amount of methylation and expression of SOCS1in BD patient's gene have been evaluated in present study as one of the main regulators of JAK / STAT pathway in order to provide the possibility of identifying possible mechanisms of these gens related to BD.

Methods and materials:

Cases and controls study group:

All cases accessed their written informed agreement for this study, and the study protocol was allowed by the ethics committee in Tabriz University of Medical Sciences, Tabriz, Iran (Permit Number: TBZMED.REC.1396.830). The study group contained of 47 Iranian patients with BD 61.7% men and 38.3% women and 61 healthy candidates (19). The analysis of BD was based on the international study group criteria for BD. (20) Features of the patients were estimated at the time of diagnosis and are summarized in **Table 2**. The control group composed of 61 age, gender, and ethnically matched healthy subjects (59% men versus 41% women) without any clinical or laboratory signs of autoimmune or inflammatory diseases.

DNA, RNA extraction and RT-PCR method:

PBMCs were separated from EDTA blood tubes by Ficoll method (Lymphodex, Inno -Train, Germany) and directly stored at −80 °C until use. DNA samples of subjects were extracted by using salting out method from the peripheral blood collected in tubes containing EDTA. Total RNA was extracted from the PBMCs according to the TRIzol method (Invitrogen, San Diego, CA), followed by reverse transcription using the reverse transcription reagent kit (Thermo Fisher scientific, USA). Then, purity and concentration of DNAs and RNAs were estimated by nanodrop (19). The entirety of total RNAs was showed by gel electrophoresis of the individual samples on a 2% agarose gel.

SOCS1 gene sequence and data about promoter were picked up from NCBI (The National Center for Biotechnology Information) and EPD (eukaryotic promoter database) databases.

ACCEPTED MANUSCRIPT

For SOCS1 mRNA sequence, the primer pairs were designed using PrimerQuest Tool, (IDT, USA and Canada) and then primer pairs of methylation sequence were designed using MethPrimer online and OLIGO software's (**Table 1**). Also, SOCS1 gene Promoter CpG islands were predicted with eukaryotic promoter database (EPD) (**Figure 1**). Then the expression of SOCS1 was measured by MIC real-time instrument (Bio Molecular Systems, AUSTRALIA). β-actin was chosen as the housekeeping gene for expression and copy number variation detection and its expression was evaluated by the following primers in **Table 1**. Fold change of SOCS1 expression were calculated using the ΔΔCt formula. All tests were performed in at three biological repeats.

Methylated DNA assessment:

In this method, genomic DNA was converted by bisulfite reaction in which unmethylated cytosine's of CpG islands are modified to uracil but methylated ones in DNA were left unmodified. Therefore, methylation and unmethylation of CpG dinucleotide was distinguished in DNA sequences.

In order to succeed in these methods, the most important step is design primers for the modified DNA. One pair of primers were designed using Primer Quest Tool and methMarker (PREMIER Biosoft, CA, USA) to amplify CpG islands of TSS (transcription start site) upstream (**Figure 1**). DNAs (0.5–2mg) were treated by the EZ DNA Methylation-Gold™ Kit (ZymoResearch, United State, cat no: D5005 & D5006) according to the manual kit protocol and subsequently, modified DNAs were used for qMS-PCR as a template. Also, in order to quantify the methylation level of this sites, following the gel electrophoresis, amounts of methylation and non-methylation sites are analyzed by real-time PCR method.

qMS-PCR amplification was carried out using 1 µl of DNA in a 10 µl PCR reaction along with primer sets. The assays were performed in three replicates and relative methylation fold change was measured for each sample with DM% method which is referred to below. PCR cycles and the timing steps include a holding cycle at 95 ° C for 15 minutes, the activation phase was performed in two steps, including DNA denaturation step of 95 °C for 10 seconds and 60 ° C for 45 seconds in 40 cycles.

The percent demethylation (DM) of SOCS1 fragments was calculated using a formula described in manufacture protocol of this kit using a ratio of amplification efficiency of the methylated samples over that of non-methylated samples:

$$DM\ \% = 100/\ [1+2^{(Ct.TG - Ct.CG)}]$$

ACCEPTED MANUSCRIPT

Statistical analysis:

Statistical analysis was performed using SPSS software version 20 (SPSS, Chicago, IL, USA). Normal distributions were tested with the Kolmogorov–Smirnov test with Lilliefors correction. Quantitative data were presented as mean ± standard deviation (SD). The differences in mRNA and serum levels of SOCS1 between control and BD groups were evaluated by Mann-Whitney U test. P-value <0.05 was considered as significant difference.

Results:

Subject Characteristics

Demographic and clinical characteristics of contributors are existing in **Table 2**. The patient group consisted of 29 males and 18 females, with a mean age of 38.02 ± 10.25 years. The control subjects included 37 males and 24 females and had a mean age of 37.4 ± 8.5 years (19). Significant difference was detected in fold change of SOCS1 expression as severe BD subgroups between cases and controls ($P < 0.05$) while was no significant difference in change fold of gene expression, between other subgroups.

As shown in the table, items that have a statistically significant difference are shown as **Bold**. SOCS1: Suppressor of cytokine signaling 1, SD: standard deviation, HLA: Human leukocyte antigen, BD: Behçet's disease.

Real-time Quantitative PCR for SOCS1 gene:

In order to compare the level of SOCS1 gene expression in two groups of case and control subjects, an independent T-test was adopted, taking into consideration the data obtained by employing kolmogorov smirnov test, was normal (P-value>0.05). The results were suggestive of significant difference between two groups (p-value<0.05). As we expected, the level of gene expression showed decline in case individuals in comparison with control group (**Figure 3**). Also in the patient subjects, we investigated the relationship between gene expression and clinical features. As the results show, the gene expression level of SOCS1 was significant difference in fold change of gene expression, between the severe BD subgroups (**table 2**).

Methylation status and mRNA expression:

ACCEPTED MANUSCRIPT

We evaluated the correlation between the promoter methylation level of the SOCS1 gene and mRNA expression. The mRNA (2.49±0.83 *versus* 2.91±0.46, $P<0.05$) of SOCS1 was significantly lower in the cases with BD than control subjects. Also, in order to compare the methylation status of SOCS1 in two groups of case and control individuals, an independent T-test was adopted, taking into consideration the data obtained by employing kolmogorov smirnov test, was normal (P-value>0.05). The results shown that there was a statistical difference between both groups in terms of methylation rate of SOCS1 gene (P-value<0.001). In line with our expectations, the methylation ratio of SOCS1 revealed increase in case individuals group in comparison with control group (4.86±1.27 versus 2.03±0.71) (**Figure 4**).

Also in the case groups, we analyzed the relationship between methylation level and clinical features. The results indicated that there was no significant difference in the level of methylation in any of the subgroups mentioned in the table (p-value> 0.05) (**table 2**).

In the figure of the CT (**Figure 5**), As initial values higher (copy number), the respective chart begins faster to rise. In this regard, most of the SOCS1 gene samples were non-methylated. Therefore, the initial values of this gene were higher and thus their CT values were lower (faster to rise in the chart). (see Figure 5).

Discussion:

Although the etiology of this disease remains unknown, it is believed that Behcet's disease begins with environmental factors such as microbial agents in people with a specific genetic context. The interaction between genetics and environmental factors can be the basis of pathogenicity in people with this disease. Recent studies have shown that there are strong evidences on the role of epigenetics in pathogenesis of autoimmune diseases in addition to genetics. Epigenetics helps to understand complex diseases (21). DNA methylation is one of the well-studied epigenetic biomarker. Epigenetic studies conducted among different populations have shown that there is a relation between demographic factors and genome methylation pattern (22).

Increasing cytokines or their signaling impairment causes various diseases such as allergies of autoimmune diseases, inflammation and cancer (23-25). Most cytokines utilize from Janus kinase signaling pathway and transcriptional activating factors. This pathway is negatively regulated by various mechanisms including SOCS proteins (cytokine signaling inhibitors) (26). SOCS protein binds to JAK or cytokine receptors and leads to inhibition of signaling events. In particular, SOCS1 and SOCS3 inhibitors are most potent JAK inhibitors because

ACCEPTED MANUSCRIPT

they contain 2 inhibitory kinase regions at N end. Studies conducted on mice with overexposed genes showed that SOCS proteins are the main physiologic and pathologic regulator in homeostasis of the immune system (27).

It has been identified that SOCS1 has tumor suppressive activity and repetition of SOCS1 gene causes inhibition of growth and induction of apoptosis in HCC (Hepatocellular Carcinoma) cells (13). Since SOCS1 hyper-methylation can activate - JAK / STAT signaling pathway, activating this pathway can directly affect the impact of various cytokines, such as IL-6 on the function of cells and consequently, lead to pathophysiology of diseases, especially diseases such as autoimmune diseases which directly affect cytokines rate (12). Therefore, the purpose of present study was to investigate the methylation amount of Socs1 gene and its role in gene expression regulation in patients with Behcet's disease.

The results of methylation and gene expression in this study showed that methylation level in patient group has been increased compared with healthy group (hypermethylated), which the increase was statistically significant. Also, the results of gene expression indicated that mean of gene expression has been decreased in patient group compared with healthy group, which the decrease was statistically significant. In another study, Davey GM et al (2006) investigated the regulating role of SOCS1 in cellular inflammation. This protein acts as a negative regulator of cytokine signaling. According to their results, this protein is able to inhibit phosphorylation and JAK function (15). In addition, SOCS1 has a complex role in T cell activation and studies have revealed that this protein plays a significant role in regulation of IL-4, IL-12, and IL-15 cytokines. Also, the abarrent expression of SOCS1 has been proven in human disease, especially in some malignancies (15). Other studies suggest that SOCS1 plays an important role in function of Treg cells by maintaining Foxp3 expression and inhibiting the production of IFNγ and IL-17a which are driven by STAT1 and STAT3, respectively (16).

The results obtained from study of Galm et al showed that SOCS1 gene silencing is probably due to its hyper-methylation in patients with myeloma. It was also identified that SOCS1 gene silencing can disrupt the negative regulation of JAK / STAT pathway. Therefore, responsiveness to cytokines is increased and supports survival and expansion of cancer cell line U266 (28). The results of other study conducted by Oshimo et al showed that methylation of SOCS1 gene has a significant relationship with lymph node metastasis, stage of tumor progression as well as reduction of SOCS1 expression in cancerous tissues. Decreased expression of SOCS1 in cancerous tissues was associated with metastatic lymph nodes and advanced tumor stage. These results suggest that deactivating transcription of SOCS1 gene through hyper-methylation mechanism is associated with progression of gastric cancer metastasis (29). However, some studies have reported conflicting results of these studies and our study. For example, Hamedi et al. have shown that the expression of SOCS has been significantly increased in PBMCs of patients with BD compared with healthy individuals (30). According to the results of various studies and our study, it is suggested that further research

ACCEPTED MANUSCRIPT

be done in order to find out the exact mechanism of SOCS genes in Behcet's disease and other autoimmune diseases. According to the results of present study and results obtained from other studies, it is possible that one of the regulatory mechanisms for gene expression in SOCS1 has been occurred by adjusting the amount of methylation.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

References

1. Vallet H, Riviere S, Sanna A, Deroux A, Moulis G, Addimanda O, et al. Efficacy of anti-TNF alpha in severe and/or refractory Behcet's disease: Multicenter study of 124 patients. J Autoimmun. 2015;62:67-74.
2. Sfikakis PP, Markomichelakis N, Alpsoy E, Assaad-Khalil S, Bodaghi B, Gul A, et al. Anti-TNF therapy in the management of Behcet's disease--review and basis for recommendations. Rheumatology (Oxford). 2007;46(5):736-41.
3. Hirohata S, Kikuchi H. Behçet's disease. Arthritis Res Ther. 2003;5(3):1.
4. Idil A, Gürler A, Boyvat A, Caliskan D, Özdemir Ö, Isik A, et al. The prevalence of Behçet's disease above the age of 10 years The results of a pilot study conducted at the Park Primary Health Care Center in Ankara, Turkey. Ophthalmic Epidemiology. 2002;9(5):325-31.
5. Yesudian P, Edirisinghe D, O'Mahony C. Behçet's disease. International journal of STD & AIDS. 2007;18(4):221-7.
6. Alipour S, Nouri M, Sakhinia E, Samadi N, Roshanravan N, Ghavami A, et al. Epigenetic alterations in chronic disease focusing on Behçet's disease. Biomedicine & Pharmacotherapy. 2017;91:526-33.
7. Kaya TI. Genetics of Behçet's disease. Pathology research international. 2011;2012.
8. Karasneh J, Gül A, Ollier WE, Silman AJ, Worthington J. Whole-genome screening for susceptibility genes in multicase families with Behçet's disease. Arthritis & Rheumatism. 2005;52(6):1836-42.
9. Robertson KD. DNA methylation and chromatin-unraveling the tangled web. Oncogene. 2002;21(35):5361.
10. Trenkmann M, Brock M, Ospelt C, Gay S. Epigenetics in rheumatoid arthritis. Clinical reviews in allergy & immunology. 2010;39(1):10-9.
11. Murray PJ. The JAK-STAT signaling pathway: input and output integration. The Journal of Immunology. 2007;178(5):2623-9.
12. Chinen T, Kobayashi T, Ogata H, Takaesu G, Takaki H, Hashimoto M, et al. Suppressor of cytokine signaling-1 regulates inflammatory bowel disease in which both IFNγ and IL-4 are involved. Gastroenterology. 2006;130(2):373-88.
13. Yoshikawa H, Matsubara K, Qian G-S, Jackson P, Groopman JD, Manning JE, et al. SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. Nature genetics. 2001;28(1):29-35.
14. Endo TA, Masuhara M, Yokouchi M, Suzuki R, Sakamoto H, Mitsui K, et al. A new protein containing an SH2 domain that inhibits JAK kinases. Nature. 1997;387(6636):921.
15. Davey G, Heath W, Starr R. SOCS1: a potent and multifaceted regulator of cytokines and cell-mediated inflammation. HLA. 2006;67(1):1-9.
16. Takahashi R, Nishimoto S, Muto G, Sekiya T, Tamiya T, Kimura A, et al. SOCS1 is essential for regulatory T cell functions by preventing loss of Foxp3 expression as well as IFN-γ and IL-17A production. Journal of Experimental Medicine. 2011:jem. 20110428.
17. Fitzpatrick DR, Wilson CB. Methylation and demethylation in the regulation of genes, cells, and responses in the immune system. Clin Immunol. 2003;109(1):37-45.
18. Ozanne SE, Constancia M. Mechanisms of disease: the developmental origins of disease and the role of the epigenotype. Nat Clin Pract Endocrinol Metab. 2007;3(7):539-46.
19. Afkari B, Babaloo Z, Dolati S, Khabazi A, Jadidi-Niaragh F, Talei M, et al. Molecular Analysis of Interleukin-10 Gene Polymorphisms in Patients with Behçet's disease. Immunology letters. 2017.
20. disease CfdoBs. International Study Group for Behçet's Disease. Lancet. 1990;335:1078-80.
21. Hatchwell E, Greally JM. The potential role of epigenomic dysregulation in complex human disease. TRENDS in Genetics. 2007;23(11):588-95.
22. Jiang R, Jones MJ, Chen E, Neumann SM, Fraser HB, Miller GE, et al. Discordance of DNA Methylation Variance Between two Accessible Human Tissues. Scientific reports. 2015;5.
23. Talaat RM, Mohamed SF, Bassyouni IH, Raouf AA. Th1/Th2/Th17/Treg cytokine imbalance in systemic lupus erythematosus (SLE) patients: Correlation with disease activity. Cytokine. 2015;72(2):146-53.

ACCEPTED MANUSCRIPT

24. Arend WP, editor Cytokine imbalance in the pathogenesis of rheumatoid arthritis: the role of interleukin-1 receptor antagonist. Seminars in arthritis and rheumatism; 2001: Elsevier.
25. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. Nature Reviews Cancer. 2004;4(1):11.
26. Cooney RN. Suppressors of cytokine signaling (SOCS): inhibitors of the JAK/STAT pathway. Shock. 2002;17(2):83-90.
27. Yoshimura A, Suzuki M, Sakaguchi R, Hanada T, Yasukawa H. SOCS, inflammation, and autoimmunity. Frontiers in immunology. 2012;3.
28. Galm O, Yoshikawa H, Esteller M, Osieka R, Herman JG. SOCS-1, a negative regulator of cytokine signaling, is frequently silenced by methylation in multiple myeloma. Blood. 2003;101(7):2784-8.
29. Oshimo Y, Kuraoka K, Nakayama H, Kitadai Y, Yoshida K, Chayama K, et al. Epigenetic inactivation of SOCS-1 by CpG island hypermethylation in human gastric carcinoma. International journal of cancer. 2004;112(6):1003-9.
30. Hamedi M, Bergmeier L, Hagi-Pavli E, Vartoukian S, Fortune F. Differential expression of suppressor of cytokine signalling proteins in Behçet's disease. Scandinavian journal of immunology. 2014;80(5):369-76.

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure captions

Figure 1. Socs1 promoter methylation**.** CpG site and island around transcription start site (TSS) were predicted by EPD and CpG island Finder software in -1000 up 1000 around primer.

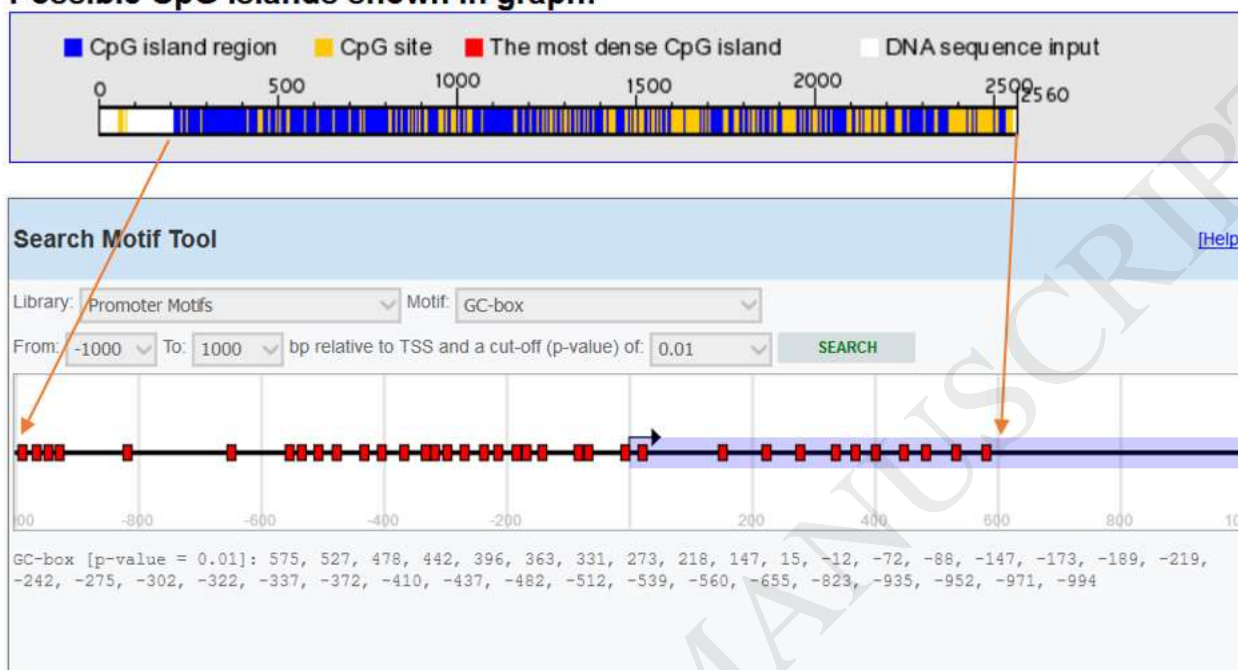
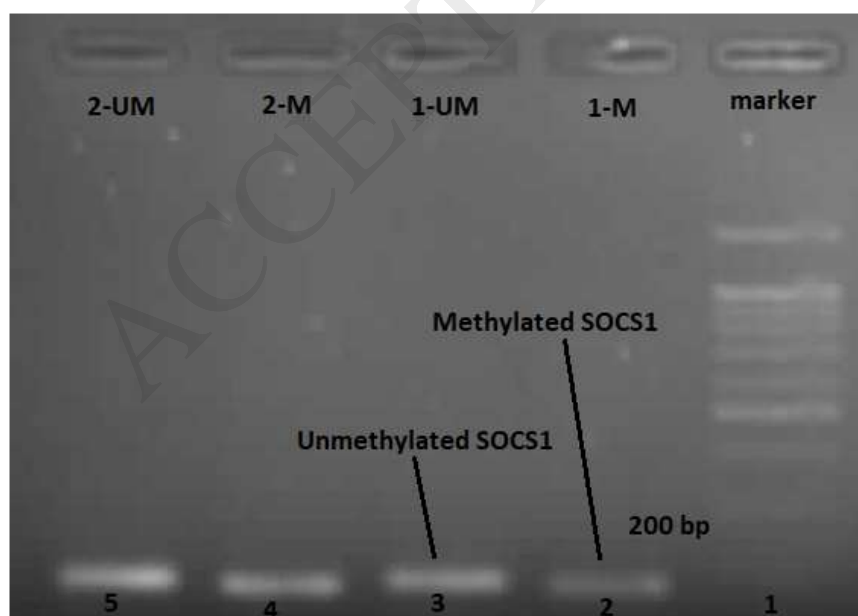


Figure 2- DNA samples loading in gel electrophoresis. Lane (1) 200 bp ladder, Samples 1,2 in lane 2 and 4 are methylated while samples 3 and 5 are unmethylated of SOCS1 promoter. As shown in the picture, the band of unmethylated samples is sharper than methylated samples.



ACCEPTED MANUSCRIPT

Figure 3- Fold change of SOCS1 expression. Regarding the average changes in the expression of the socs1 gene in the patients with BD and the healthy groups, the amount of it is comparable to that of the healthy group in the patient group, which indicates that SOCS1 gene expression was declined among the cases in the patient group.

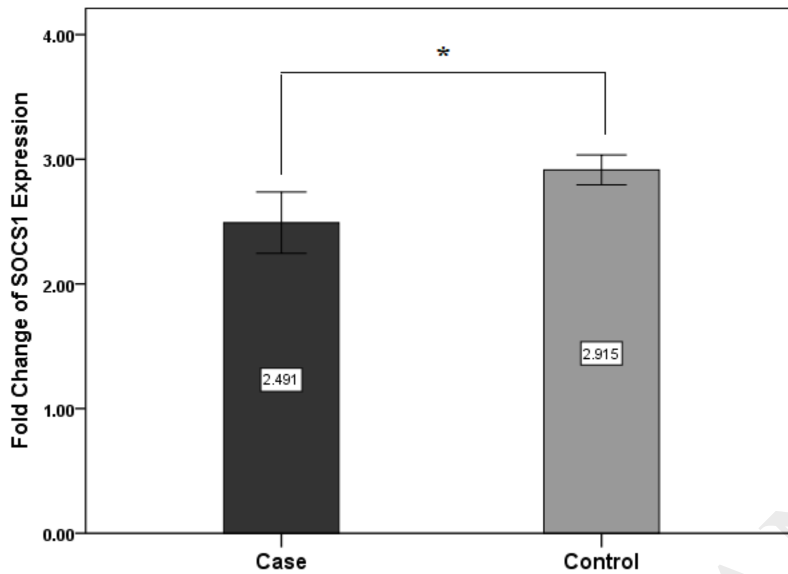
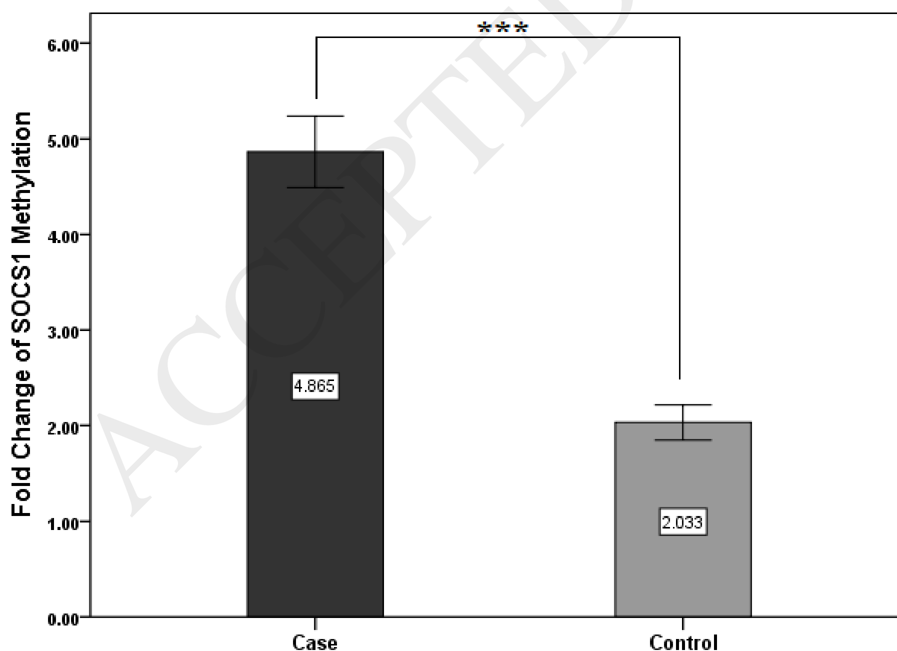


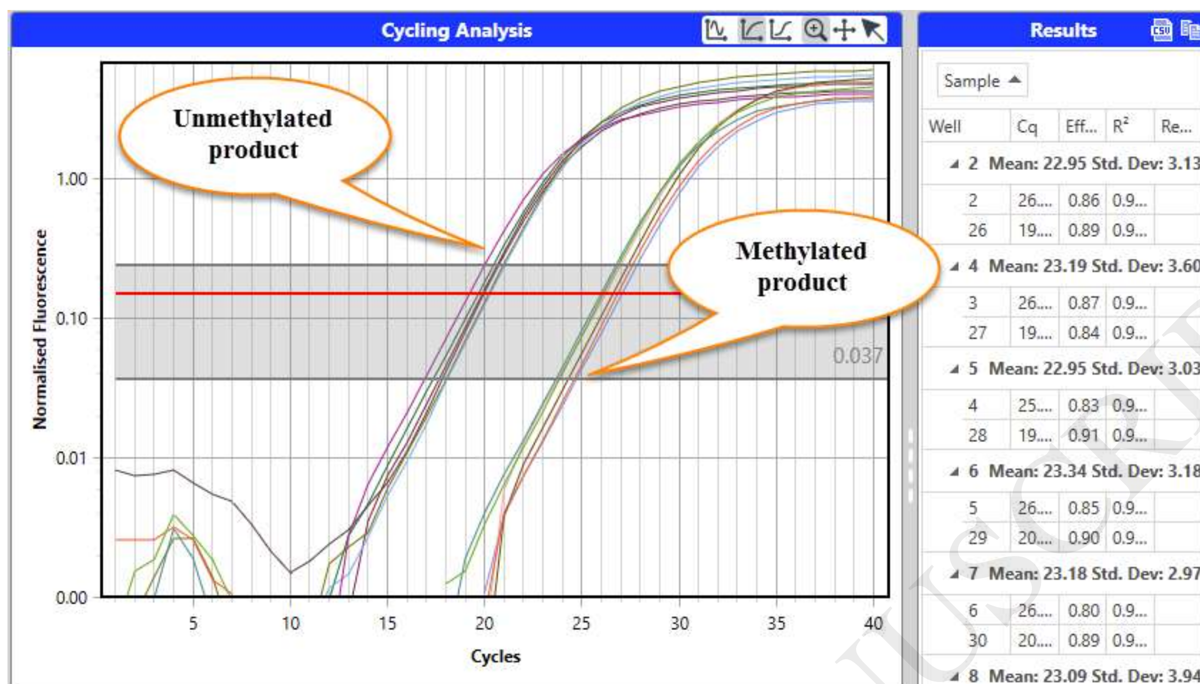
Figure 4- Change fold of SOCS1 methylation. As shown in the chart, the fold change of SOCS1 methylation in the patient group is significantly higher than the control group.



ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Figure 5- CT curve (Cycling threshold) in samples with methylated and non-methylated primers.



ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table

Table 1- PCR Primers AND Product size

		Target size
QMS-PCR FOR SOCS1	FM-SOCS1: GTTCGTCGGTTTTATTTAGGTCGTTTTTC RM-SOCS1: CGATTCTAACTCCAACCGTCCG	165
	FU-SOCS1: GGGTTTTTGTTGGTTTTATTTAGGTTGTTTTTT RU-SOCS1: CAACCAATTCTAACTCCAACCATCCA	173
SOCS1 for expression	F: SOC1: CTTCTGTAGGATGGTAGCACAC R:SOCS1: AGGAAGAGGAGGAAGGTTCT	98
Beta-actin	F: b-actin GGTGAAGGTGACAGCAGT R:b-actin TGGGGTGGCTTTTAGGAT	154

ACCEPTED MANUSCRIPT

ACCEPTED MANUSCRIPT

Table 2- Clinical profile of patients with SOCS1 gene expression and its methylation

Characteristics and Clinical features expression	Frequency	Methylation level of SOCS1 expression (mean ± SD)	P-value	Fold change of SOCS1 expression (mean ± SD)	P-value
Age					
<45	32 (69%)	5.02±1.27	0.207	2.34±0.85	0.08
≥45	15 (31%)	4.5±1.2		2.8±0.71	
Gender					
Male	66(61.1%)	3.31±1.91	0.685	2.71±0.68	0.668
female	42(38.9%)	3.18±1.39		2.76±0.68	
HLA-B5-					
Positive	17 (65.4%)	5.02±0.87	0.265	2.77±0.65	0.685
Negative	9 (34.6%)	4.54±1.2		2.89±0.71	
HLA-B51					
Positive	8 (46.7%)	4.98±1.14	0.888	2.54±0.69	0.265
Negative	7 (53.3%)	4.91±0.88		2.95±0.67	
HLA-B27					
Positive	2 (8.3%)	3.59±2.39	0.572	2.81±0.49	0.891
Negative	22 (91.7%)	4.94±0.89		2.72±0.82	
Oral aphtha					
Positive	45 (96%)	4.83±1.28	0.466	2.49±0.82	0.953
Negative	2 (4%)	5.51±0.85		2.45±1.41	
Genital ulcer					
Positive	24 (51%)	4.8±1.51	0.744	2.48±0.95	0.956
Negative	23 (49%)	4.92±0.98		2.49±0.71	
Pathergy					
Positive	8 (24%)	4.61±1.31	0.388	2.42±0.59	0.371
Negative	25 (76%)	4.97±0.91		2.71±0.84	
Sever B.D					
Positive	26 (55%)	4.85±1.35	0.941	2.29±0.91	**0.05**
Negative	21 (45%)	4.88±1.19		2.73±0.67	
Severe eye involvement					
Positive	10 (22%)	5.37±1.62	0.181	2.49±0.92	0.999
Negative	36 (78%)	4.76±1.14		2.49±0.83	

ACCEPTED MANUSCRIPT