ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
April 2015; 14(2):179-187.

Investigating the Effect of rs3783605 Single-nucleotide Polymorphism on the Activity of *VCAM-1* Promoter in Human Umbilical Vein Endohelial Cells

Fatemeh Dadgar Pakdel[1,2], Mohammad Keramatipour[2], Farshid Noorbakhsh[1], Saeed Talebi [2], and Mohammad Vodjgani[1]

[1] *Department of Medical Immunology, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[2] *Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

Received: 20 January 2014; Received in revised form: 3 July 2014; Accepted: 3 August 2014

ABSTRACT

The interaction between immune cells and endothelial lining of blood vessels is vital in many processes such as inflammatory and immune responses as well as cancer cell metastasis. The expression level of *VCAM-1* is regulated by many factors including the promoter activity that is possibly affected by the single nucleotide polymorphisms (SNPs) present in the promoter. There are previous reports suggesting an important role for rs3783605 at -420 position in the pathogenesis of VCAM1-associated diseases. This is possibly due to the effect of this SNP on promoter activity and gene expression. Therefore, present study was designed to investigate the effect of rs3783605 on the activity of *VCAM-1* gene promoter in human umbilical vein endothelial cells (HUVEC).

In this study, two appropriate expression vectors containing *VCAM1* promoter with different alleles of rs3783605 were constructed to express the Green Fluorescent Protein (GFP). Expression vectors were transfected into HUVECs and their EGFP expression level was assessed by the fluorescent microscopy and real-time PCR.

Bright green fluorescence was seen in the HUVECs transfected by expression vector containing *CMV* promoter. The expression level in the cells transfected by vector containing promoter with A allele of rs3783605 was 0.14888 folds and G allele was about 0.37851 folds of cells transfected by vector having CMV promoter ($p<0.001$). Moreover, HUVECs transfected by G allele of rs3783605 showed about 2-fold higher transcriptional activity compared with the A allele, ($p=0.049$).

Our findings showed that rs3783605 polymorphism may play a role in VCAM-1 gene expression. Therefore, it is likely that it may have an important role in the pathogenesis of VCAM1-associated diseases and tumor metastases.

Key words: Human Umbilical Vein Endohelial Cells; Interleukin 17E; Polymorphisms, Promoter; Single Nucleotide; Vascular Cell Adhesion Molecule-1

Corresponding Authors: Mohammad Keramatipour, MD, PhD and Mohammad Vodjgani, PhD;
Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences (TUMS), Tehran, Iran. Tel/Fax: (+98 21) 8895 3005, E-mail: keramatipour@sina.tums.ac.ir.
Department of Medical Immunology, School of Medicine, Tehran University of Medical Sciences (TUMS), Tehran, Iran. Tel: (+98 21) 6641 9536, E-mail: Vojganim@sina.tums.ac.ir

INTRODUCTION

The vascular cell adhesion molecule 1 (VCAM-1) is the immunoglobulin like transmembrane protein that is induced on the surface of endothelial cells by inflammatory cytokines such as TNF-α after inflammation. It binds to very late antigen 4 (VLA-4)

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

integrin on the surface of monocytes, T and B cells, basophils and eosinophils.[1,2]

The migration of immune cells to the inflammatory sites and the interaction of VLA-4 on these cells with VCAM-1 on the endothelium can lead to different inflammatory processes.[3] This molecule has important roles in the pathogenesis of several diseases such as rheumatoid arthritis, experimental autoimmune encephalomyelitis, nephrotoxic nephritis, skin allergic reactions and asthma.[3,4] In addition to these roles, VCAM-1 is important in tumor metastases.[3] ETS (E26 transformation-specific) transcription factors have conserved DNA binding domains that recognize DNA sequence GGAA/T.[5-7] The involvement of ETS genes in cancer and cellular proliferation and metastasis have been reported.[5,7] ETS2 is the end effector molecule of mitogen-activated protein kinase (MAPK) pathway.[5] *VCAM-1* is a target gene that its expression is affected by ETS transcription factors.[5]

Several polymorphisms have been identified in the *VCAM-1* promoter that can affect the *VCAM-1* expression. There are previous reports suggesting an important role for rs3783605 at -420 position in the pathogenesis of VCAM1-associated diseases, possibly due to its effect on gene expression. The rs3783605 is located close to the binding site of ETS2 transcription factor on the VCAM-1 promoter. This might be an explanation for the effect of rs3783605 on expression level of VCAM-1 gene. Therefore, present study was designed to investigate the effect of rs3783605 on the activity of VCAM-1 gene promoter in Human Umbilical Vein Endothelial Cells (HUVEC).

MATERIALS AND METHODS

Polymerase chain reaction and Site Directed Mutagenesis

To find out the promoter sequence of *VCAM-1* the promoter database was searched. Necessary promoter elements including the positions of the transcription initiation site, core promoter elements and transcription factor binding sites were elucidated (Figure 1).

About 500bp upstream of the transcription start site was amplified using PCR. To obtain different alleles of rs3783605 on amplified promoter fragments, site directed mutagenesis was done simultaneously with PCR. To achieve this, two forward and one reverse primers were used with the following sequences. More details are presented in Figure 2.

VCAM-1 forward-1: 5’-GGC AGA TCT CTT TTA GAA TTG CAA ACA TAT TTC-3’

VCAM-1 forward-2: 5’-GGC AGA TCT CTT TTA GAA TTG CAA ACG TAT TTC-3’

VCAM-1 reverse: 5’-GCA GCT GGG AGG GTA TTC AGC TCC T-3’

Figure 1. The VCAM-1 promoter sequence. ATG: translation initiation site, +1: transcription starting site The transcription factors binding sites and A-420G SNP are shown (2).

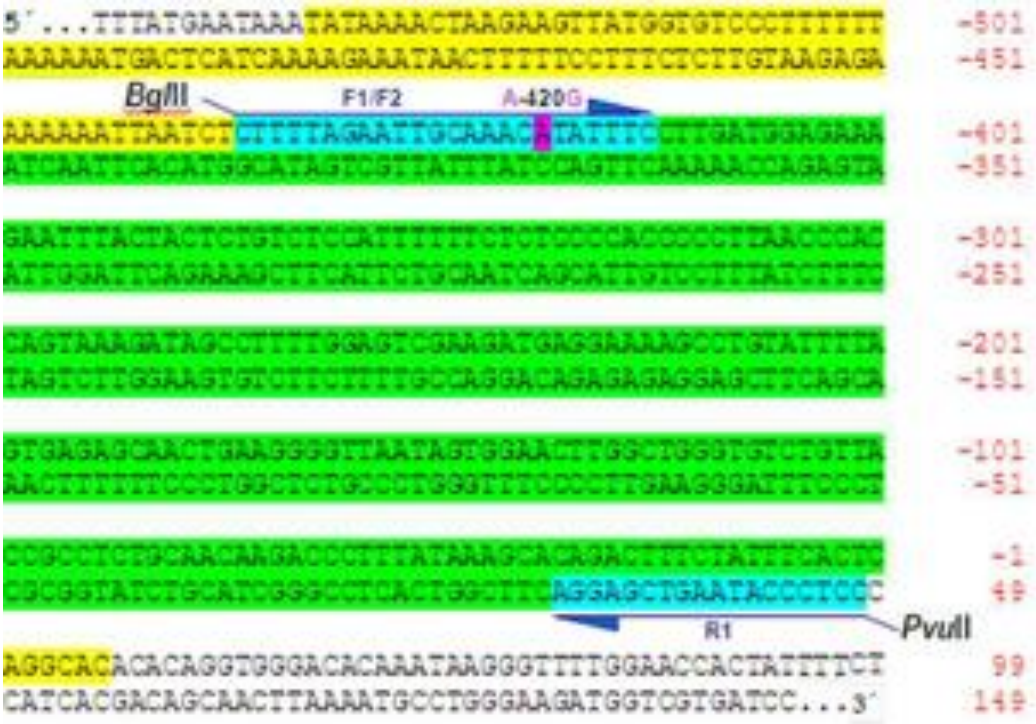

Figure 2. The VCAM-1 promoter sequence and the position of designed primers. F1: VCAM-1 forward-1, F2: VCAM-1 forward-2, R1: VCAM-1 reverse

PCR reaction was performed using master mix (2x) from Ampliqon company (Ampliqon A/S, Denmark). PCR products were run on 1% agarose gel (Figure 3). Resulted fragments were then purified from gel using Expin Gel SV Purification kit (GeneAll Biotechnology Co., South Korea) according to the manufacturer's protocol and the sequence were analyzed using sanger sequencing (Pishgam Biotech Co., Iran).

Vectors

The pTZ57R/T TA cloning vector (Thermo Scientific, USA) was used to clone the purified PCR products. Vector map is shown in figure 4. The PCR products were ligated into the vector as described in following section. After confirmation of having the correct fragment in the vector, another step of subcloning was performed, taking the promoter fragment out of the vector and ligating it into the pcDNA-GFP vector that was previously made in our lab. This step was done using BglII & PvuII in a double digestion reaction to obtain the promoter fragment first, and then the ligation reaction was performed to insert the fragment into a pre-digested pcDNA-GFP expression vector. The map for pcDNA-GFP vector is shown in figure 5.

TA Cloning in the pTZ57R/T Vector, Transformation and Screening of the Clones

3µl of Vector pTZ57R/T (0.17 pmol ends) was mixed with 6µl of the 5X Ligation Buffer, 90ng of the Purified *VCAM-1* Promoter Fragment (Approx. 130.54pmol ends) and 1µl of the T4 DNA Ligase and then nuclease-free water was added to this mixture up to total volume of 30µl. The mixture was vortexed

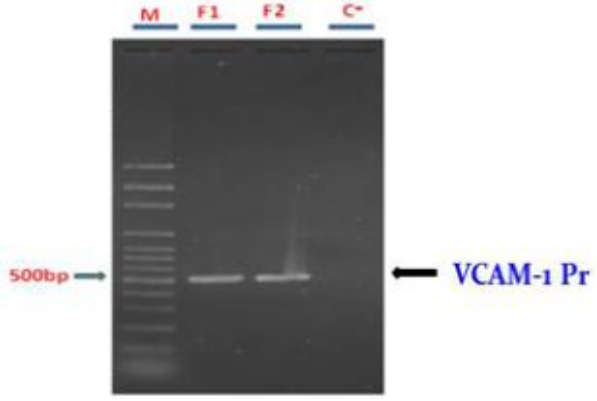

Figure 3. The electrophoresis of amplified *VCAM-1* promoter on the 1% agarose gel. M: 100bp DNA ladder. F1: amplified fragment by F1 primer. F2: amplified fragment by F2 primer. C- : negative control

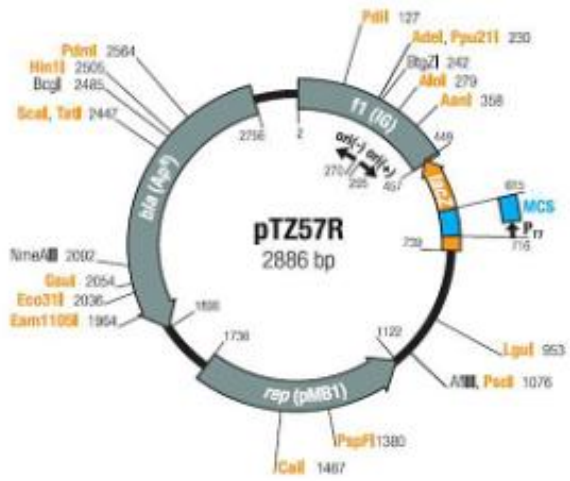

Figure 4. The pTZ57R/T TA cloning vector map. (http:// www.fermentas.com/catalog/kits/kitinstaclone.htm)

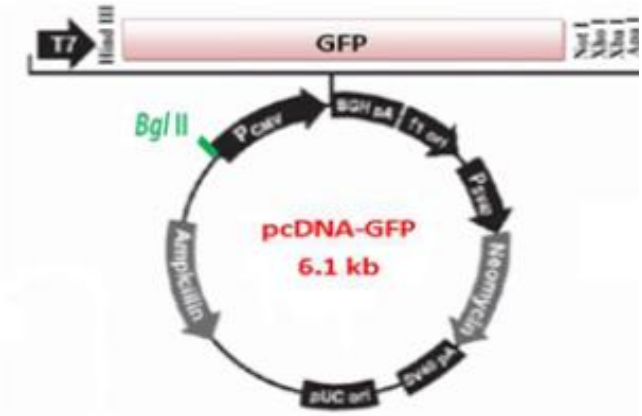

Figure 5. The pcDNA-GFP vector map (14).

briefly and centrifuged for 3-5 second. The ligation mixture was incubated at room temperature (22°C) for 1 hour. 2.5µl of the ligation mixture was used directly for bacterial transformation.

For preparation of competent cells, we used fresh bacterial colonies. LB agar plates, supplemented with ampicillin, X-Gal and IPTG were used and incubated in a 37°C incubator for at least 20 min before plating. 1.5 ml of C-medium was used for each of the two transformations. The bacterial culture was transferred to 1.5 ml of pre-warmed C-medium and was mixed. Then the tubes were incubated at 37°C for 2 hours in a shaker and the bacterial cells were pelleted by centrifugation. We mixed cells in 300 µl of T-solution and incubated on ice for 5 min. Then they were centrifuged for 1 min and the supernatant was discarded. Pelleted cells were resuspended in 120µl of T-solution (prepared according to the manufacturer's protocol) and incubated 5 min on ice. Then 2.5µl of ligation mixture was added into new microcentrifuge tubes and then was chilled on ice for 2 min. 50µl of the cells was added to each tube, mixed and incubated on ice for 5 min, then plated on pre-warmed LB plates. The plates were incubated overnight at 37°C.

We used plasmid mini prep Kit (GeneAll Biotechnology Co., South Korea), according to the manufacturer's protocol. After minipreparation, they were confirmed by PCR and sequencing. PCR was done by M13F and M13R (5 pmol/µI). The results of gel electrophoresis are shown in the Figure 6.

Subcloning in the pcDNA-GFP Vector, Transformation and Screening of the Clones

For preparation of the insert, we cut plasmids by BglII & PvuII (Thermo Scientific, USA) double digestion. 6µl of each restriction enzyme with 145ng/µl of DNA and 7µl of the Red Buffer (10X) were mixed and distilled water was added up to 70µl. Tubes were incubated for 16h at 37°C.

For preparation of the vector, pcDNA-GFP vector was digested by HindIII restriction enzyme (Thermo Scientific, USA). 5µl of HindIII was mixed with 20 µl of the pcDNA-GFP Vector 3100 ng/µI) and 10µl of the Red Buffer (10X). Then distilled water was added

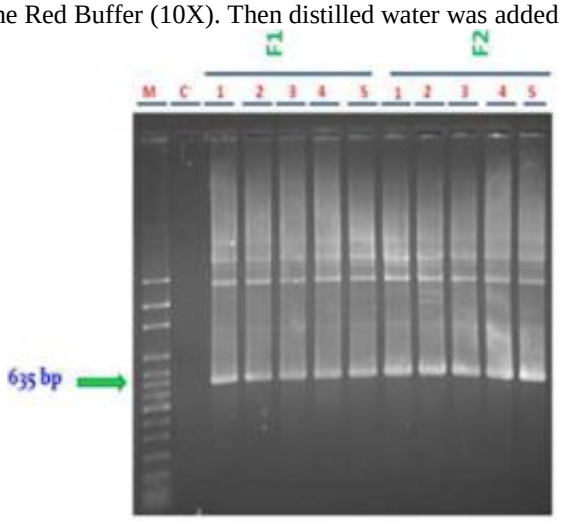

Figure 6. Screening of pTZ57R/T-VCAM1 Promoter Clones by PCR. M: 100bp DNA ladder. C-: negative control

F1: Clone containing A allele of rs3783605 in the VCAM-1 promoter. F2-3: Clone containing G allele of rs3783605 in the VCAM-1 promoter. All of the clones are containing proper sequence.

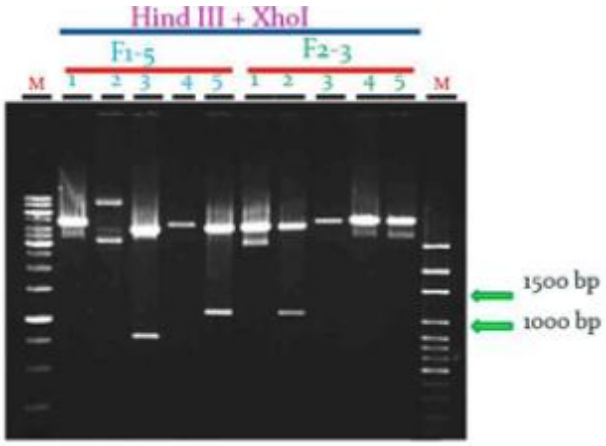

Figure 7. Screening of pcDNA-GFP/VCAM-1 Pr Clones. Hind III & Xho I: Restriction enzymes.
F1-5 (1-5): Clones that may be containing A allele of rs3783605 in the VCAM-1 promoter. F2-3 (1-5): Clones that may be containing G allele of rs3783605 in the VCAM-1 promoter. F2-3-2 and F1-5-5 likely are containing proper sequence.

up to 100µl. Tubes were incubated for 16h at 37°C. DNA was purified by gel purification kit (GeneAll Biotechnology Co., South Korea).

The End-filling reaction was performed by using Klenow Fragment. 2µl of the Klenow (4U/µl) (Thermo Scientific, USA) was mixed with 56µl of the pcDNA-GFP Vector (106.2 ng/µl), 3µl of the dNTP (5Mm), 8µl of Klenow Buffer and then distilled water was added up to 80µl. Tubes were incubated for 1h at 37°C and for 10 min in 75ºC for enzyme inactivation. DNA was purified by gel purification kit (GeneAll Biotechnology Co., South Korea).

This fragment was subsequently digested by *Bgl*II restriction enzyme (Thermo Scientific, USA). 25µl of *Bgl* II was mixed with 70µl of the pcDNA-GFP Vector (106.2 ng/µI) and 10µl of the Orange Buffer (10X) and then distilled water was added up to 100µl. Tubes were incubated for 16h at 37°C. DNA was purified by gel purification kit (GeneAll Biotechnology Co., South Korea).

In the final step, prepared inserts were ligated into the pcDNA-GFP vector. 10.5µl of pcDNA-GFP Vector (7.9ng/µl) from each allele was mixed with *VCAM-1* Promoter (9ng/µl), 1µl of T4 DNA Ligase and 4µl of the Buffer (5X) (Thermo Scientific, USA). Distilled water was added up to 20µl. 2 tubes from each reaction were prepared and the tubes were incubated overnight at 4 °C.

For transformation, 5µl of the ligation reaction was added to 50µl of the XL1 blue competent cells. Tubes were incubated on ice for 30min and were heat shocked for 60-90s. Afterwards, the tubes were incubated on the ice for 2min and 950µl of Luria-Bertani (LB) broth was added. Then it was incubated at 37ºC for 1h with shaking. 200µl of the mixture was spread on LB agar supplemented with ampicillin (100µg/ml) and the plate was incubated overnight at 37ºC.

After subculture, mini preparation was done by using mini-prep kit (GeneAll Biotechnology Co., South Korea). The clones were confirmed by digesting with XhoI and HindIII as well as DNA sequencing (Figure 7).

Cell Culture, Transfection and Fluorescence Microscopy

HUVECs were purchased from Pasteur institute of Iran and cultured in DMEM/Ham's F12 medium containing 10% fetal bovine serum, 100 IU/ml penicillin and 100µg/ml streptomycin (Gibco co., USA) at 37ºC, 5% CO2 in 75 cm2 flasks. After three passages, when the cultured cells reached to 70-90% confluency, we harvested the cells by trypsin dissociation. After cell counting with Neubauer chamber, we seeded about $7x10^4$ cells in each well of 6 well plate, in 4 ml of growth medium 24 hours prior to transfection and then HUVECs were transfected by TurboFect transfection reagent (Thermo Scientific, USA) according to the manufacturer's protocol. We mixed 6µl of Turbo Fect reagent with the 4µg diluted DNA and analyzed transgene expression 24 hours later. After 24 hours, the fluorescence activity was monitored and then cells harvested by trypsin-EDTA dissociation and the pellete of cells were transferred to -80°C for subsequent RNA extraction.

Real-Time PCR

Total RNA was isolated from HUVECs using Ribospin kit (GeneAll Biotechnology Co., South Korea) and then cDNA was synthesized by M-MLV cDNA Synthesis Kit (Enzynomics, South Korea) according to the manufacturer's protocol. For quantitative PCR (qPCR), RealQ PCR 2X master mix with green dye without ROX (Ampliqon A/S, Denmark) was used.

The cDNA level of EGFP (the gene of interest) and GAPDH (the housekeeping gene as a control) from each sample were measured and then analyzed.

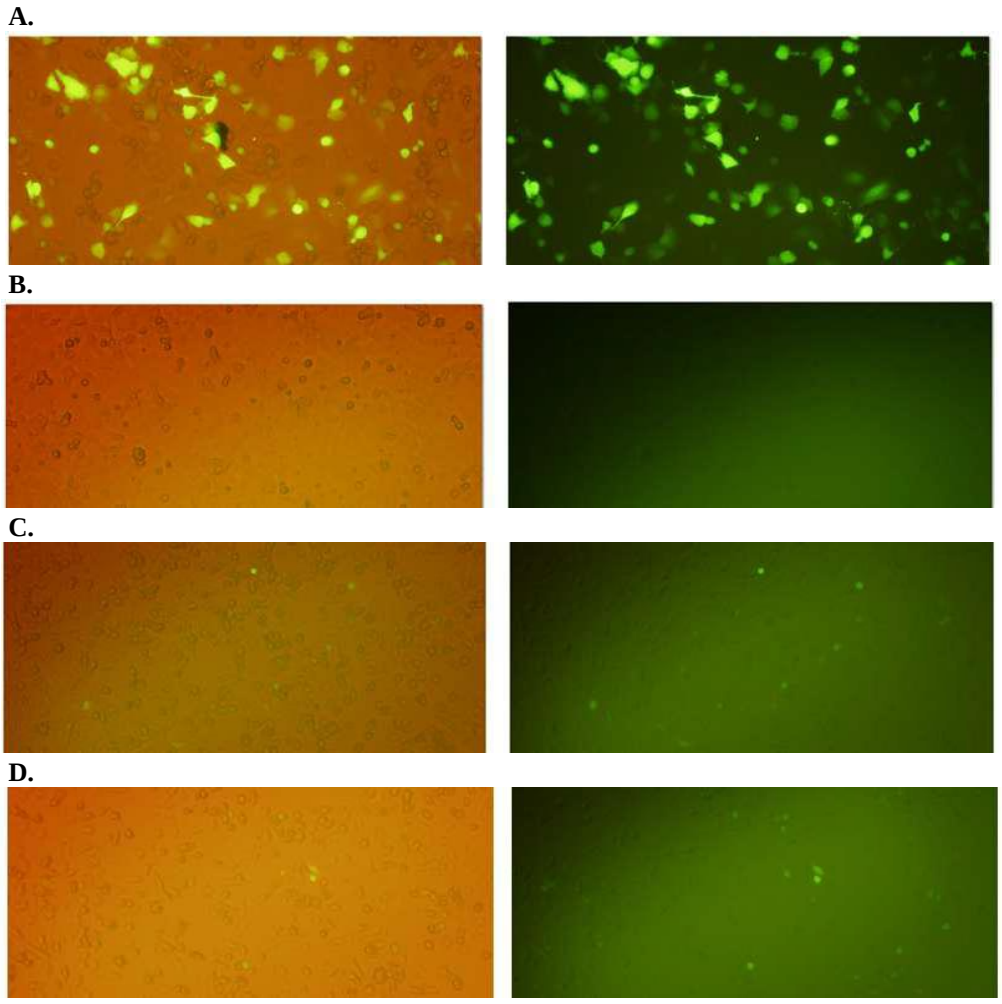

Figure 8. The fluorescent microscopy results of EGFP expression in HUVECs. A. The cells transfected by vector containing EGFP reporter gene and the CMV promoter. B. The cells transfected by vector having EGFP gene without promoter (negative control). C. HUVECs transfected by EGFP reporter gene encompassing the VCAM-1 promoter having A allele of rs3783605. D. HUVECs transfected by vector comprising EGFP reporter gene and the VCAM-1 promoter containing G allele of rs3783605.

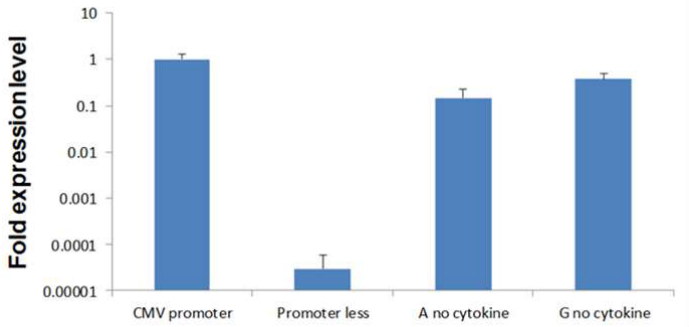

Figure 9. The fold expression level of HUVECs transfected by the vector containing A or G allele of rs3783605 and EGFP reporter genes as well as vector having EGFP gene without promoter compared to the HUVECS transfected by CMV promoter vector comprising EGFP reporter gene in unstimulated state after 7 hours. *P* value comparisons for the promoter less, A & G alleles vs. CMV promoter without stimulation were 0.001.

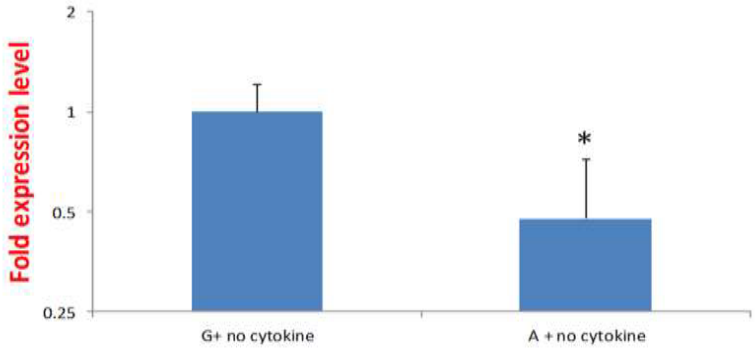

Figure 10. The fold expression level of HUVECs transfected by the vector containing *VCAM-1* promoter having A allele of rs3783605 compared to the cells transfected by the *VCAM-1* promoter having G allele of rs3783605. * $p< 0.05$.

Statistical Analysis

Data were analyzed on the basis of the relative expression method by Relative Expression Software Tool for Rotor-Gene (REST-RG, version 3). It is a calculation software for the relative expression in real-time PCR using pairwise fixed reallocation randomization test and it does error estimation of the calculated ratio using a Taylor's series.

RESULTS

In this research, we investigated EGFP expression in the HUVECs which were transfected by two allele of rs3783605, A and G, without cytokine stimulation. Moreover, we analyzed the EGFP expression level of A and G allele of rs3783605 comparing with vector containing Cytomegalovirus promoter as well as promoter less vector.

The EGFP expression was assessed by fluorescent microscopy (Fig.8). As it can be seen in figure 8, the expression level in HUVECs containing CMV vector was much higher than others and bright green fluorescence was seen. The fluorescent intensity in cells comprising promoter-less vector was zero. The cells containing the G or A alleles of rs3783605 expressed the EGFP protein at low levels and in some cells green fluorescence was seen but assessment of entire cells showed that cells containing the G allele had higher intensity compared with cells containing the A allele.

In real time PCR, our obtained results indicated that EGFP expression level in the HUVECs transfected by promoter-less vector is about 0.00001 folds of cells with *CMV* promoter vector which is statistically significant (p value=0.001). Its expression level in the cells transfected by the A allele of rs3783605 was approximately 0.14888 folds and the HUVECs comprising the G allele is about 0.37851 folds of cells with CMV promoter vector (see Fig.9).

Moreover, our results showed that HUVECs transfected by the G allele of rs3783605 led to about 2-fold higher transcriptional activity compared with the A allele which was statistically significant (p=0.049) (Figure 10).

DISCUSSION

Results from this study demonstrated that the VCAM-1 promoter activity is affected by the single nucleotide polymorphisms (SNPs) present in the promoter. We studied the transcriptional activity of the VCAM-1 promoter affected by rs3783605 A>G. We found that this SNP is involved in different levels of VCAM-1 expression.

Previous studies on the structural analysis of the VCAM-1 promoter revealed important transcription factor binding sites in the VCAM-1 promoter that have a key role in VCAM-1 expression.[2] Another study showed that rs3783605 is located in ETS2 binding site and therefore may be involved in the pathogenesis of VCAM-1-associated diseases including asthma, atherosclerotic lesions, multiple sclerosis, thromboembolic diseases, multiple myeloma, insulin-dependent diabetes mellitus and breast cancer.[1,6,7,9,10] This association might be due to the effect of this variation on the promoter activity and VCAM-1 expression.

Our work is consistent with previous reports

showing the role of rs3783605 on the activity of VCAM-1 promoter, although the details of our findings might be somewhat different.

One important factor that plays a role in the outcome of such studies is the promoter fragment that is being analyzed. The promoter activity may change from experiment to experiment depending on the regulatory sequences present in the selected fragments.

Figures 1 and 2 show the promoter fragment that was used in our study. This fragment contains ETS2 binding site as well as rs3783605, therefore it is possible to assume that the effect of this variation on ETS2 transcriptional activity can be reasonably addressed by this promoter fragment. This fact itself is enough to show the importance of our work because ETS2 is named as one of the most important transcription factors involved in regulation of VCAM-1 expression.

Transcription factor ETS2 is the downstream signaling factor that is activated in mitogen-activated protein kinase (MAPK) pathway.[5] The VCAM-1 is the target gene of ETS transcription factors including ETS2 and some cytokines including TNF-α can lead to activation of ETS2 and then induction of VCAM-1 expression.[6]

All of the transcription factors contain different domains that have different functions. The ETS transcription factors have two domains: the transactivation domain (TA) and the DNA binding domain or ETS domain. In some ETS transcription factors such as ETS2, other domains have been described that contain the regulatory region, with positive or negative regulatory functions that affect the transactivation domain, and have inhibitory domains (ID) that affect the DNA binding property of ETS factor.[13] Accordingly, the interaction between these multifunctional modular domains in different transcription factors has synergistic repression or activation effect on the gene expression.[13] In addition to its effect on DNA binding, the interactions between proteins regulate the target gene selection and transcriptional function of ETS transcription factors.[12] Previous studies have shown that interaction between transcription factors such as ETS2 and AP1 is enhanced by specific DNA motifs which indicated other regulatory mechanisms involved in gene expression.[13] Other studies have suggested cis-regulatory elements and motifs that are involved in DNA binding of these transcription factors in combinational form and so involved in activation of gene expression.[11] Moreover, this specific combination of transcription factors leads to cell type specific responses to the different cytokine stimulations.[12] Thus, this SNP is likely to affect all of these properties.

Results from this study indicate that ETS2 and therefore its binding site on the VCAM-1 promoter have an important role in VCAM-1 expression. According to the above, the rs3783605 may affect the DNA binding of ETS2 singly or DNA binding of ETS2-transcription factors in combinational forms. This leads to synergistic repression or activation of gene expression as well as interaction affinity between these transcription factors. Hence, we can conclude from this data that rs3783605 A>G may affect the VCAM-1 associated disease severity as well as their progression. Therefore, we suggest a more comprehensive study on the transcriptional activity of this variant considering its interactions with different cytokines that are possibly involved in the regulation of VCAM-1 promoter activity and expression.

ACKNOWLEDGEMENTS

This work was supported by a research grant from Tehran University of Medical Sciences, Deputy of Research (grant number: 11931-30-04-89). The authors are very thankful for this financial support.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the materials discussed in this manuscript.

REFERENCES

1. Idelman G, Taylor JG, Tongbai R, Chen RA, Haggerty CM, Bilke S, et al. Functional profiling of uncommon VCAM1 promoter polymorphisms prevalent in African American populations. Hum Mutat 2007; 28(8):824-9.
2. Neish AS, Williams AJ, Palmer HJ, Whitley MZ, Collins T. Functional Analysis of the Human Vascular Cell Adhesion Molecule 1 Promoter. J Exp Med 1992; 176(6):1583-93.
3. Postigo AA, Teixidó J, Sánchez-Madrid F. The alpha 4 beta 1/VCAM-1 adhesion pathway in physiology and disease. Res Immunol 1993; 144(9):723-35.
4. Hamzaoui A, Ammar J, El Mekki F, Borgi O, Ghrairi H,

Ben Brahim M, et al. Elevation of serum soluble E-selectin and VCAM-1 in severe asthma. Mediators Inflamm 2001; 10(6):339–42.

5. Hsu T, Trojanowska M, Watson DK. Ets proteins in biological control and cancer. J Cell Biochem 2004; 91(5):896–903.
6. Cheng C, Tempel D, Den Dekker WK, Haasdijk R, Chrifi I, Bos FL, et al. Ets2 determines the inflammatory state of endothelial cells in advanced atherosclerotic lesions 2011; 109(4):382-95.
7. Han B, Mehra R, Dhanasekaran SM, Yu J, Menon A, Lonigro RJ, et al. A fluorescence in situ hybridization screen for E26 transformation-specific aberrations: identification of DDX5-ETV4 fusion protein in prostate cancer. Cancer Res 2008; 68(18):7629-37.
8. O'Hanlon DM, Fitzsimons H, Lynch J, Tormey S, Malone C, Given HF. Soluble adhesion molecules (E-selectin, ICAM-1 and VCAM-1) in breast carcinoma. Eur J Cancer 2002; 38(17):2252-7.
9. Kamper E.F, Kopeikina L.T, Karajiannis Ch, Gourgiotis D, Karpathios Th, Stavridisl J.C. Tetranectin soluble P. selectin and VCAM-1 in the plasma of children with insulin-dependent diabetes mellitus. Fibrinolysis & Proteolysis 1999; 13 (3):148-53.
10. Gimenez MA, Sim JE, Russell JH. TNFR1-dependent VCAM-1 expression by astrocytes exposes the CNS to destructive inflammation. J Neuroimmunol 2004; 151(1-2):116-25.
11. De Val S, Chi NC, Meadows SM, Minovitsky S, Anderson JP, Harris IS, et al Combinatorial regulation of endothelial gene expression by ets and forkhead transcription factors. Cell 2008; 135(6):1053–64.
12. Li R, Pei H, Watson DK. Regulation of Ets function by protein - protein interactions. Oncogene 2000; 19(55):6514-23.
13. Basuyaux JP, Ferreira E, Stéhelin D, Butticè G. The Ets transcription factors interact with each other and with the c-Fos/c-Jun complex via distinct protein domains in a DNA-dependent and -independent manner. J Biol Chem 1997; 272(42):26188-95.
14. Izadi M, Abiri M, and Keramatipour M. Producing a Mammalian GFP Expression Vector Containing Neomycin Resistance Gene. Avicenna J Med Biotech 2009; 1(1): 33-6.