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Prediction of Response to Methotrexate Treatment in RA using mRNA and miRNA biomarkers

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BACKGROUND

Methotrexate (MTX) is one of the most prescribed long-term effective disease-modifying anti-rheumatic drugs and therefore considered as the standard medication for the treatment of Rheumatoid Arthritis (RA). Nonetheless, about 40-50% of the patients does not show adequate improvement under MTX therapy, while some patients even suffer from adverse and toxic side effects like pulmonary, hepatic, renal or bone marrow abnormalities. The usage of therapy-specific predictive biomarkers would be an option to minimize such effects, to determine the most promising therapy for each individual patient subgroups and last but not least to reduce socio-economic costs.

OBJECTIVES

This study aimed at defining predictive mRNA and miRNA biomarkers in whole blood samples (PAXgene) from 52 RA patients for the response to future treatment with the anchor drug MTX.

METHODS

Sample acquisition

Whole blood samples collected from 52 active RA patients before initiated MTX treatment were obtained from two independent clinical studies. Classification of RA patients into good (***R***), moderate (***MR***) and non-responders (***NR***) was performed according to DAS28 and EULAR response criteria after 12-14 weeks of MTX treatment. Only patients with no or low steroid dose supplementation were analyzed. Patient characteristics and statistics are summarized in the ***Supplementary data handout***.

RNA purification and processing

Extraction of intracellular total RNA and miRNA was performed according to PAXgene Blood Kit protocol (Qiagen). For mRNA expression analyses extracted RNA samples were 'globin depleted' using the GLOBINclear Kit (Life Technologies), followed by mRNA amplification employing GeneChip 3'IVT Express Kit and hybridized onto GeneChip 133 Plus 2.0 Arrays (Affymetrix). For miRNA expression analyses extracted total-RNA samples were labeled using the FlashTag Biotin Labeling Kit (Genisphere) and hybridized onto GeneChip miRNA 2.0 Arrays according to the manufacturer protocol. Labeling of mRNA/miRNA microarrays was carried in a GeneChip Fluidics Station. Hierarchical clustering of candidate genes to discriminate response to MTX was performed using the Genesis software.

Expression profiling of predictive gene candidates using microarray anlyses

Differential mRNA gene expression was performed by Affymetrix profiling using samples from 26 R, 13 MR, and 13 MR. Generated mRNA signal data were normalised and evaluated using the Affymetrix Expression Console (MAS5.0) and the BioRetis online database algorithm (BioRetis). Gene expression change calls and fold-change (FC; ≤ / ≥ 1.5) expression values were used as selection criteria to select mRNA candidate genes. MicroRNA expression data from 18 R, 13 MR, and 8 NR prior to treatment with MTX.were background corrected (RMA), quantile normalised and median polished utilizing the miRNA QC-Tool. Selection of predictive miRNA biomarkers was performed by FC-values (≤ / ≥ -1.5) and p- values ≤0.05.

Validation of biomarkers predictive biomarkers using qPCR

Independent validation of obtained differential mRNA expression results was performed by quantitative real time PCR (qPCR) for 30/32 predictive mRNA candidate genes employing standardised RT² primers (Qiagen; Germany) and Power SYBR Green (Life Technologies) in a StepOne Plus Real-Time Cycler (Life Technologies). For two of the predictive candidate genes no commercial RT² Primer Assays were not available. Ribosomal phosphoprotein large P0 (RPLPO) was used as reference control and GAPDH was used as inter-run calibrator using an in parallel runned standardized human reference RNA (Agilent). Amplification efficiencies, corrected delta-delta-Ct values, and statistical evaluation of identified mRNA biomarkers predictive for success of MTX therapy was performed with the REST2009 software (Qiagen).

RESULTS

Patient characteristics

All 52 patients included in this study showed active RA with a DAS28 score >5,0 prior to treatment with MTX. The mean disease duration of RA patients was 1.9 months (SD=2.1; SEM=0.5) in the HITHARD study group and 3.8 months (SD=1.5; SEM=0.3) in patients included in the own observational study. No or only low correlation ($p<0.05$) was found between the future response to MTX and gender, disease duration, the initial visual analog scale values, c-reactive protein, erythrocyte sedimentation rate, tender joint count and swollen joint counts, the initial DAS28 score, as well as RF and ACPA (***Supplementary data handout***).

Selection of mRNA predictors

For robust statistical interpretation of gene expression profiles at first only definite R and NR were used for predictor gene selection. Initial comparison of R and NR (FC ≤ / ≥ -2.0) revealed a set of 14 candidate genes. However, no clear separation between R and NR was obtained. Interestingly, 9 of these genes were gender related (*) and one of them was HLA-DRB4 which was considered as pivotal pre-selection criterion master gene (***Fig. 1***).

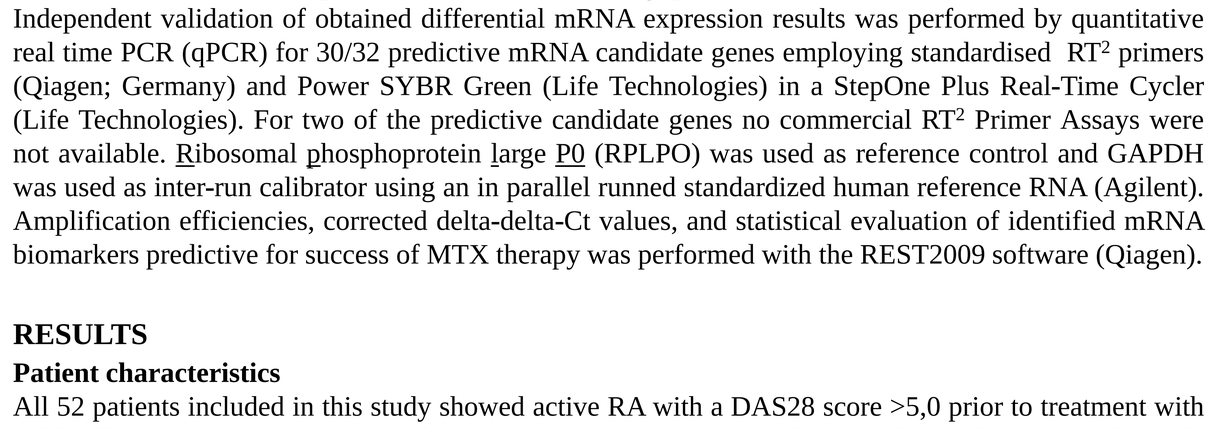

Figure 1: Hierarchical clustering of mRNA predictive candidate genes using PAXgene samples
Comparison of R, MR and NR gene expression profiles (FC ≤ / ≥ 2) obtained from 52 RA patients revealed 14 genes. Candidate genes were clustered hierarchically. However, no clear separation was obtained. Ten of these gene candidates were gender related. Non-gender related genes are indicated by an asterisk (*).

After separation of the patients into HLA-DRB4 positive (n=29) and negative (n=23) subgroups two predictive mRNA gene sets (n=16 each) were defined (Change call 80%, FC FC ≤ / ≥ 1.5). Hierarchical cluster analyses using these marker gene panels resulted in a clear discrimination of R and NR. and revealed sensitivity and specificity rates of 92.9% and 100% in the HLA-DRB4 positive patient subgroup (***Fig. 2A***) and sensitivities & specificity rates of 100% in the HLA-DRB4 negative RA patient subgroup (***Fig. 2B)***.

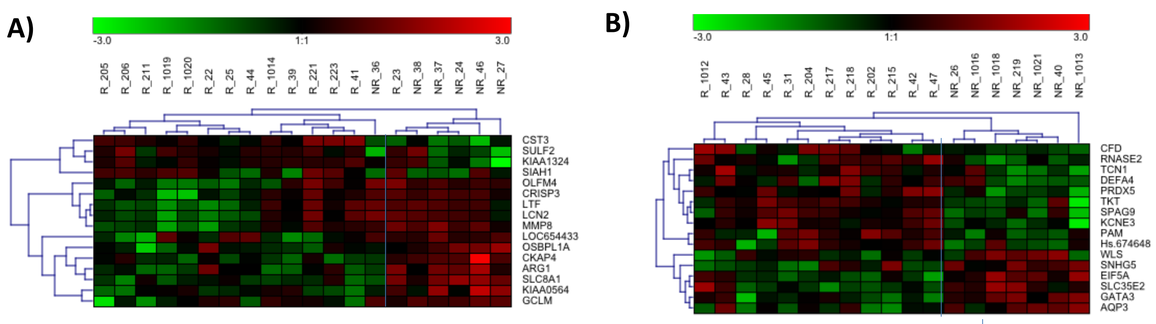

Figure 2: Hierarchical clustering of predictive mRNA biomarkers of the HLA-DRB4 positive and negative RA patient subgroups
Hierarchical clustering with subgroup specific gene expression values of the HLA-DRB4 positive **(A)** and negative subgroup **(B)** was performed (sensitivity and specificity rates: 100%).

Integration of corresponding mRNA expression data from MR to the hierarchical cluster analyses confirmed these results. In the HLA-DRB4 positive patient subgroup (***Fig. 3A***) sensitivity and specificity rates of 83.3% and 100% were obtained, the separation of R, MR, and NR in the HLA-DRB4 negative RA patient subgroup (***Fig. 3B***) resulted in a 100% sensitivity and specificity.

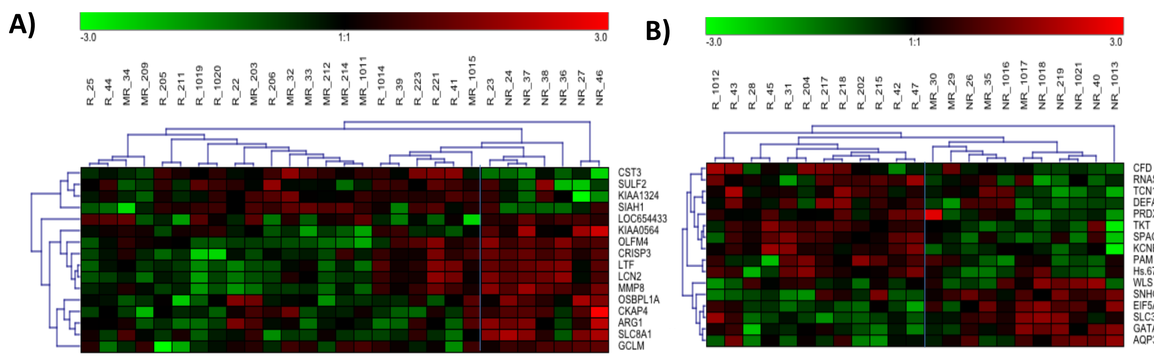

Figure 3: Hierarchical clustering of predictive mRNA biomarkers of the HLA-DRB4 positive and negative RA patient subgroups
As indicated in Fig.1, the same predictive mRNA genes specific for the HLA-DRB4 positive **(A)** and the negative subgroup **(B)** were analysed by hierarchical clustering (Sensitivity and specifity rates are given above).

Validation of MTX predictor genes using qPCR

Real time qPCR was performed independently to validate the predictive gene sets of the HLA-DRB4 positive and negative subgroups. qPCR and microarray data correlated well for 22 of the 30 tested genes confirming a robust translatability. Statistically highly significant discrimination of MTX response were obtained by qPCR of the followong candidate genes: OLFM4 ($p=0.005$), LCN2 ($p=0.007$), MMP8 ($p=0.008$), SLC8A1 ($p=0.010$), SNHG5 ($p=0.013$), and KIAA0564 ($p=0.036$) (***see: supplementary data handout).***

Selection of micro RNA predictors

Analyses of micro-RNA (miRNA) gene expression signals resulted in 6 selected gene candidates. Hierarchical clustering allowed a good separation MTX responders and non-responders (***Fig. 4***). One NR and one R clustered incorrectly.

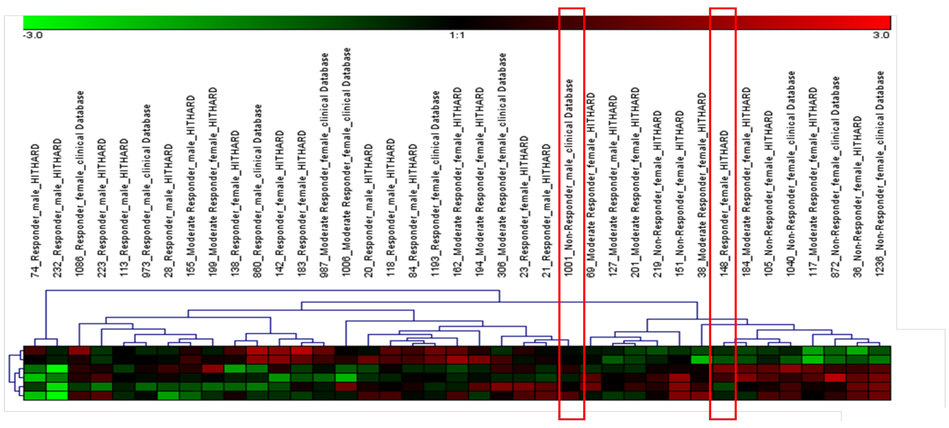

Figure 4: Hierarchical clustering of miRNA biomarkers predictive for success of MTX treatment
Six miRNA candidate genes to predictive the future success of MTX treatment were selected. Hierarchical cluster of miRNA expression signals (39 patient samples) allowed a good separation of R (left panel) and NR (right panel).

CONCLUSION

An early prediction of successful therapy outcome, in particular MTX provides the opportunity for individually customized medications. Identification of specific predictive response marker genes such as CD11c for anti-TNF monotherapy[1,2] leads to a substantial interest to define predictive biomarkers for MTX monotherapy[3,4], as well. Combination of those prediction markers is a next approach to calculate the outcome of FDA recommended standard combination therapy. We presume that MTX therapy outcomes can be predicted by the defined RNA gene sets after inclusion of RA subgrouping criteria, thus constituting a big step towards the achievements of "personalized medication" **i)** even after initiation of RA, **ii)** without unwanted risks of toxification or adverse reactions, allowing **iii)** significant reduction of socio-econmic costs.

References:

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