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Genomic stratification by expression of HLA-DRB4 alleles identifies differential innate and adaptive immune transcriptional patterns - A strategy to detect predictors of methotrexate response in early rheumatoid arthritis

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ABSTRACT

Effective drug selection is the current challenge in rheumatoid arthritis (RA). Treatment failure may follow different pathomechanisms and therefore require investigation of molecularly defined subgroups. In this exploratory study, whole blood transcriptomes of 68 treatment-naïve early RA patients were analyzed before initiating MTX. Subgroups were defined by serologic and genetic markers. Response related signatures were interpreted using reference transcriptomes of various cell types, cytokine stimulated conditions and bone marrow precursors. HLA-DRB4-negative patients exhibited most distinctive transcriptional differences. Preponderance of transcripts associated with phagocytes and bone marrow activation indicated response and transcripts of T- and B-lymphocytes non-response. HLA-DRB4-positive patients were more heterogeneous, but also linked failure to increased adaptive immune response. RT-qPCR confirmed reliable candidate selection and independent samples of responders and non-responders the functional patterning. In summary, genomic stratification identified different molecular pathomechanisms in early RA and preponderance of innate but not adaptive immune activation suggested response to MTX therapy.

1. Introduction

In the last decade treatment options for rheumatoid arthritis have improved dramatically. However, response still remains difficult to predict by clinical and routine laboratory markers. Even genome wide biomarker studies [1,2] and autoantibody screenings so far did not meet the high expectations for routine application. This does not discredit the technical methods or question their sensitivity. The main problem lies in the heterogeneity of the disease itself with many different genetic associations, most dominantly in the HLA-locus [3,4]. Extensive knowledge has been accumulated for the HLA-DRB1 encoded shared epitope (SE) alleles [5,6], which contribute to distinct pathomechanisms in RA and which may thereby interfere with drug response [7–9]. An additional HLA-DR locus, HLA-DRB4 is present on nearly all haplotypes containing HLA-DRB1*04 alleles, as well as on those containing HLA-DRB1*07 and *09 alleles [8]. In contrast to the 1860 currently known alleles for the HLA-DRB1 locus [10], its paralogue HLA-DRB4 is very restricted in variability of alleles (n = 17) and frequency of carriers and typically is present in individuals positive for the HLA-DRB1*04 SE variants [11,12]. HLA-DRB4 expression may also influence RA immunopathology and development of ACPAs [13,14]. Interestingly, all oligonucleotides of the 209728_at probeset on HG-U133 Plus 2.0 GeneChip microarrays detect sequences specific for the constant 3′ region of HLA-DRB4 (www.affymetrix.com) and thus can conveniently provide information on RA-relevant HLA genetics when applying this technology.

In line with the influence of HLA, the key molecule at the interface between innate and adaptive immunity, RA is divided into sero-positive and sero-negative disease. Presence of adaptive patterns like rheumatoid factor (RF) and/or antibodies against citrullinated proteins (ACPA) may influence disease severity and drug responsiveness [7,15–18].

Given that all possible treatment outcomes from good to non-response occur in each genetic or serologic sub-entity, each of these

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may bear its own spectrum of pathophysiological characteristics. Therefore, subdividing patients according to genetics or potentially modulatory conditions prior to screening for response prediction appears obvious to focus on and elaborate well-defined molecular mechanisms.

On this background, we applied transcriptional profiling as the currently most reasonable approach for a global screen and processed whole blood to cover all potential phenotypic changes from innate to adaptive immunity. Early RA seemed most attractive for being unmodified by DMARDs, and comparison of responders with non-responders before initiation of therapy appeared as best option to concentrate on disease heterogeneity and not on drug effects. As non-responders to MTX are more likely to respond to biologics, which have dramatically changed RA outcome, this comparison according to differential outcome after methotrexate (MTX) therapy focused on a very fundamental molecular phenomenon, which may exist already at clinical onset or develop during the course of the disease when MTX efficacy deteriorates.

2. Materials and methods

2.1. Patient recruitment and sample collection

Sixty-eight patients with early active RA (ACR/EULAR classification criteria [19]) and naïve to DMARDs were recruited in the multicenter study HITHARD [20] (n = 29) and in the biomarker study ArthroMark (www.arthromark.de/) at the Charité (n = 21 for pattern selection, n = 18 for independent validation). Clinical characteristics and EULAR responses [21] were assessed at baseline and at an average of 3.7 months after start of MTX treatment (15 mg/week as a standard, continuous dose). Statistical evaluation of clinical and laboratory data was performed by Wilcoxon-rank test. Studies were approved by the Berlin State and the Charité University Ethics Committees.

2.2. Preparation of total RNA

Blood was collected in PAXgene® tubes (PreAnalytiX, Hombrechtikon, Switzerland), incubated for 24 h at 20 °C on an overhead rotator and stored at −20 °C. After RNA extraction (blood RNA kit; Qiagen, Hilden, Germany), quantity and quality was determined by NanoDrop-1000® spectrophotometry and Bioanalyzer-2100®.

2.3. Microarray analyses

After globin reduction (GLOBINclear™; Life Technologies, Darmstadt, Germany), reverse transcription and in vitro amplification (GeneChip® 3'IVT Express; Affymetrix, High Wycombe, UK), labeled cRNA (50 μg/ml) was fragmented and hybridized onto U133-Plus-2.0 microarrays for 18 h at 45 °C and stained (Fluidics Station 450). CEL-files were extracted from raw data (Affymetrix 3000 scanner) using the GCOS software.

2.4. Statistical analyses of microarray

Signals were generated by RMA and MAS5.0 algorithms and quantile normalized. Group comparisons were performed with Qlucore (Lund, Sweden) for ANOVA and PCA, with R-packages (Limma, glmnet) for Limma and Lasso and with BioRetis (http://www.bioretis-analysis.com) for MAS5.0 group comparisons [22]. Eventually, MAS5.0 algorithms were applied. The top 100 genes increased and decreased by frequency of change calls and mean of signal log ratios (SLR) and represented by the probeset with the highest mean signal were selected. PAM was applied for classification and cross-validation. Specificity, sensitivity and likelihood ratio were determined for quality assessment. Hierarchical clustering of log transformed and z-normalized signal intensities (z-values) was performed in Genesis [23]. Response was scored in each sample as average of z-values for genes increased minus average of z-values for genes decreased in responders. Receiver Operating Characteristics (ROC) and Area Under the Curve (AUC) were calculated in MS-Excel.

Functional analysis was performed with own* and public reference transcriptomes of blood cell populations (GSE17639, GSE23618, GSE58173*), bone marrow precursors (GSE19599), G-CSF-effects on whole blood (GSE7400) and monocytes stimulated with LPS, TNF and IFNs (GSE38351*) were processed by MAS5.0, quantile normalized and averaged for each defined condition. Functional scores were calculated as median of log-transformed and z-normalized signals from all genes of each functional pattern [24]. For validation of independent samples, MAS5.0 based group comparisons were performed with the corresponding HLA-DRB4^{-} group of the initial selection study as reference for non-response and response patterns in the previously established top 100 genes increased and decreased in responders.

2.5. Ingenuity pathway analysis (IPA)

IPA was applied for gene interaction and ontology analyses (Ingenuity, Redwood City, CA, USA). Highest-scoring neighborhood analysis was performed with 200 genes increased and decreased in MTX responders versus non-responders of the HLA-DRB4$^{+/-}$ subgroups.

2.6. RT-qPCR validation

Gene candidates were validated by quantitative PCR (qPCR) employing commercial RT^2-Primer Assays (Qiagen) and Power SYBR® Green (Life Technologies). For two genes no commercial RT^2-Primer Assays were available. ACTA1, GAPDH, HPRT1, and RPLP0 were tested as housekeeping reference using BestKeeper. GAPDH served as inter-run calibrator with the Universal Human Reference RNA (Agilent). Amplification efficiencies and corrected ΔΔCt-values were calculated as described [25].

3. Results

3.1. Patients and descriptive statistics

Fifty patients were grouped into responders (n = 26), moderate responders (n = 11) and non-responders (n = 13) according to EULAR DAS28 response between baseline and after 3.7 months of MTX therapy (Table 1, Supplemental Table S1). No significant differences of routine parameters were found between responders and non-responders at baseline. Only DAS28 and dependent parameters significantly differed after 3.7 months as consequence of classification. Correspondingly, DAS28 reduction revealed inverse correlations with DAS28-associated parameters after treatment ($-0.71 > R > -0.93$) but not with any clinical or laboratory parameter at baseline ($-0.43 < R < 0.40$).

3.2. Testing genetic and immunological subgroups for molecular patterns of response

Pre-analysis of array data identified MAS5.0/BioRetis algorithms as most reliable and suggested heterogeneous properties of the patient population (Supplemental Fig. S1, Supplemental Tables S2 and S3). Assuming impact by immunological or genetic characteristics on pathomechanisms and treatment outcome, patients were grouped either by gender, RF, ACPA, SE or the haplotype-specific HLA transcripts DRB4 (209728_at) and DQA1 (203290_at) [26]. In each subgroup, responders and non-responders were compared and genes ranked by frequency of change call and fold change. For each subgroup comparison, the best 100 genes increased and 100 decreased in responders were selected. PAM classification including cross-validation revealed highest sensitivity, specificity, likelihood ratio, and predictive values in the subset of HLA-DRB4^{-} patients (Table 2).