



A Novel MTHFR Isoform-based Biomarker for RA and SLE

Tímea Ocskó¹, Attila Balog², László Kovács², Zsolt Bánfai³, Gyula Hoffmann⁴, Tibor T. Glant¹ and Tibor A. Rauch^{1,5*}

¹Department of Orthopedic Surgery, Rush University Medical Center, Chicago, USA

²Department of Rheumatology and Immunology, Szent-Györgyi Albert Clinical Center, Szeged, Hungary

³Department of Medical Genetics, University of Pécs, Pécs, Hungary

⁴Department of Comparative Anatomy and Developmental Biology, University of Pécs, Pécs, Hungary

⁵Department of Medical Biology, Medical School, University of Pécs, Pécs, Hungary

Abstract

Objective: Disease- and drug-related biomarkers are the basis of personalized medicine by guiding patient-specific clinical decisions. The methylene-tetrahydrofolate reductase (MTHFR) gene-associated C677T polymorphism has garnered particular attention because it can lead to an amino acid change resulting in a catalytically compromised enzyme. Here, we provide an alternative interpretation of C677T-associated MTHFR phenotypes that does not exclude the original hypothesis but rather places it in a different context. Our duon-based theory has practical implications as it facilitates the development of a new predictive biomarker for Rheumatoid Arthritis (RA) and Systemic Lupus Erythematosus (SLE).

Methods: The new MTHFR promoter was identified using the 5'RACE method and functionally characterized in transient expression studies. Western blotting and confocal microscopy were used to investigate the subcellular localization of MTHFR isoforms. Expression of MTHFR transcript variants was monitored by qRT-PCR and a gene expression index (Li/Si score) was calculated from the C_i values.

Results: A new MTHFR isoform was identified, driven by a novel promoter region that overlaps with the site of the C677T polymorphism. Quantitative monitoring of the catalytically active and the catalytically inactive isoforms' expression revealed that the proportion of MTHFR isoforms could be altered in PBMCs in a disease-specific manner. The calculated Li/Si scores were found to be characteristic for specific subgroups of RA and SLE patients.

Conclusion: Differential expression of MTHFR isoforms provides a foundation on which a predictive biomarker (Li/Si score) could be developed for RA and SLE reflecting disease susceptibility and drug response.

Keywords: MTHFR; C677T polymorphism; Duon; Rheumatoid Arthritis (RA); Systemic Lupus Erythematosus (SLE)

Introduction

Methylene-tetrahydrofolate reductase (MTHFR) plays a role in the folate cycle by catalyzing the conversion of 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, which can be used in various metabolic events such as DNA and protein biosynthesis and epigenetic modifications [1]. The MTHFR gene gained more interest when high plasma homocysteine levels were found in patients with coronary heart disease [2] and the hyperhomocysteinemia was attributed to altered MTHFR activity caused by SNPs [3]. Since then, the field has rapidly expanded and over 4000 publications have focused on MTHFR polymorphism-implicated diseases including RA and SLE [4]. These publications were based on the assumption that diminished MTHFR activity results from the C677T polymorphism-associated protein structure change but ignored the fact that reduced activity could only be detected after preincubating MTHFR proteins at non-physiological high temperatures [5]. However, recent genome-wide studies reshaped the understanding of SNPs' role in pathogenesis; it was found that the vast majority of the SNPs are located in non-coding regions, therefore, their disease-promoting effects could be only explained by assuming that gene regulatory elements (i.e., Transcription factor (TF) binding sites) were affected [6,7]. In addition, the discovery of *duons*, TF binding sites in protein-coding regions, has practical implications for the interpretation of genetic variations [8,9]. Indeed, close to 15% of disease-associated SNPs identified by GWASs were located within *duons* and might be directly involved in pathological mechanisms by compromising both regulatory and/or structural functions of proteins. We hypothesized that the C677T polymorphism is within a *duon*, which would significantly affect the expression level of the *MTHFR* gene in addition to altering the amino acid sequence of MTHFR protein.

Molecular biomarkers are measurable indicators of normal and pathogenic processes and might also have the potential to predict favorable responses to therapy [10]. Rheumatology has traditionally relied on biomarkers and some of them have been incorporated into disease classification criteria including erythrocyte sedimentation rate, C-reactive protein, rheumatoid factor, anticitrullinated protein antibodies, various anti-nuclear antibodies, anti-topoisomerase I (Scl-70) antibody and anti-synthetase antibody [11]. Several novel molecular and clinical biomarkers have recently been identified, which can help in differential diagnosis, disease subset definition, or predicting the progression of organ damage [12]. Inflammatory rheumatic diseases are typically complex syndromes, therefore, patients in the same disease category can be genetically heterogeneous and therapeutic agents are usually effective on specific subsets of patients, which underpins the importance of biomarker-driven personalized therapy [13]. DMARD responsiveness-specific biomarkers have not been identified, and although whole-blood transcriptome analysis revealed promising expression panels for anti-TNF therapy responsiveness, no candidate biomarkers have progressed to becoming clinical diagnostic factors.

*Corresponding author: Tibor A. Rauch, Department of Orthopedic Surgery, Rush University Medical Center, 1735 W. Harrison Street, Chicago, IL 60612, USA, Tel: (312) 942-5766; Fax: (312) 942-8828; E-mail: tibor_rauch@rush.edu

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Accordingly, there is an obvious need for biomarkers that can predict the response to immunosuppressive treatments and to particular therapeutic agents. Methotrexate (MTX) is a first-line treatment option for newly diagnosed RA patients, but approximately 30% of RA patients develop MTX-related adverse drug events (ADEs), provoking discontinuation of MTX therapy [14]. In the context of MTX toxicity, the predictive biomarker potential of C677T polymorphisms has been investigated, but has resulted in contradictory conclusions [15].

Here, we report the discovery of a novel predictive biomarker (Li/Si score) for RA and SLE, which can be informative regarding disease susceptibility and drug responsiveness, which can help guide clinical decisions.

Materials and Methods

Cell culture

The K562 cell line (ATTC CCL-243) was cultured in DMEM medium complemented with 10% fetal bovine serum. The cells cultures were maintained in a humidified incubator at 37°C with 5% CO₂ in air.

Study subjects

All patients and control individuals were recruited under an IRB-approved protocol in Hungary. Signed informed consent was obtained from each subject.

Controls: Healthy adults aged 19–65 years were recruited from the local community. Exclusion criteria were any chronic conditions including hypertension, allergy, diabetes, obesity and smoking. Control subjects had no infections and did not take NSAID one month before sampling.

Patients: RA patients were diagnosed according to the latest ACR/EULAR classification criteria [16] and MTX therapy and medium dose glucocorticoid therapy was initiated. Twenty-three of the 32 RA patients received a variety of DMARDs, containing at least one of the following components: MTX, leflunomide, sulfasalazine or chloroquine. Eleven of the 32 RA patients received biologic therapy including anti-tumor necrosis factor (TNF) or IL6 receptor antagonist. SLE patients were diagnosed and classified according to the updated ACR97 criteria [17] at the time of sampling. All SLE patients had a moderate or a severe disease activity. SLEDAI scores were calculated at the time of sampling. Patients were treated with the following medications at onset of the SLE diagnosis and the two years after the diagnosis respectively: methylprednisolone (100%, 78.3%), chloroquine (100%, 78.3%), azathioprine (40.9%, 18.2%), cyclophosphamide (54.5%, 0%), methotrexate (27.3%, 13.7%), cyclosporine (4.5%, 4.5%), and mycophenolate mofetil (13.6%, 9.1%), epratuzumab (0%, 4.5%), belimumab (0%, 4.5%), ustekinumab (0%, 4.5%).

Total RNA isolation, cDNA synthesis and quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

Peripheral blood mononuclear cells (PBMCs) were separated on a Ficoll gradient for RNA and DNA isolation. All of these procedures were conducted as previously described, but using CFX real-time PCR machine (Bio-Rad, Hercules, CA) for quantitative studies. Normal human tissue RNA samples were purchased as FirstChoice® Human Total RNA Survey Panel (Ambion, Austin, TX). Isoform-specific qRT-PCR primers were: Tr.v1F: ACATCTGTGTGGCAGGTTAC, Tr.v1R: GGAGTGGTAGCCCTGGAT and Tr.v2F: GTCATCCCTATTGGCAGGTTAC; Tr.v2R: GGAGTGGTAGCCCTGGAT.

Determination of Li/Si score

Li/Si score calculation is similar to the fold difference calculation in qRT-PCR experiments. Accordingly $Li/Si\ score = 2^{Ct\ of\ Long\ MTHFR\ isoform - Ct\ of\ Short\ MTHFR\ isoform}$ values correspond to qRT-PCR measured threshold cycles. For example; if a Long MTHFR isoform's Ct value is 27 and a Short MTHFR isoform's Ct value is 29 then Li/Si score is $2^{27-29} = 0.25$. This particular Li/Si score implies that the short MTHFR isoform's expression level is ¼ of the long MTHFR isoform.

Genotyping of subjects

Nested PCR was employed to amplify C677T polymorphism carrying regions and PCR fragments were directly sequenced and the genotype was determined using Finch TV chromatogram viewer (Geospiza, Inc., Seattle, WA).

Rapid Amplification of cDNA Ends (5'RACE)

5'RACE was conducted on 1 µg of total RNA isolated from K562 cells using FirstChoice RLM-RACE kit (Thermo-Fisher Scientific, Waltham, MA). Amplified 5' MTHFR transcript ends were cloned, sequenced, and data were deposited into NCBI GenBank (JQ429447).

Western blotting

Sub-cellular protein fractions were prepared using Nuclei EZ Prep nuclei isolation kit (Sigma-Aldrich, Saint Louis, MO). Western blotting was performed as described before. C-terminal specific anti-MTHFR antibody (GTX88281, Lot. No. 821604005) was purchased from GeneTex (Irvine, CA).

Immunofluorescence microscopy

K562 cells were fixed and incubated with IM7.8.1-Alexa Fluor 488 conjugated antibody to detect membrane-bound CD44⁺. After the cells were blocked and stained with C-terminal specific anti-MTHFR antibody (GTX88281). Rabbit anti-goat IgG-Texas Red (H+L) (Vector Laboratories, Burlingame, CA) was used as a secondary antibody to detect intracellular MTHFR. Nuclear counterstaining was performed with DAPI. A Zeiss LSM 700 confocal microscope and ZEN 2.3 imaging software were used to detect and analyze subcellular localization of MTHFR isoforms.

Luciferase reporter gene assays

The two different C677T alleles carrying intronic promoters were cloned into pGL4.19 Luciferase reporter vector (Promega, Madison, WI). Recombinant constructs were electroporated into 5x10⁶ K562 cells and cells were lysed in 400 µL of 1X Cell Culture Lysis Reagent (Promega, Madison, WI) after 24 hours. Luciferase activity was measured by using 20 µL of lysate and 100 µL of Luciferase Assay Reagent (Promega, Madison, WI) in Single Tube Luminometer (Titertek-Berthold, Pforzheim, Germany).

Statistical analysis

Fisher's exact test was used for calculation of statistical significance of small sample sizes [18].

Results

MTHFR could be considered as a gatekeeper enzyme for epigenetic processes [1]; therefore, we investigated how epigenetic signals could affect its own expression. Epigenetic histone signals, downloaded

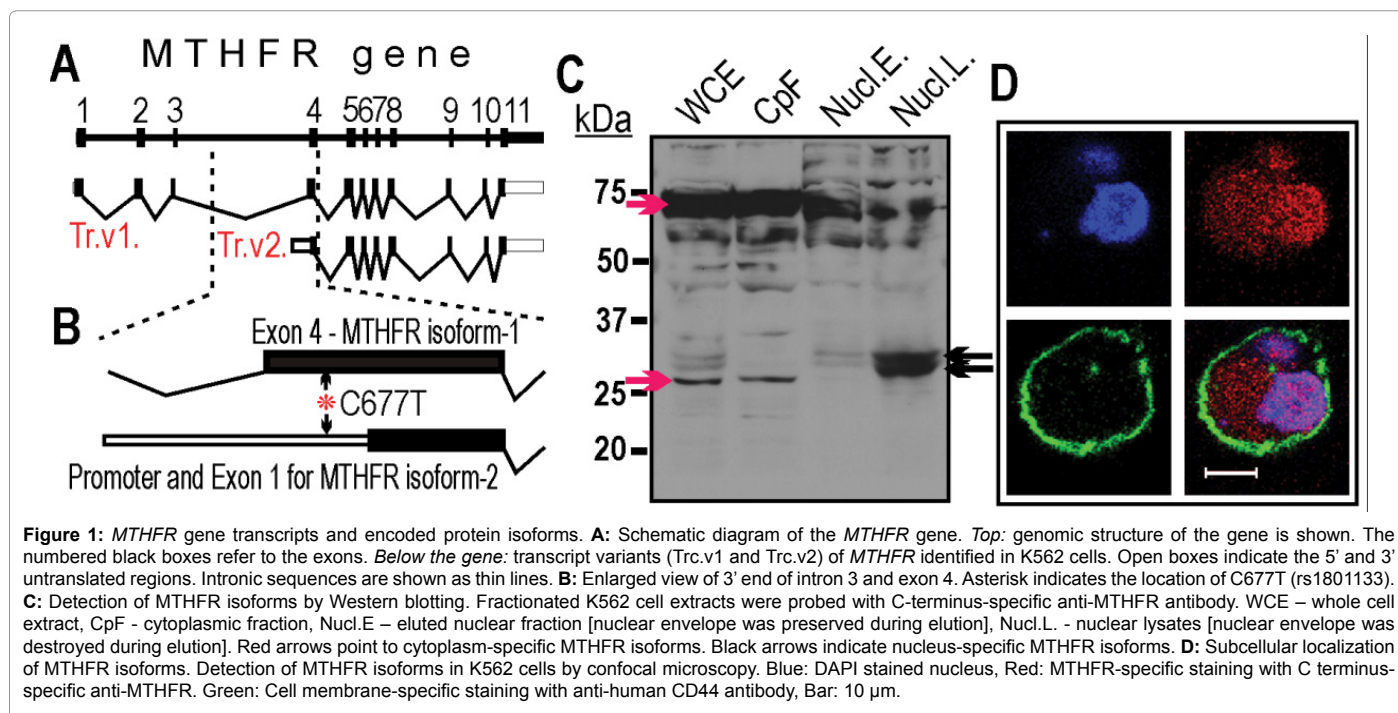
from the ENCODE database [19], that are known for defining transcriptionally active promoter regions [20] forecasted the presence of a new supplementary promoter for *MTHFR*. Interestingly, the predicted promoter harbors the C677T polymorphism (Figure 1A), which implies that it might affect its activity. By employing 5'RACE method [21] we identified a new *MTHFR* transcript variant (Trc.v2), which was initiated from the forecasted promoter region (i.e., in *MTHFR* intron 3) (Figure 1B). Thus, the first three exons were missing from the Trc.v2 transcript and it could encode only a truncated *MTHFR* isoform carrying the regulatory domain of the enzyme [22]. Localization of the short *MTHFR* isoform was investigated in subcellular fractions by Western blotting using a C-terminus specific antibody. The full length *MTHFR* isoform dominated in the cytoplasmic fraction but the short *MTHFR* isoform could be detected in nuclear lysates (Figure 1C). Nuclear localization of the short *MTHFR* isoform was confirmed by confocal microscopy (Figure 1D). The tissue-specific expression of the new *MTHFR* isoform was explored in 20 normal human tissue samples using isoform-specific qRT-PCR. The short *MTHFR* transcript variant (Trc.v2) was ubiquitously expressed together with the full-length *MTHFR* coding variant (Trc.v1). The absolute expression levels of the two isoforms varied among tissues. Therefore, to compare *MTHFR* isoform expression between tissues (and later between patients), we introduced the *Li/Si score* that reflects the fold expression difference between the long (Li) and the short (Si) transcript variants. According to these data, the newly described *MTHFR* isoform is expressed at low levels in all *normal* tissues together with the full length *MTHFR* isoform (Figure 2A).

Next, we compared the transcript variants' (Trc.v1 and Trc.v2) expression in PBMCs isolated from 32 newly diagnosed (treatment naive) patients with active RA, 23 newly diagnosed (treatment naive) patients with active SLE, and 22 healthy age-matched controls. *Li/Si* scores were calculated from qRT-PCR values and compared to clinical phenotypes. By using *Li/Si* scores, control individuals, RA and SLE patients could be divided into 3 classes. Class-defining threshold values were determined on the basis of the observed *Li/Si* scores in 20 normal

tissue samples (Figure 2A) and the 22 control PBMC samples (Figure 2B). Accordingly, *Class A* individuals had *Li/Si* scores <0.65, *Class B* included individuals with *Li/Si* scores between 0.65 – 2.0 and *Class C* individuals had *Li/Si* scores >2.0. We found that low *Li/Si* scores (*Class A*) were characteristic for control individuals (82%, $p < 0.01$) (Figure 3A). The intermediate *Li/Si* score (*Class B*) was exclusively characteristic of RA (46.8%, $p < 0.01$, OR: 8.438, 95% CI: 2.118 - 8.813) and SLE (65.2%, $p < 0.01$, OR: 9.643, 95% CI: 2.362 - 11.049) patients (Figure 3B). In addition, we found that RA and SLE patients with intermediate *Li/Si* scores (*Class B*) were prone to ADEs, including hepatotoxicity and gastrointestinal complaints for RA, and gastrointestinal complaints, fever, and hepatitis for SLE patients.

RA patients with intermediate *Li/Si* scores had a 19% chance ($p < 0.01$, OR: 27, 95% CI: 2.503 - 20.328) of developing ADE, while SLE patients had 39% chance ($p < 0.01$, OR: 36, 95% CI: 3.453 - 37.524) (Figures 3B and 3C). Interestingly, the subjects with high *Li/Si* values (*Class C*) were relatively frequent among controls (13.6%, $p = 0.0986$, OR: 0.25, 95% CI: 0.058, 0.00795) and RA patients (28.1%, $p < 0.05$, OR: 5.062, 95% CI: 1.196 - 2.966) but scarce among SLE patients (4.3%, $p = 1$, OR: 0.643, 95% CI: 0.061 - 0.014) (Figure 3).

Next, we genotyped all subjects by sequencing the C677T polymorphism-carrying region and investigated how allele frequencies and *Li/Si* scores correlated with diseases. We found that T allele-carrying individuals were over-represented among control subjects (77.27%) compared to RA (40%, $p < 0.05$) or SLE (34.7%, $p < 0.01$) patients, but further correlation could not be found between C667T alleles and diseases. These findings indicated that the C677T polymorphism itself has a limited potential as a biomarker, but the *Li/Si* scores could indicate susceptibility to diseases (i.e., RA and SLE) and ADE (Figure 3). We also investigated whether a single C to T nucleotide switch that simulate C667T polymorphism had effect on reporter gene (i.e., Luciferase) activity in transient expression studies. The C-allele carrying promoter



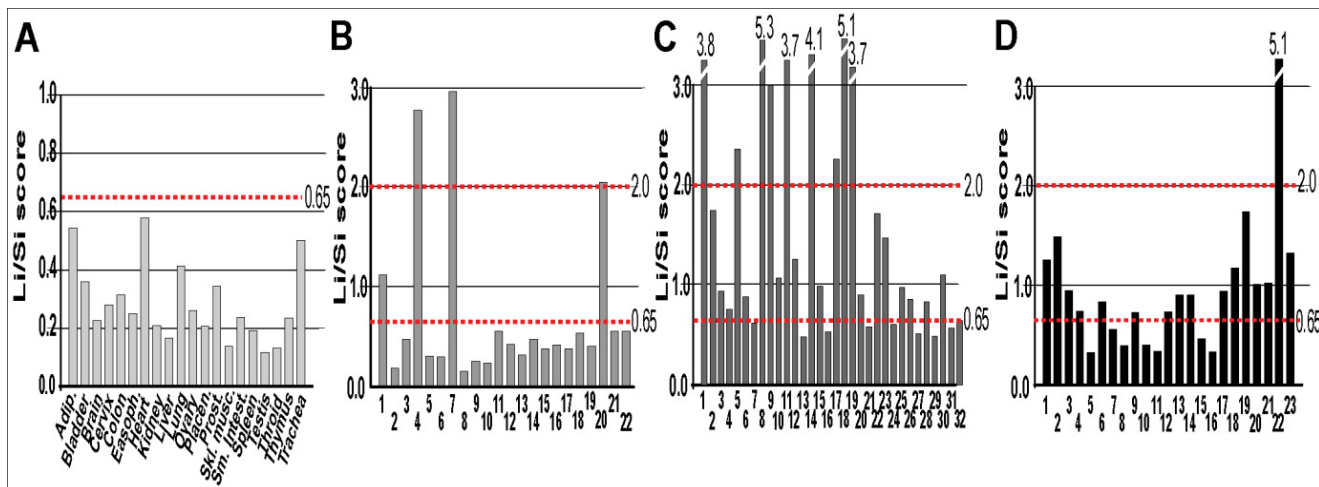


Figure 2: Expression of *MTHFR* isoforms in human tissue and PBMC samples. Isoform-specific PCR Ct values were used to calculate the Li/Si scores. The Li/Si score represents the relative expression difference (fold-difference) between the long *MTHFR* (Li) and short *MTHFR* isoform (Si). The expression level of the long isoform is considered 1 in each case. Li/Si scores in **A**: normal human tissue samples, **B**: PBSCs of control human subjects, **C**: PBSCs of RA patients and **D**: PBSCs of SLE patients. Dotted lines mark the class-defining 0.65 and 2.0 Li/Si scores described in the Results.

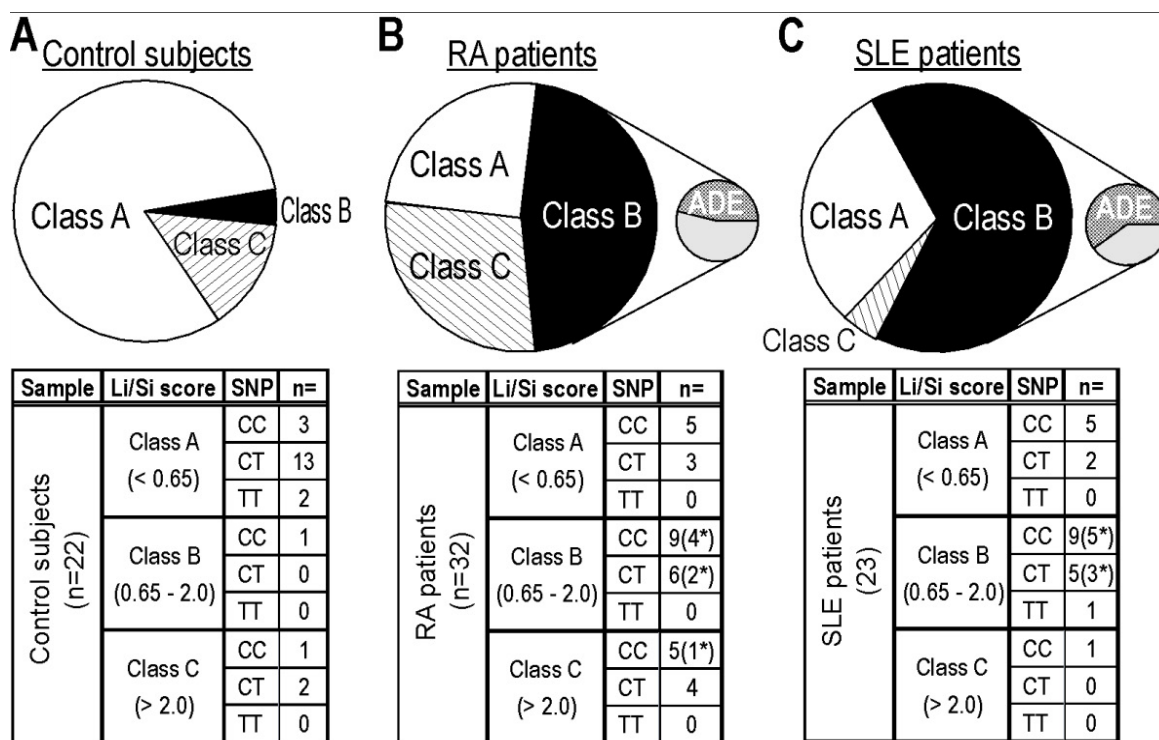


Figure 3: Li/Si scores and SNP genotypes in human PBMC samples. Pie-of-pie charts demonstrate Li/Si score and ADE distribution in human subjects. SNP distributions are summarized under the corresponding pie-of-pie charts. SNP: actual genotype of C677T polymorphism. n: number of the investigated subjects. Asterisks label patients with ADEs.

provoked 20% ($p < 0.05$) higher Luciferase activity than the T-harboring allele indicating allele-dependent promoter activity.

Discussion

Epigenetic histone signal profiles predicted the existence of an intragenic *MTHFR* promoter, which proved to be active in subsequent studies (Figure 1). The novel *MTHFR* promoter was located in intron

3, and the corresponding transcript encoded a truncated *MTHFR* isoform with unknown function in the nucleus. What makes the intronic *MTHFR* promoter even more interesting is that the C677T polymorphism resides in exon 4 and overlaps with this promoter (Figure 1B). Transient expression studies revealed that the T-allele was associated with low expression of the novel transcript variant (Trc.v2), which is characteristic for the control (healthy) individuals. Accordingly,

the low Trc.v2 expression is protective against the investigated diseases and C677T is part of a bona fide *duon* since it encodes an internal part of the full-length MTHFR, and as part of the intronic promoter, it affects the expression of the truncated MTHFR isoform.

By exploring Li/Si scores in 22 control individuals and 55 patients (32 RA and 23 SLE) we took the first steps toward evaluating Li/Si score as a potential biomarker. On the basis of the observed Li/Si scores, we defined 3 levels (low, intermediate and high Li/Si score classes), which can be linked to disease susceptibility. Notably, the current threshold values could be further specified after extended studies for larger patient populations. Although healthy individuals dominantly possess low Li/Si scores (*Class A*), similar Li/Si values could also be detected in RA (25%, $p < 0.01$, OR: 0.074, CI: 0.019, 0.0009) and SLE (30.4%, $p < 0.01$, OR: 0.097, CI: 0.024, 0.001) patients (Figure 3), which can be attributed to the polygenic nature of diseases implicated more decisive risk regions. RA and SLE. Intermediate Li/Si scores (*Class B*) were found to be exclusive for RA and SLE patients, and a subpopulation of these subjects were also prone to ADEs. Specifically, patients with intermediate Li/Si scores tend to develop hypersensitivity to DMARDs, including MTX, azathioprine, chloroquine and naltrexone. The link between Li/Si score and MTX toxicity can be explained by the fact that this drug inhibits the folate cycle in which MTHFR plays a role, and the disease-associated imbalance of MTHFR isoform expression might also trigger ADEs. However, the other DMARDs, including azathioprine, chloroquine and naltrexone, are involved in different pathways, and it is unclear how Li/Si scores relate to the adverse reactions to these drugs.

Conclusion

Application of the Li/Si score might help guide clinical decisions regarding therapeutic intervention in several ways by identifying patients with a high tolerance toward DMARDs (*Class A and Class C*) who might benefit from an increased dose. On the other hand, individuals with potential ADEs (*Class B*) could be treated more carefully to minimize DMARD induced toxicity. Since ADE prone individuals constitute a subpopulation among patients with intermediate Li/Si scores (*Class B*), it will be essential to identify additional factors that could be used to discriminate between the DMARD sensitive and insensitive subjects. The Li/Si score in its current form has shown potential as a predictive biomarker in RA and SLE patient populations. To be widely used as a biomarker, the Li/Si score must be assessed in an extended patient cohort, and further studies should be conducted to validate the threshold values of Li/Si score-based classifications. The discovery and preliminary validation of Li/Si score in this study might have an impact on the use of DMARDs in rheumatic disorders and that the Li/Si score has the potential to become the first non-serological predictive test in RA and SLE.

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Author's Contributions

Study conception and design is done by A.B. and T.A.R., Acquisition

analysis and interpretation of data is done by T.O., A.B., L.K., Z.B., G.H., T.T.G. and T.A.R., Study supervision is done by A.B. and T.A.R. and T.A.R. wrote the first draft and all other authors amended the manuscript.

Competing Interests

The authors declare that they have no competing interests.

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