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Analysis of the epigenetic regulation of TNF receptor superfamily 25 (TNFRSF25) in rheumatoid arthritis

Bahareh Golbargi[a], Ali Najafi[b,*], Shirin Mastury[a], Farinaz Golbargi[c]

[a] Department of Medicine, Tehran medical Sciences Branch, Islamic Azad University, Tehran, Iran
[b] Molecular Biology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran
[c] Department of Pharmacology, Islamic Azad University Of Pharmaceutical Sciences Branch, Tehran, Iran

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ABSTRACT

Epigenetic changes in certain genes have been shown to strongly contribute to the development of Rheumatoid Arthritis (RA) and the behavior of involved cell types. Microarray methylation datasets were used to analyze DNA methylation in RA. Differentially methylated CpGs were selected using the bioinformatics tools and significant CpG islands were further experimentally analyzed. TNFRSF25 was selected based on differentially methylated CpGs island analysis. The results revealed that the promoter region of the TNFRSF25 gene was consistently non-methylated in PBMCs from healthy controls, in RA patients, and in the synovial fluid of RA patients. Therefore, CpG motifs probably play an essential role in the expression and can be used to distinguish between RA patients and healthy individuals.

1. Introduction

Rheumatoid Arthritis (RA) is a chronic inflammatory disorder that damages the joints (Maeshima et al., 2016; Ambatipudi et al., 2018). A variety of cell types are involved in RA pathogenesis, including several lymphocyte subsets, dendritic cells, osteoclasts, and synovial fibroblasts (SFs) (de la Rica et al., 2013). Rheumatoid arthritis synovial fibroblasts (RASF) are the most common cell type at the site of invasion (de la Rica et al., 2013; Klein et al., 2012; Turner et al., 2018). RASFs show characteristics typically associated with an aggressive phenotype, such as up-regulation of proto-oncogenes, specific matrix-degrading enzymes, adhesion molecules, and cytokines (de la Rica et al., 2013). RASFs are also characterized by their ability to secrete cytokines, chemokines, and joint-damaging enzymes. In addition, RASFs are resistant to apoptosis, leading to synovial hyperplasia, and have invasive and migratory properties that could lead to the spread of the disease to unaffected joints (Klein et al., 2012). Despite extensive research efforts, the underlying cause of RA, and the mechanism responsible for the aggressive rheumatoid phenotype are unknown (Klein et al., 2012), even though several studies have indicated the role of abnormal tumor suppressor gene structure and function (Alamanos and Drosos, 2005; Nakano et al., 2013a). Nonetheless, environmental factors and genetic predisposition (de la Rica et al., 2013) through epigenetic deregulation are associated with the etiopathology of RA (Liu et al., 2016; Webster et al., 2018). Lately, aberrant epigenetic changes have been shown to strongly contribute to the progression of RA by affecting different aspects of the disease such as modifying behavior and gene expression of various cell types, especially the resident synovial fibroblasts (SF) in the affected joint(s) (Klein et al., 2012; Firestein, 2018). Epigenetic changes are defined as stable and heritable changes in gene expression that do not involve changes to the underlying DNA sequence (Liu et al., 2016; Plant et al., 2016; Wu et al., 2016). DNA methylation, histone modification, chromatin remodeling (Wu et al., 2016), or microRNA mediated (miRNAs) changes are the predominant mechanisms; of these, DNA methylation is the most investigated (Quintero-Ronderos and Montoya-Ortiz, 2012). DNA methylation plays a key role in controlling gene expression and can potentially contribute to immune dysregulation (Nakano et al., 2013a; Karouzakis et al., 2018). Importantly, alterations in DNA methylation patterns have been observed in autoimmune diseases like RA (Karouzakis et al., 2018; Nakano et al., 2013b). DNA methylation refers to the addition of a methyl (CH_3) group to cytosine-phosphate-guanine (CpG) sites, i.e., where a cytosine follows a guanine in the DNA sequence. The cytosine in these CpG sites is converted into 5-methylcytosine by DNA methyltransferases (Kim et al., 2016). Such methylation at the CpG sites located at or near the promoter suppresses gene transcription, especially when the CpGs are

Abbreviations: TNFRSF25, TNF receptor superfamily 25; RA, Rheumatoid arthritis; RASF, Rheumatoid arthritis synovial fibroblasts; miRNA, microRNA; CpG, cytosine-phosphate-guanine; GEO, Gene Expression Omnibus; DEGs, Differentially Expressed Genes; GRN, gene regulatory network

* Corresponding author.
E-mail address: najafi74@bmsu.ac.ir (A. Najafi).

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clustered as CpG islands (de la Rica et al., 2013). Differential DNA methylation (Nakano et al., 2013a) might alter gene expression (Nakano et al., 2013b) and synoviocyte function (Nakano et al., 2013a; Nakano et al., 2013b). In RA, the FLS function is potentially related to several factors, including characteristic DNA methylation signatures and dysregulation of genes encoding proteins such as sentrin, PTEN, p53 (Maeshima et al., 2016), and TNFRSF25(DR3) (de la Rica et al., 2013). Thus, DNA methylation is now widely recognized as being critical for maintaining the function of immune cells like FLS (Sun et al., 2016). Further, it has been demonstrated that epigenetic changes such as DNA methylation could alter FLS gene expression (Maeshima et al., 2016; de la Rica et al., 2013), which in turn plays an important role in the pathogenesis of RA (Wu et al., 2016).

An evaluation of the whole epigenome, using high-throughput technology, has yielded large and valuable datasets that can be used for discovering new gene regulatory mechanisms. Additionally, bioinformatic tools provide an opportunity to analyze defined pools of data for gene selection and biomarker discovery. Therefore, combining an in-silico study with an experiment can help validate and confirm a hypothesis (Braun, 2014; Najafi et al., 2014; Xie and Ahn, 2010).

Thus, the aim of this study was to use an in-silico top-down approach to identify the epigenetic marker regions in rheumatoid arthritis. Subsequently, one of the key markers (TNFRSF25) was tested in the samples from rheumatoid arthritis patients and control subjects for confirmation. It is possible that the identification of such cell-specific targets of epigenetic dysregulation can also serve as clinical markers for the disease onset, progression, and response to treatment (de la Rica et al., 2013; Quintero-Ronderos and Montoya-Ortiz, 2012).

2. Materials and methods

2.1. Data collection and primary processing

Scientific publications and public microarray databases such as the Gene Expression Omnibus (GEO) and ArrayExpress were reviewed and the microarray datasets GSE46650 and GSE46364 were selected because they contained information on DNA methylation in rheumatoid arthritis. Raw data from 22 Fibroblast-like synoviocyte samples were included in this study; the data were generated using the Illumina Human Methylation 450 BeadChip platform (GPL13534 and GPL16304). The raw data were downloaded locally in soft file format. Pre-processing and differentially methylated CpGs selection analyses were conducted using the geWorkbench software. Based on differentially methylated CpGs regions, related genes were extracted as DEGs (Differentially Expressed Genes).

2.2. Network reconstruction and analysis

The gene regulatory network (GRN) of the DEGs was reconstructed using the STRING database (Szklarczyk et al., 2014) to predict functional association among the DEGs. The STRING interaction database was derived from four major sources: genomic context, high-throughput experiments, co-expression, and the previous knowledge. Briefly, the gene list was submitted online or through a related plugin in the Cytoscape package (Shannon et al., 2003) and the interactions between the genes were retrieved and displayed as interaction networks.

The reconstructed networks were imported into Cytoscape software and analyzed using the Network Analyzer Plugin of the Cytoscape package. Topological parameters such as the number of nodes, edges and connected components, characteristic path length, the number of shortest paths, average number of neighbors, and centrality measures, including network diameter, radius and clustering coefficient, centralization, density, and heterogeneity, were also calculated by the Network Analyzer plugin. Only critical genes were screened and one important gene identified in-silico phase was further analyzed experimentally.

Table 1
The quality of DNA samples obtained from blood and synovial fluid determined by OD 260/280.

Sample	OD (260 nm)	OD(280 nm)	Concent. (ng/μL)	260/280
Control 1	0.569	0.308	28.5	1.85
Control 2	0.584	0.311	29.2	1.88
Patient 1	0.561	0.310	28.0	1.81
Patient 2	0.316	0.174	15.8	1.81
Synovial fluid 1	1.400	0.730	70	1.92
Synovial fluid 2	0.473	0.251	23.7	1.89

2.3. Patients and controls

Because RA is an autoimmune disease can be controlled with medication and joint replacement in this disease is rarely done, for this reason the blood samples were used. Blood samples were obtained from two patients with RA and two healthy control subjects. The patients were both anti-CCP and RF positive. Synovial fluid samples were also obtained from the RA patients and were non-septic.

All samples were collected from patients admitted to the Baqiyatallah Hospital (IRAN, Tehran) in 2016.

2.4. Extraction of genomic DNA

DNA was extracted from serum and synovial fluid samples using the YTA Genomic DNA Extraction Mini Kit (Cat No: YT9040) according to the manufacturer's instructions. DNA quantity and quality were estimated using spectrophotometry and agarose gel electrophoresis, respectively. (Table 1).

2.5. Sodium bisulfite conversion of non-methylated cytosines in DNA

The methylation status of a DNA sequence can be determined using the sodium bisulfite method wherein the treatment of DNA with sodium bisulfite converts cytosine residues to uracil but leaves methylated cytosines unaffected (Fig. 1).

The methylation status was analyzed using the EpiTect Bisulfite Kit (Qiagen, Cat No: 59104) according to the manufacturer's instructions.

2.6. Methylation-specific polymerase chain reaction (MSP)

The promoter region of the selected gene was amplified by PCR using the sodium bisulfite-treated DNA samples and methylation-specific primer sets. The amplification products were detected by electrophoresis on a 2% agarose gel. The gels were visualized in a UV transilluminator. The primer pairs used for amplifying methylated and non-methylated DNA are detailed in Table 2.

The PCR amplification reaction (15 μL) contained 0.5 μL of template, 20 pmol/μL of each primer (0.5 μL), 100 mM $MgCl_2$ (1.5 μL), 3.5 μL 7% DMSO, and 7 μL Master Mix (Taq 2× Master Mix Red, 1.5 μL $MgCl_2$, Amplicon). The PCR conditions were initial heating to 95 °C for 5 min, subsequent amplification for 30 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 40 s, and a final incubation at 72 °C for 5 min. This procedure results in the conversion of non-methylated cytosine to thymine, whereas methylated cytosine is unaffected.

3. Results

3.1. Data analysis

An Infinium Human Methylation 450 BeadChip was used to determine the methylation status of 485,512 loci in GSE46364 (platform: GPL16304, 11 samples) and 485,577 loci in GSE46650 (platform: GPL13534, 12 samples) in FLS. The loci were removed from subsequent analysis if any of the probes for a locus could not be distinguished from

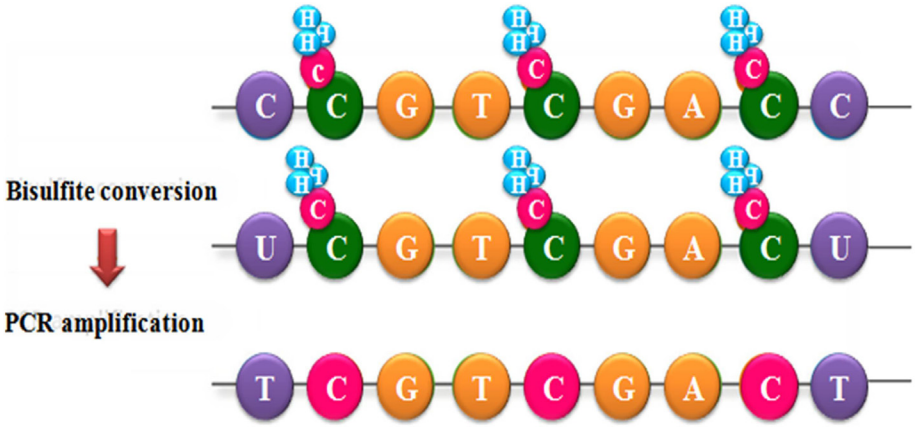

Fig. 1. Sodium bisulfite conversion is the gold standard for DNA methylation analysis. After treatment with sodium bisulfite, non-methylated cytosine residues are converted to uracil whereas 5-methylcytosine (5mC) remains unaffected.

the background (p-value < 0.01) or if not enough beads were present on the chip for the exact measurement of their methylation level. After filtering, 61,944 loci from GSE46650 and 6093 loci from GSE46364 were available for further analysis. To assess the methylation status of both the RA patients and controls, methylation scores for all filtered loci within a sample were summed, and any significant difference between them was assessed using Student's t-test (p-value < 0.01). The list of genes that were differentially methylated in RA patients and controls (Supplementary file) implied that these genes can potentially be regulated through DNA methylation. This list included many genes associated with RA. A total of 178 genes were screened and, through a stepwise subtraction processes, four candidate genes were selected, namely TNFRSF25, HOXA5, INPP5A, and HUS1B as they showed a considerable increase in methylation frequency in RA than in controls. Of these four genes, the best candidate was TNFRSF25 (DR3) as the bioinformatics results indicated that TNFRSF25 is hypermethylated in RASFs. TNFRSF25 is widely associated with RA pathogenesis and progression. The methylation status of this promising gene was next validated by methylation-specific PCR (MSP) in each sample.

3.2. Methylation status of the TNFRSF25 (DR3) promoter

The MSP for the promoter region of the TNFRSF25 (DR3) gene was performed with specific primers that would yield a band of 192 bp for the methylated sequence or a band of 190 bp for the non-methylated sequence. The PCR amplification of the CpG island with methylation-specific and unmethylation-specific primers revealed that the amplified region was consistently non-methylated in PBMCs and synovial fluid from both healthy controls and patients with RA. (Fig. 2A, B).

4. Discussion

Rheumatoid arthritis (RA) is an autoimmune disease of unknown cause (Takami et al., 2006). In recent years, epigenetic modifications, including DNA methylation, have been reported to strongly contribute to the development of RA by modifying gene expression levels and behavior of synovial fibroblasts (SF) (Klein et al., 2012). As epigenetic modifications function as an integrator of environmental factors and genetic difference, the detailed epigenetic analysis may facilitate a better understanding of the development and progression of RA (Fujio, 2015). Previous reports show the existence of genes with altered DNA methylation in RASFs (de la Rica et al., 2013). DNA methylation is a form of epigenetic modification and is an interesting approach for identifying biomarkers of treatment response as DNA is comparatively more stable than messenger RNA and most proteins, can be altered by drug therapy, and is amenable to high-throughput whole genome typing (Plant et al., 2016). Methylation abnormalities have been associated with many diseases, including RA, where expression or suppression of key genes allows cells to escape normal cytostatic controls. Local inflammation can also alter the expression of the enzymes responsible for initiating and maintaining DNA methylation (Nakano et al., 2013a).

In this study, we identified four genes that were dysregulated in rheumatoid arthritis due to alterations in DNA methylation patterns (de la Rica et al., 2013). The genes HOXA5, INPP5A, HUS1B in the peripheral blood of RA patients (Park et al., 2013) and TNFRSF25 in the synovial cells of RA patients (Takami et al., 2006) emerged as possible epigenomic biomarkers. These data strongly support the notion that DNA methylation patterns are distinct in RA and normal FLS and that their role in the pathophysiology of RA and their potential as diagnostic or prognostic markers should be further investigated (Park et al., 2013). These genes play a key role in inflammation, matrix regulation, cell trafficking, leukocyte recruitment, and immune response, and could represent novel therapeutic targets (Nakano et al., 2013a; Nakano et al., 2013b) (Table 2).

The differently methylated genes could modify FLS gene expression and contribute to the pathogenesis of RA. Rheumatoid FLS exhibit a unique aggressive phenotype that contributes to joint destruction, (Nakano et al., 2013a) and a study has shown that dysregulated apoptosis (programmed cell death) may be one of the causes of RA pathogenesis (Takami et al., 2006). Multiple CpG motifs exist in the promoter region of TNFRSF25 (DR3) and apoptotic signaling pathways are often the targets for epigenetic gene silencing (Takami et al., 2006). The CpG island in the TNFRSF25 (DR3) gene promoter is specifically

Table 2
The primer pairs used for methylation-specific polymerase chain reaction (MSP) for TNFRSF25 gene.

Feature	Forward Methylated Primer	Reverse Methylated Primer	Forward unMethylated Primer	Reverse unMethylated Primer
Sequences	GTTTTATTTGGTTTGTTCGTTGTC	CGTACTCTCTACCCGTCGTAA	TTTATTTGGTTTGTTTGTTGTTGTT	ACTCCATACTCTCTACCCATCATAA
Primer Length	24 mer	21 mer	25 mer	25 mer
Annealing temperature	54 °C	54 °C	54 °C	54 °C
GC content	33.3%	52.4%	24.0%	40.0%
product Length	192 bp		190 bp	

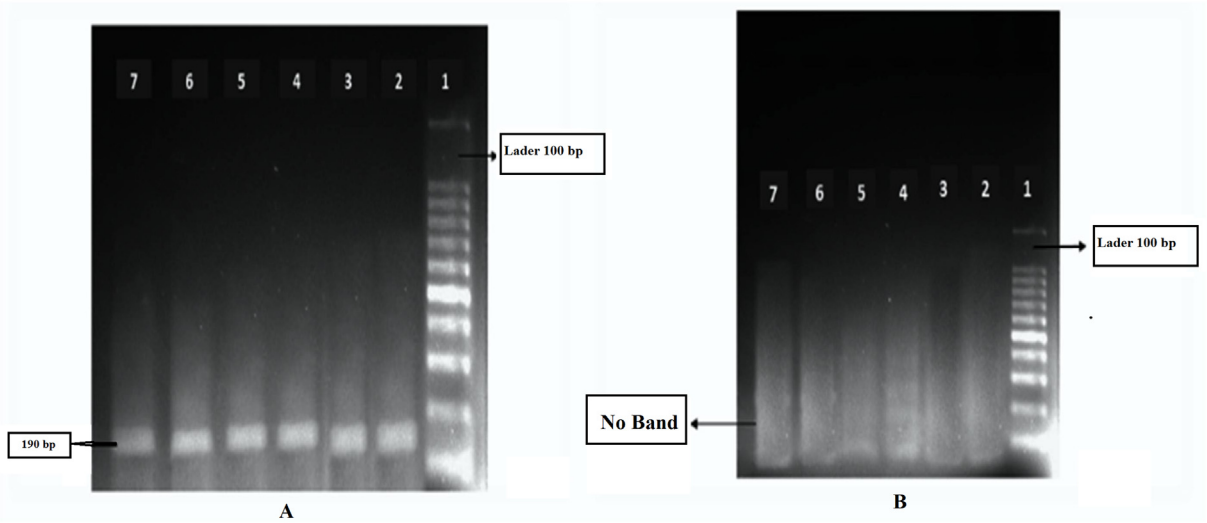

Fig. 2. Methylation status of the TNFRSF25 gene promoter, as determined by methylation-specific polymerase chain reaction (PCR). (Fig. 2A): Amplifying non-methylated DNA. (Fig. 2B): Amplifying methylated DNA. Lane 1: DNA ladder 100 bp; Lane 2: Patient 1 (blood sample); Lane 3: Patient 2 (blood sample); Lane 4: healthy Control 1 (blood sample); Lane 5: healthy Control 2 (blood sample); Lane 6: Patient 1 (Synovial fluid sample); and Lane 7: Patient 2 (Synovial fluid sample).

Table 3
The critical genes with individual differentially methylated loci in RA.

Gene name	Differentially methylated loci	Previously reported RA implication
TRAF2 (de la Rica et al., 2013)	Hypomethylated	
CD74 (de la Rica et al., 2013)	Hypomethylated	Initiates MIF signal transduction (levels related with RA course) (de la Rica et al., 2013)
HOX (de la Rica et al., 2013)	Hypomethylated	
S100A14 (de la Rica et al., 2013)		Involved in invasion through MMP2 (elevated in RA plasma) (de la Rica et al., 2013)
ST14 (Nakano et al., 2013a), ADAM32 (Nakano et al., 2013a), CDK14 (Nakano et al., 2013a), STK24 (Nakano et al., 2013a)	Hypomethylated	
ITGA6 (Nakano et al., 2013a)	Hypomethylated	Focal adhesion' pathway (Nakano et al., 2013a)
DPP4 (de la Rica et al., 2013)	Hypermethylated	DPP4 encodes a serine protease, which cleaves a number of regulatory factors, including chemokines and growth factors. DPP4 inhibitors have recently emerged as novel pharmacological agents for inflammatory disease (de la Rica et al., 2013). Its inhibition increases cartilage invasion by RASF (de la Rica et al., 2013).
TMEM51 (de la Rica et al., 2013)	Hypermethylated	
TNFRSF25 (de la Rica et al., 2013; Klein et al., 2012; Quintero-Ronderos and Montoya-Ortiz, 2012; Meda et al., 2011; Strietholt et al., 2008)	Hypermethylated	Apoptosis (de la Rica et al., 2013; Klein et al., 2012; Quintero-Ronderos and Montoya-Ortiz, 2012; Meda et al., 2011; Strietholt et al., 2008) promoter of TNFRSF25, which is downregulated inducing resistance to apoptosis (Szklarczyk et al., 2014; Shannon et al., 2003).
HOXA5 (Nakano et al., 2013a), INPP5A (Nakano et al., 2013a) HUS1B (Nakano et al., 2013a), RXRA (Nakano et al., 2013a)	Hypermethylated	

methylated to down-regulate the expression of the DR–3 protein in rheumatoid synovial cells, which may then resist apoptosis of the RA synovial cells. Thus, it is possible that the apoptosis-inducing death receptor Fas plays an important role in RA. In contrast, we found that the TNFRSF25 (DR3) promoter region was almost completely non-methylated in PBMCs from both RA patients and healthy individuals (Takami et al., 2006). This observation implies that the choice of tissue for analysis and time of sampling are crucial as methylation levels vary substantially across tissues and time (McGregor et al., 2016) (Table 3).

In the current report, we focused on the contribution of DR–3 in the pathogenesis of RA as it is a member of the apoptosis-inducing tumor necrosis factor (TNF) receptor superfamily that includes Fas. The promoter region near the translation start site contains GATA, Oct–1, and two Sp1 binding sites, but does not have either the TATA or the CAAT box. TNFRSF25 (DR3) is located on chromosome 1 (1p36.2) and contains 10 exons. Therefore, CpG motifs probably play an essential role in the expression of the TNFRSF25 (DR3) gene (Takami et al., 2006). The methylation level of TNFRSF25 (DR3) promoter is used to distinguish between RA patients and healthy individuals, and is a highly sensitive and specific diagnostic marker for RA (Zhao et al., 2016). The three other genes identified through bioinformatics, their descriptions, and related methylated regions, are listed in a supplementary file (Supplementary file 1).

Available data raise the hope that, in future, detailed knowledge of epigenetic regulatory mechanisms in RA will help develop new treatment strategies and improve the therapeutic efficacy and treatment outcome.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.genrep.2019.100424.

Declaration of Competing Interest

All authors declared that they have no conflicts of interest.

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