Abstract

Treatment strategies blocking tumor necrosis factor (anti-TNF) have proven very successful in patients with rheumatoid arthritis (RA), showing beneficial effects in approximately 50-60% of the patients. However, a significant subset of patients does not respond to anti-TNF agents, for reasons that are still unknown. The aim of this study was to validate five single nucleotide polymorphisms (SNPs) of PTPRC, CD226, AFF3, MyD88 and CHUK gene loci that have previously been reported to predict anti-TNF outcome. In addition, two markers of RA susceptibility, namely TRAF1/C5 and STAT4 were assessed, in a cohort of anti-TNF–treated RA patients, from the homogeneous Greek island of Crete, Greece. The RA patient cohort consisted of 183 patients treated with either of 3 anti-TNF biologic agents (infliximab, adalimumab and etanercept) from the Clinic of Rheumatology of the University Hospital of Crete. The SNPs were genotyped by TaqMan assays or following the Restriction Fragments Length Polymorphisms (RFLPs) approach. Disease activity score in 28 joints (DAS28) at baseline and after 6 months were available for all patients and analysis of good versus poor response at 6 months was performed for each SNP. None of the 7 genetic markers correlated with treatment response. We conclude that the gene polymorphisms under investigation are not strongly predictive of anti-TNF response in RA patients from Greece.

Introduction

Rheumatoid Arthritis (RA) is an autoimmune disorder characterized by chronic and destructive inflammation in synovial joints, exhibiting a highly variable disease course [1]. Conventional disease-modifying antirheumatic drugs (DMARDs), most commonly methotrexate (MTX), remain the cornerstone of RA treatment [2]. However, patients for whom MTX produces an inadequate response are treated with biological agents which inhibits inflammatory cytokines (tumor necrosis factor (TNF)-α and interleukin 6 (IL-6) [3], deplete B-cells or inhibit T-cell activation. A major impediment to successful management is that 30–40% of patients have inadequate response and the reasons for this remain largely unknown. Anti-TNF agents work through the inhibition of the interaction between TNFα and its receptors, thus inhibiting the
downstream signaling [4]. Five of these drugs, namely adalimumab, infliximab, etanercept, golimumab and certolizumab are approved for use in RA [2]. Ideally, clinicians would like to have predictors of response, in order to select the appropriate agent in individual basis. Reliable predictors (biomarkers, clinical predictors) are clinically important to avoid potential side effects from agents that will not have clinical benefit and will be an important first step as we move towards an era of stratified medicine [5].

Genetic markers could be useful in daily practice because they do not vary with time, and analysis can be carried out using samples derived from patient’s blood. Many pharmacogenetic studies dealing with anti-TNF response have included genes participating in various signaling pathways that regulate key immune and inflammatory processes. These studies were conducted following either the candidate-gene approach [6–11] or the emerging approach of genome-wide association studies (GWAS) [12]. Furthermore, research has focused on analyzing genes that are associated with the development of RA as potential predictors of anti-TNF efficacy [13–15]. Independent replication of findings in other populations will now be required to provide support that these associations are true positives. This study aimed to confirm whether five SNP markers, found in previous studies to predict responses to anti-TNF treatment of RA patients, are also associated with responses to therapy in a genetic homogeneous Greek population. Additionally, two genes that have previously been shown to correlate with RA development, namely STAT4 rs7574865 and TRAF1/C5 rs1081848 [16–18] were selected for investigation as putative markers of anti-TNF response due to the role of these genes in TNF signaling [19,20].

Materials and Methods

Patients with RA who had received anti-TNF infusions in the Clinic of Rheumatology of the University Hospital of Crete, satisfying the American College of Rheumatology (ACR) criteria [21], were included (40 males, 143 females [78.1%]). TNFα blockade therapy was given to these patients following unsuccessful treatment with at least one disease modifying anti- rheumatic drug (DMARD). Clinical response was determined according to the European League Against Rheumatism (EULAR) criteria [22]. Information on age, gender, anti–cyclic citrullinated peptide (anti-CCP) antibody positivity, rheumatoid factor (RF), anti-TNF start and follow-up dates (treatment duration), DAS28 scores at baseline and at 6 months follow-up was recorded. The study was approved by the local Ethics Committee for medical research of the University Hospital of Heraklion, Crete, and was carried out in compliance with the declaration of Helsinki. Informed consent of the patients was oral because the samples were analyzed as anonymous ones and the aforementioned ethics committee approved this procedure.

We selected a panel of single nucleotide polymorphism (SNP) markers mapping to five recently suggested treatment-response associated genes (PTPRC, CD226, AFF3, MyD88 and CHUK) and 2 RA susceptibility loci (TRAF1-C5 and STAT4) for genotyping in a genetic homogeneous cohort of patients treated with anti-TNF agents. Allelic discrimination of the PTPRC rs10919563, CD226 rs763361, AFF3 rs10865035, MyD88 rs7744 and CHUK rs1159174 SNPs was carried out using commercially available Taqman assays on a 7000 Real-Time PCR system (catalogue ID:C_31565763_10, C_1464836_20, C_2099360_10, C_3094830_10 and C_1345902_10, respectively) from Applied Biosystems (Foster City, CA, USA). Amplification and genotyping for the TRAF1/C5 rs1081848 (Sdu) and STAT4 rs7574865 (HpaI) was performed by restriction fragments lengths polymorphism by using protocols developed from our group and published previously [23]. Genotypes were scored blindly, and analysis of all ambiguous samples was repeated. Moreover, 10% of the samples were amplified twice for checking the accuracy of the results.

Summary descriptive statistics are presented as mean +/- SD, or frequencies, as appropriate. SNP variants and other categorical variables were compared between the responders and non-responders by means of chi-square or Fisher’s exact test. Continuous parameters were compared between these two groups with independent samples t-test. IBM-SPSS was used for the analyses, and all statistical tests were carried out at the two-sided 5% level of significance. Power analysis was conducted using the Quanto software (available at: http://hydra.usc.edu/gxe) at a two-sided level of p<0.05, assuming a log-additive genetic model, the sample size available, the observed allele frequencies in the general population and an estimated effect (odds ratio) of 1.2. No adjustment was made for anti-TNF drug type in these analyses, as the aim was to identify drug class effects.

### Table 1. Cohort characteristics of 183 RA patients treated with anti-TNF therapy.

|                      | Responders | Non-Responders | p-value |
|----------------------|------------|----------------|---------|
| Number               | 106        | 77             |         |
| Sex (% female)       | 74.5%      | 83.1%          | 0.205   |
| Age, mean ± SD       | 59.26 ± 11.87 | 59.52 ± 10.72 | 0.881   |
| RF positive          | 46.9%      | 45.9%          | 1.00    |
| ACPA positive        | 59.43%     | 45.45%         | 0.231   |
| Erosions             | 30%        | 20.8%          | 0.257   |
| Start DAS, mean ± SD | 6.22 ± 1.22 | 6.095 ± 1.47   | 0.524   |
| End DAS, mean ± SD   | 4.05 ± 1.21 | 6.17 ± 1.29    | <0.01   |
| Improvement           | 2.18 ± 0.96 | 0.07 ± 0.97    | <0.01   |
| Infliximab treated % | 73 (68.9%) | 49 (63.6%)     | 0.526   |
| Etanercept treated % | 10 (9.4%)  | 13 (16.9%)     | 0.175   |
| Adalimumab treated % | 23 (21.7%) | 15 (19.5%)     | 0.854   |
| Number of previous biologic therapies, median (IQR) | 1 (1-2) | 1 (1-1) | 0.490 |
| Methotrexate (MTX) usage | 63.2% | 55.8% | 0.360 |
| Mean dose of MTX (mg/week) | 15.3 ± 3.6 | 13.8 ± 4.7 | 0.080 |
| Prednisolone usage    | 20.8%      | 14.3%          | 0.330   |
Table 2. Genetic association of seven SNPs with the response to treatment with anti-tumor necrosis factor agents in patients with RA from Crete.

| Marker | Gene   | Number | MAF Responders | Genotype counts | p | OR (95% CI) |
|--------|--------|--------|----------------|-----------------|---|-------------|
|        |        |        | Responders     | Non-responders  |    |             |
| rs10919563 | PTPRC | 106    | 0.146          | 0.12            | 76/29/1 | 0.64 | 1.22 (0.54-2.72) |
| rs7574865  | STAT4  | 106    | 0.24           | 0.30            | 55/51/0 | 0.23 | 1.345 (0.73-2.48) |
| rs10865035 | AFF3   | 106    | 0.47           | 0.42            | 31/50/25 | 0.29 | 1.26 (0.72-2.18) |
| rs7744     | MyD88  | 106    | 0.66           | 0.45            | 92/14/0 | 0.5 | 0.67 (0.19-2.29) |
| rs10818488 | TRAF1/C5 | 106  | 0.42           | 0.45            | 27/86/11 | 0.67 | 0.91 (0.52-1.57) |
| rs11591741 | CHUK   | 106    | 0.37           | 0.32            | 42/48/15 | 0.32 | 1.273 (0.71-2.26) |
| rs763361   | CD226  | 106    | 0.44           | 0.43            | 34/51/21 | 1 | 1.015 (0.58-1.76) |

Discussion

The use of anti-TNF biological agents has transformed the management of RA, although a substantial proportion of treated patients demonstrate either partial or no response to these therapies. The present investigation is the first study of genetic predictors of anti-TNF response performed in Greece to date. The cohort of the Cretan RA patients analyzed in the present work is a part of the Hellenic Biologic Registry for Rheumatic Diseases that collects data from all patients who receive biologic agents in 7 Rheumatology Centers of Greece. Considering that independent replication is required to confirm definitively the association of SNPs with response to anti-TNF therapy, we performed the present study focusing on the genetic homogeneous population of Crete, which shares a common genetic and cultural background and a common environment, while it is characterized by good genealogical and clinical records and low migration rates, thus contributing to an increased reliability of the data collected [23–25]. However, our study failed to detect any association between seven SNPs and response to anti-TNF therapy.

SNPs that have been associated with response to anti-TNF therapy confer modest effects, thus, in order to detect subtle effects, studies require adequate power. The current study had 24% power to detect a difference in DAS28 of ≥ 0.6 (a clinically meaningful change) for allele frequencies of >5% (detailed power values are not presented). One possible explanation for our results may therefore be that our study is underpowered to detect modest effects. Of note, the allelic frequencies of the genes analyzed for association with drug response in the Cretan population are not markedly different than those in other European populations i.e. PTPRC MAF 0.15 in Crete vs. 0.12, AFF3 0.53 vs. 0.50, CD226 0.44 vs. 0.48, STAT4 0.24 vs. 0.25 [7,9,10]. These findings emphasize on the lack of any substantial genetic differences between Cretan and other European populations although various previous reports reported some distinct population-specific differences [26–28]. The two RA susceptibility loci analyzed, TRAF1/C5 and STAT4, may also need further investigation. However, genes contributing to disease susceptibility are not necessarily associated with treatment response, as shown previously in the case of the well established RA susceptibility locus mapping to PTPN22 [8]. Interestingly, another RA risk SNP of TRAF1/C5,
the rs3761847, was found to be associated with anti-TNF treatment response in a Southern European population [29]. The rs 6427528 CD84 SNP, which was reported recently that may serve as a useful biomarker for response to etanercept treatment in RA patients of European ancestry [30], will also be investigated in the Cretan population in an attempt to clarify its putative role in this cohort.

Despite substantial effort seen the last few years in the study of genetic markers of anti-TNF treatment response, only few associations have been identified [10]. Furthermore, the genetic associations reported previously are modestly effecting, in contrast, for example, to the large genetic effects seen in studies focusing on the role of VKORC1 and CYP2C9 genes with response to warfarin therapy [31]. It is therefore unlikely that markers of anti-TNF response with large effect exist and we will need to analyse thousands of individuals before reproducible finding will begin to be reported. This will require large national and international collaborations and sharing of datasets.

In conclusion, we present data that does not support any positive association between carriage of alleles for any of the seven SNPs examined and response to anti-TNF therapy in RA patients. However, larger studies are needed to definitively exclude the association of the SNPs under investigation in the Greek population.

Author Contributions

Conceived and designed the experiments: GNG DP AB IF. Performed the experiments: MIZ EM IF PS PR DP. Analyzed the data: GNG MIZ GC PS DTB FCG. Contributed reagents/materials/analysis tools: PS DTB GNG PR AB FCG. Wrote the manuscript: GNG DP MIZ AB GC DTB FCG.

References

1. Molines IB, Schett G (2011) The pathogenesis of rheumatoid arthritis. N Engl J Med 365: 2205-2219 (Review) doi:10.1056/NEJMra1004965. PubMed: 22150039.
2. NICE. Rheumatoid arthritis-drugs for treatment after failure of a TNF inhibitor. Available: http://guidance.nice.org.uk/TA195.
3. Lipsky PE, Van der Heijde DM, St Clair EW, Furst DE, Breedveld FC et al. (2000) Infliximab and methotrexate in the treatment of rheumatoid arthritis. N Engl J Med 343: 1594–1602. doi:10.1056/NEJM200011033432202. PubMed: 11096168.
4. Feldmann M, Brennan FM, Williams RO, Woody JN, Maini RN (2004) The transfer of a laboratory based hypothesis to a clinically useful therapy: the development of anti-TNF therapy for rheumatoid arthritis. Best Pract Res Clin Rheumatol 18: 59-80. doi:10.1016/j.berh.2003.09.010. PubMed: 15123038.
5. Trusheff MR, Burgess B, Hu SX, Long T, Averbuch SD et al. (2011) Quantifying factors for the success of stratified medicine. Nat Rev Drug Discov 31: 817-833. PubMed: 22037040.
6. Criswell LA, Lunn RF, Turner KN, Woehl B, Zhu Y et al. (2004) The influence of genetic variation in the HLA–DRB1 and LTA–TNF regions on the response to treatment of early rheumatoid arthritis with methotrexate or etanercept. Arthritis Rheum 50: 2750–2756. doi:10.1002/art.20469. PubMed: 15457442.
7. Cui J, Saevarsdottir S, Thomson B, Padyukov L, van der Helm-van Mil AH et al. (2010) Rheumatoid Arthritis Risk Allele PTPRC Is Also Associated With Response to Anti-Tumor Necrosis Factor Therapy. Arthritis Rheum 63: 1569-1561. PubMed: 20987874.
8. Potter C, Hyrich KL, Tracey A, Lunt M, Plant D et al. (2009) Association of rheumatoid factor and anti-cyclic citrullinated peptide positivity, but not carriage of shared epitope or PTPN22 susceptibility variants, with antitumour necrosis factor response in rheumatoid arthritis. Ann Rheum Dis 68: 69-74. PubMed: 18375541.
9. Tan RJ, Gibbons L, Potter C, Hyrich KL, Morgan AW et al. (2010) Investigation of rheumatoid arthritis susceptibility genes identifies association of AFF3 and CD226 variants with response to anti-tumour necrosis factor treatment. Ann Rheum Dis 69: 1029-1035. doi:10.1136/ard.2009.118406. PubMed: 20444755.
10. Plant D, Prajapati R, Hyrich KL, Morgan AW, Wilson AG et al. (2012) Biologics in Rheumatoid Arthritis Genomics and Genomics Study Syndicate, Barton A. Replication of association of the PTPRC gene with response to anti-tumor necrosis factor therapy in a large UK cohort. Arthritis Rheum 64: 665-670. doi:10.1002/art.33381. PubMed: 21952740.
11. Prajapati R, Plant D, Barton A (2011) Genetic and genomic predictors of anti-TNF response. Pharmacogenomics 12: 1571-1588 (Review) doi: 10.2217/pgs.11.114. PubMed: 22044414.
12. Plant D, Bowes J, Potter C, Hyrich KL, Morgan AW et al. (2011) Genome-wide association study of genetic predictors of anti-tumor necrosis factor treatment efficacy in rheumatoid arthritis identifies associations with polytomies at seven loci. Arthritis Rheum 63: 645-653. doi:10.1002/art.30130. PubMed: 21061259.
13. Barton A, Eyre S, Ke X, Bowes J, Fanoultsooupolou K et al. (2009) Identification of AF4/FMR2 family, member 3 (AFF3) as a novel rheumatoid arthritis susceptibility locus and confirmation of two further panautoimmune susceptibility genes. Hum Mol Genet 18: 2518–2522. doi:10.1038/hmg/ddp177. PubMed: 19395276.
14. Haller JP, Maier LM, Cooper JD, Plagnol V, Hinks A et al. (2009) CD225 Gly307Ser association with multiple autoimmune diseases. Genes Immun 11: 5–10. doi:10.1038/gene.2009.85. PubMed: 18971939.
15. Plant D, Flynn E, Mbarek H, Dieudé P, Cornelis F et al. (2010) Investigation of potential non-HLA rheumatoid arthritis susceptibility loci in a European cohort increases the evidence for nine markers. Ann Rheum Dis 69: 1548–1553. doi:10.1136/ard.2009.121020. PubMed: 20498205.
16. Kurreeman FA, Padyukov L, Marques RB, Schrodi SJ, Seddighzadeh M et al. (2007) A candidate gene approach identifies the TRAF1/C5 region as a risk factor for rheumatoid arthritis. PLOS Med 4:e278. doi:10.1371/journal.pmed.0040278. PubMed: 17880261.
17. Plenge RM, Saetland M, Padyukov L, Lee AT, Remmers EF et al. (2007) TRAF1-C5 as a risk locus for rheumatoid arthritis-a genomewide study. N Engl J Med 357: 1199-1209. doi:10.1056/NEJMoa073491. PubMed: 17804836.
18. Remmers EF, Plenge RM, Lee AT, Graham RR, Hom G et al. (2007) STAT4 and the risk of rheumatoid arthritis and systemic lupus erythematosus. N Engl J Med 357: 977-986. doi:10.1056/NEJMoa073003. PubMed: 17804842.
19. Tatsikos EN, Louaini D, Dunn IF, Sannikova TY, Davidson L et al. (2007) TNF signaling in TRAF1-deficient mice. Immunity 15: 647-657. doi:10.1016/S1074-7613(01)00207-2. PubMed: 11672546.
20. Wajant H, Henkler F, Scheuch P (2001) The TNF-receptor-associated factor family: scaffold molecules for cytokine receptors, kinases and their regulators. Cell Signal 13: 389-400. doi:10.1016/S0950-0722(01)00016-0. PubMed: 21384837.
21. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF et al. (1988) The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 31: 315-324.
22. van Gestel AM, Prevoo ML, van ’t Hof MA, van Rijswijk MH, van de Putte LB et al. (1996) Development and validation of the European League Against Rheumatism response criteria for rheumatoid arthritis: comparison with the preliminary American College of Rheumatology and the World Health Organization/International League Against Rheumatism criteria. Arthritis Rheum 39: 34–40. doi:10.1002/art.1780390105. PubMed: 8546736.
23. Zervou MI, Sidirooulos P, Petrizzi E, Vagziouarakis V, Krasoudaki E et al. (2008) Association of a TRAF1 and a STAT4 gene polymorphism with increased risk for rheumatoid arthritis in a genetically homogeneous population. Hum Immunol 69: 567-571. doi:10.1016/j.jhimmun.2008.06.006. PubMed: 18622578.
24. Zervou MI, Goulielmou GS, Castro-Giner F, Tosca AD, Krueger-Krasagakis S (2009) STAT4 gene polymorphism is associated with psoriasis in the genetically homogeneous population of Crete, Greece. Hum Immunol 70: 738-741. doi:10.1016/j.jhimmun.2009.05.008. PubMed: 19500629.
25. Mantaka A, Goulielmos GN, Koulentaki M, Tsagournis O, Voumvouraki A et al. (2012) Polymorphisms of genes related to endothelial cells are associated with Primary Biliary Cirrhosis (PBC) patients of Cretan origin. Hum Immunol 73: 829-835. doi:10.1016/j.humimm.2012.05.003. PubMed: 22609442.

26. Patrinos GP, Kollia P, Loutradi-Anagnostou A, Loukopoulos D, Papadakis MN (1998) The Cretan type of non-deletional hereditary persistence of fetal hemoglobin [A gamma-158C--->T] results from two independent gene conversion events. Hum Genet 102: 629-634. doi: 10.1007/s00439004-0753. PubMed: 9703422.

27. Spanaki C, Plaitakis A (2004) Bilineal transmission of Parkinson disease on Crete suggests a complex inheritance. Neurology 62: 815-817. doi:10.1212/01.WNL.0000113720.71587.88. PubMed: 15007141.

28. Apostolakis S, Baritaki S, Krambovitis E, Spandidos DA (2005) Distribution of HIV/AIDS protective SDF1, CCR5 and CCR2 gene variants within Cretan population. J Clin Virol 34: 310-314. doi:10.1016/j.jcv.2005.01.010. PubMed: 16286055.

29. Canhão H, Rodrigues A, Santos MJ, Carmona-Fernandes D, Costa J et al. (2012) TRAF1/C5 locus is associated with response to anti-TNF in rheumatoid arthritis. J Transl Med 10 (Suppl 3): 31. doi: 10.1186/1479-5876-10-S2-A31. PubMed: 22369209.

30. Cui J, Stahl EA, Saevarsdottir S, Miceli C, Diogo D et al. (2013) Genome-wide association study and gene expression analysis identifies CD84 as a predictor of response to etanercept therapy in rheumatoid arthritis. PLOS Genet 9: e1003394. PubMed: 23555300.

31. Bodin L, Verstuyft C, Tregouet DA, Robert A, Dubert L et al. (2005) Cytochrome P450 2C9 (CYP2C9) and vitamin K epoxide reductase (VKORC1) genotypes as determinants of acenocoumarol sensitivity. Blood 106: 135–140. doi:10.1182/blood-2005-01-0341. PubMed: 15790782.