



Baseline adenosine receptor mRNA expression in blood as predictor of response to methotrexate therapy in patients with rheumatoid arthritis

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Abstract

Methotrexate (MTX) reduces inflammation by increasing extracellular adenosine levels in rheumatoid arthritis (RA) patients. Adenosine acts via G-protein coupled receptors; ADORA1, ADORA2a, ADORA2b and ADORA3. We studied if baseline expression of whole blood adenosine receptors can predict response to MTX. RA patients [American College of Rheumatology/European-League-Against-Rheumatism (EULAR) 2010 criteria], Disease modifying anti-rheumatic drug (DMARD) naïve with active disease [Disease Activity Score 28 (DAS28) > 3.2] were enrolled. Blood samples were collected at baseline ($n = 100$) and at 4 months after therapy ($n = 50$). Patients were treated with MTX monotherapy. Based on EULAR response, patients were categorized into three groups i.e. good, moderate and non-responders. Adenosine receptors gene expression (ADORA1, ADORA2a, ADORA2b and ADORA3) in whole-blood RNA was measured using real-time PCR. HPRT1 was used as housekeeping gene. Receptor expression at baseline was correlated with response to MTX. All values are expressed as median (interquartile range). Hundred patients [87% females; age 40 (18) years]; duration of disease 24 (24.75) months; DAS28 4.7 (1.25) were enrolled. Fifty-one were classified as good, 28 moderate and 21 as non-responders. No expression of ADORA1 and ADORA2b was detected. Significant difference was observed in the expression levels of ADORA3 between good vs non-responder ($P = 0.03$) and moderate vs non-responder ($P = 0.002$). On ROC curve analysis, ADORA3 with cut-off value of less than -0.60 (ΔCt) predicted non-response to MTX treatment (AUC: 0.7, $P = 0.006$). ADORA3 mRNA levels in whole blood may serve as a biomarker of response to MTX.

Keywords Adenosine receptor · Adenosine receptor 3 · Rheumatoid Arthritis · Biomarkers · Methotrexate

Introduction

Rheumatoid arthritis is a chronic, systemic autoimmune disease characterized by synovial proliferation and pro-inflammatory cytokine-mediated joint destruction. To minimize severe joint damage leading to functional disability, early diagnosis and treatment are crucial in patients with

RA [1]. Methotrexate is the first line of therapy prescribed in RA patients and approximately one-third of the patients do not respond to this drug [2, 3]. It is important to identify a marker which can predict response to MTX therapy in the initial phase of the disease. Early treatment of the disease has been shown to reduce joint damage and improve functional status in patients with RA.

The mechanism of action of MTX is complex. One of the major mechanism of action of MTX is to increase the extracellular adenosine levels [4]. Adenosine acts via adenosine receptors expressed on inflammatory cells. Adenosine receptors are G-protein coupled receptors categorized into four subclasses: adenosine A1 receptor (ADORA1), adenosine 2a receptor (ADORA2a), adenosine 2b receptor (ADORA2B) and adenosine 3 receptor (ADORA3) [5]. The adenosine receptors, ADORA2a and ADORA2b are associated with Gs protein inducing cAMP production while ADORA1 and

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ADORA3 with Gi protein which inhibits cAMP production [6].

Polyglutamated form of MTX inhibits aminoimidazole carboxamido adenosine ribonucleotide (AICAR) transformylase increasing the concentration of AICAR [7–9]. The accumulation of AICAR causes inhibition of enzymes, such as adenosine monophosphate (AMP) deaminase and adenosine deaminase which elevates the concentration of adenosine intracellularly [7]. In animal models of RA, MTX treatment leads to accumulation of AICAR and the AICAR levels are associated with increased concentration of extracellular adenosine [10, 11]. The speculated mechanism for the increase in extracellular concentration of adenosine is through sequential dephosphorylation of ATP (adenine nucleotides) by ecto-5'-nucleotidase [12]. In RA patients, synovial cAMP levels inversely correlated with disease activity, erythrocyte sedimentation rate (ESR) and IL-18 levels [13]. Thus, adenosine is a key player in mediating anti-inflammatory response on treatment with MTX.

Adjuvant-induced arthritis development was inhibited on the infusion of adenosine into the knee joint of rats and the severity of collagen-induced arthritis was reduced in response to an adenosine receptor agonist [14, 15]. The use of ADORA2A antagonist or adenosine deaminase abrogates the anti-inflammatory effect of MTX in vivo in carrageenan-induced mouse air pouch inflammation [11]. The use of caffeine, nonselective adenosine receptors antagonist, is associated with poor response to MTX [16, 17]. In ADORA2a knockout mice model of peritonitis, MTX treatment was shown to increase adenosine levels in the exudates [18]. Moreover, response to MTX was reduced in CD73 knockout mice as compared to wild type highlighting the importance of extracellular production of adenosine [19]. The CD73 protein is an ecto-5'-nucleotidase and it converts adenosine monophosphate (AMP) into adenosine [19].

The expression of adenosine receptors is increased on immune cells in RA patients [20, 21]. Therefore, it is possible that RA patients with higher expression of adenosine receptors are more likely to respond to MTX therapy. In this study, we have explored if mRNA expression of four adenosine receptors in whole blood cell of RA patients at baseline could predict response to MTX therapy.

Materials and methods

Patients

RA patients fulfilling the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) 2010 classification criteria [22] were included in the study. All had Disease Activity Score at 28 joints (DAS28) > 3.2. Patients were either MTX naïve or had

received MTX > 6 months ago and had not taken any other disease modifying anti-rheumatic drug (DMARD). Patients with any contraindications to MTX treatment such as chronic liver disease, chronic kidney disease, pregnancy, lactation were excluded from the study. The study was approved by Institutional Ethics Committee of SGPGIMS (IEC number: 2016-169-PHD-EXP) and all patients provided a written, informed consent.

At baseline (BL), clinical details about disease, physician and patient global assessment, health assessment questionnaire (HAQ), and DAS28 score were assessed. At baseline, a blood sample was collected for adenosine receptor analysis ($n = 100$). All patients were started on weekly 10 mg MTX, the dose was escalated every 2 weeks by 2.5 mg till they achieved DAS28 < 2.6, or reached a maximum tolerated dose with upper limit of 25 mg/week. At the end of 4 months, besides assessing disease activity, a blood sample ($n = 50$) was drawn again for estimation of adenosine receptors mRNA expression levels. Patients were classified into three groups, good (GR), moderate (MR) and non-responders (NR) based on European league of associations of Rheumatology (EULAR) criteria [22]. According to EULAR criteria for response, the decrease in DAS28 from baseline as well as the absolute value of DAS28 at present time is used to classify the patient as non, moderate or good responder. If the decrease in DAS28 is more than 1.2 and the DAS28 now is < 3.2 it is classified as good response and if it is more than > 3.2 it is moderate response. If the decrease is between > 0.6–< 1.2 and the present DAS28 is less than 5.1 it is classified as moderate response and if the present DAS28 is more than 5.1 it is classified as no response. If the decline in DAS28 is less than 0.6 it is classified as no response.

Isolation of RNA from blood

Blood was collected in EDTA coated vials (BD vacutainer 5 ml; BD Diagnostics, San Diego) and stored in aliquots (0.25 ml blood in 0.75 ml Trizol, Life Technologies Inc., USA) at -80°C . Total RNA was extracted using chloroform-isopropyl alcohol extraction method. In brief, sample was brought to room temperature (RT) and vortexed for 15 min. Lysed solution was centrifuged ($10,000\times g$, 10 min, 4°C). To the supernatant, 200 μl chloroform was added and mixed for 15 min by inverting the tube and held for 10 min at RT. The tube was centrifuged ($12,000\times g$, 15 min, 4°C) and the aqueous layer was collected and mixed with 500 μl of isopropyl alcohol by gently inverting the tube (10 min). The mixture was centrifuged ($12,000\times g$, 10 min, 4°C) and the pellet was washed twice by adding 75% ethanol (1 ml) followed by centrifugation ($7500\times g$, 5 min, 4°C). Finally, the pellet was air dried (30 min) and re-dissolved in 30 μl RNase-free water. Concentration and purity of RNA were

determined using Nano-Drop spectrophotometer (Nanodrop Inc., Wilmington, DE, USA).

Adenosine receptor quantification using RT-qPCR

For adenosine receptor analysis, 1 µg of total RNA was reverse transcribed to complementary DNA (cDNA) using random reverse transcription (RT) primers, 100 mM each dNTP, 50 units/µl MultiScribe reverse transcriptase enzyme, RNase inhibitor and RT buffer (Applied Biosystems, Carlsbad, CA, USA). The reaction mixture (10 µl) was incubated in a thermocycler (Mastercycler PCR system, Eppendorf, USA) at 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min; and then maintained at 4 °C. To check for DNA contamination a negative control reaction (without reverse transcriptase enzyme) was setup for 10 samples.

Quantification of adenosine receptors was done by real-time PCR performed on resulting cDNA via TaqMan receptor-specific primers and TaqMan Fast advanced PCR Master Mix with UNG (Applied Biosystems, Carlsbad, CA, USA). No expression of the adenosine receptors was observed in the negative controls indicating no DNA contamination.

The reaction mixture was prepared according to manufacturer's protocol and incubated in LightCycler® 480 II Real-Time System (Roche Applied Science, Penzberg, Germany) at 50 °C for 2 min, 95 °C for 2 min and 40 cycles of 95 °C for 1 s and 60 °C for 20 s. Expression levels of adenosine receptors; ADORA1 (assay ID Hs00181231_m1), ADORA2a (assay ID Hs00169123_m1), ADORA2b (assay ID Hs00386497_m1) and ADORA3 (assay ID Hs04194761_s1) were normalized with respect to HPRT1 (assay ID:Hs02800695_m1) and GAPDH (assay ID Hs02786624_g1) gene. The values were expressed as ΔC_t , i.e., the C_t value of adenosine receptor gene was subtracted from the C_t value of housekeeping genes. Fold change calculation after treatment was calculated as follows: ΔC_t value (HPRT1 mean – adenosine receptor C_t mean), $\Delta \Delta C_t$ value (Follow-up (FU) ΔC_t – BL ΔC_t) and fold change as $2^{-(\Delta \Delta C_t)}$.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., USA). Difference at baseline levels of adenosine receptors between GR, MR and NR and between BL and FU samples were assessed using Mann–Whitney U test. Paired analysis of BL and FU sample was done using Wilcoxon matched-pairs signed rank test. Correlation of response status was done with age, duration of disease, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and baseline DAS28 using Spearman's rank correlation. Association of response status with gender and RF positivity was determined using Chi-squared test. The mRNA levels

of ADORA2a and ADORA3 was correlated with baseline DAS28 to see any influence on disease activity.

Logistic regression analysis was done to assess the effect of gender, baseline DAS28, baseline ADORA3 levels and duration of disease on response status of RA patients. Receiver operating characteristic (ROC) curve analyses for blood ADORA3 at baseline was performed and area under curve (AUC) was calculated. The cut-off was determined using Youden index. P value less than 0.05 was considered to be statistically significant. All data are expressed as median and interquartile range (IQR).

Results

Patients characteristics

Hundred patients (87 females) with median age of 40 (IQR 18) years, duration of disease 24 (IQR 24.75) months and DAS28 4.7 (IQR 1.25) were enrolled (Table 1).

Baseline level of adenosine receptors in whole blood RNA of patients

The mRNA expression of ADORA1 and ADORA2b in whole blood RNA was not detectable by qPCR. The median (IQR) for the expression levels of ADORA2a and ADORA3 in 100 patients, respectively, were -0.24 (0.92) and -0.18 (2.74) with HPRT1 and -5.265 (0.67) and -4.91 (2.27) with GAPDH. Since similar results were obtained with both the housekeeping genes, data normalized with HPRT1 was analysed further.

Moderate correlation of ADORA3 was observed with ADORA2a ($r=0.48$, $P<0.05$). The expression levels of ADORA2a and ADORA3 did not correlate with baseline clinical variables such as duration of disease and DAS28.

Baseline difference in expression of ADORA2a and ADORA3 in good, moderate and non-responders to MTX

Most patients improved with methotrexate therapy (Table 2). Out of 100 patients, 51 were classified as GR, 28 as MR and 21 NR to MTX. The median baseline levels of CRP were higher in responders [0.46 (1.47)] as compared to non-responders [0.31 (0.49)] ($p=0.03$). No difference was observed in baseline values of RF, DAS28, ESR, duration of disease, age and gender between R vs NR groups.

The median levels of ADORA2a in GR, MR and NR are -0.11 (0.93), -0.24 (0.68) and -0.4 (1.72), respectively. The expression levels of ADORA3 in GR, MR and NR are as follows, 0.27 (2.56), 0.63 (2.32) and -1.15 (1.89), respectively. The expression of ADORA2a and ADORA3

were reduced in NR as compared to GR and MR. However, significant difference was observed between GR vs NR ($P=0.02$) and MR vs NR ($P=0.003$) in the expression levels of ADORA3 (Fig. 1).

In ROC curve analysis patients were categorized into two groups i.e. responder (GR + MR) and non-responder,

ADORA3 showed a good AUC of 0.7 with 95% confidence interval (CI) of 0.59–0.80 ($P=0.006$). The ADORA3 mRNA expression cut-off value of less than -0.60 (ΔCt), with a sensitivity of 63.6% and specificity of 61.9% predicts non-response to MTX (Fig. 2). The positive predictive value is 86.2% and the negative predictive value is 31%.

Table 1 Baseline characteristics of Rheumatoid Arthritis patients

Characteristics	Total ($n=100$)	Responder		Non-responders ($n=21$)	P value (responders vs. non-responders)
		Good responders ($n=51$)	Moderate responders ($n=28$)		
Female/male, n	87/13	40/11	27/1	20/1	0.21
Age, year	40 (18)	39 (22)	39.5 (16)	45 (19.5)	0.48
Duration of disease in months	24 (24.75)	12 (30)	24 (28.5)	36 (60)	0.17
Tender joint count	11 (8.5)	10 (6)	13.5 (8)	11 (12)	0.88
Swollen joint count	8.50 (7)	7 (6)	10.5 (8.5)	9 (10)	0.92
Health assessment questionnaire	1.50 (1)	1.4 (1.12)	1.69 (0.5)	1.69 (0.88)	0.85
C-reactive protein, mg/dl	2.19 (3.36)	2.12 (2.68)	2.29 (5.75)	2.17 (3.29)	0.03
Disease activity score (DAS28-CRP)	4.7 (1.25)	4.41 (0.8)	5.17 (1.13)	5.2 (1.91)	0.09
IgM-RF-positive (%)	84 (84%)	44 (86.3%)	22 (78.6%)	18 (86%)	0.92

Male, female and RF positivity are expressed as number and percentage in parenthesis. Other variables are represented as median and IQR in parenthesis

Table 2 Effect of methotrexate therapy on disease variables ($n=100$)

Characteristics	Baseline	4 months after therapy	P values
Tender joint count	11 (8)	3 (6)	<0.05
Swollen joint count	8 (7)	2 (5)	<0.05
C-reactive protein	2.15 (3.34)	0.42 (1.49)	<0.05
Disease activity score (DAS28-CRP)	4.64 (1.17)	2.80 (1.39)	<0.05
Health assessment questionnaire	1.5 (0.88)	0.63 (0.94)	<0.05

All values are given as median with interquartile range in the bracket

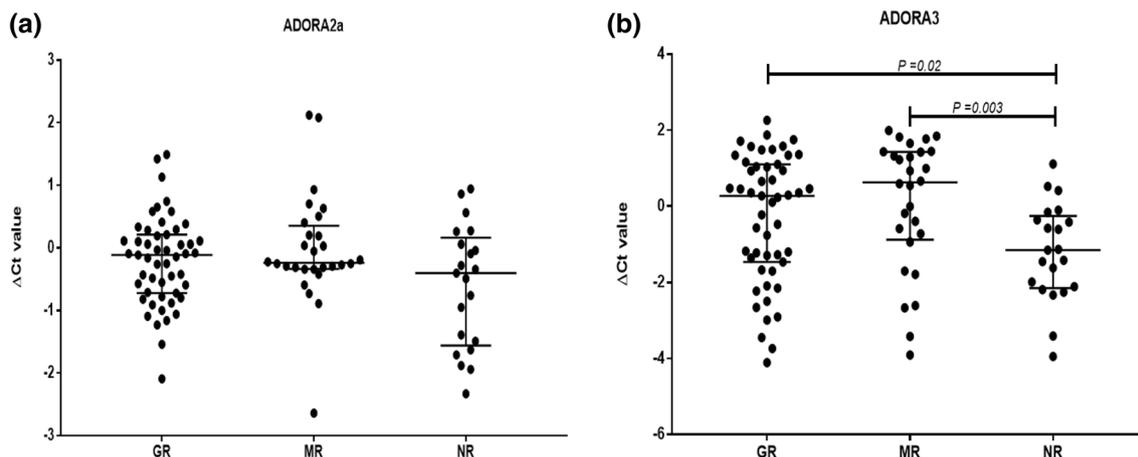


Fig. 1 Baseline whole blood mRNA expression of ADORA2a (a) and ADORA3 (b) in three groups i.e. good responder ($n=51$), moderate responder ($n=28$) and non-responder ($n=21$) group of RA patients

by qPCR. $*P<0.05$, as determined by Mann–Whitney U test. ΔCt delta threshold cycle, GR responder, MR moderate responder, NR non-responder

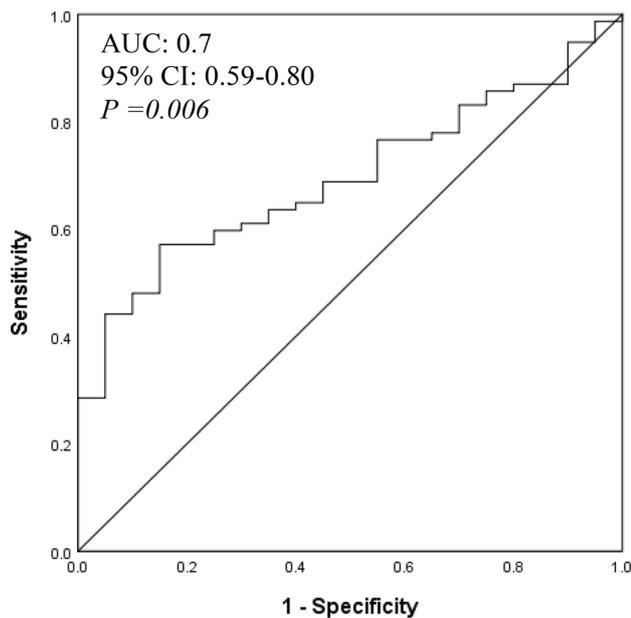


Fig. 2 Receiver operating characteristic (ROC) curve analyses of ADORA3 as predictor of response to MTX. The area under curve (AUC) is 0.7 with 95% confidence interval (CI) of 0.59–0.80

Significant correlation of duration of disease ($r=0.28$, $P=0.004$) and BL_DAS28 ($r=0.32$, $P=0.001$) was observed with response at 4 months. A binary logistic regression analysis was performed to determine the effect of gender, duration of disease, BL_DAS28 and ADORA3 on the likelihood of response to MTX therapy in RA patients. The model could correctly classify 84% of the cases. Duration of disease ($P=0.029$) and ADORA3 ($P<0.03$) were significantly associated with the response status of the patient (Supplementary Table 1).

Effect of MTX on levels of ADORA2a and ADORA3

The mRNA expression levels of ADORA2a and ADORA3 were upregulated 4 months after MTX therapy (Fig. 3). The median mRNA expression of ADORA2a and ADORA3 at BL – 0.24 (0.93), – 0.18 (2.74) and FU 0.34 (0.87), 0.73 (2.70), respectively. Change in relative expression of ADORA2a and ADORA3 are as follows. ADORA2a: fold change 1.79, $P=0.0001$ and ADORA3: fold change 2.39, $P=0.0003$.

Discussion

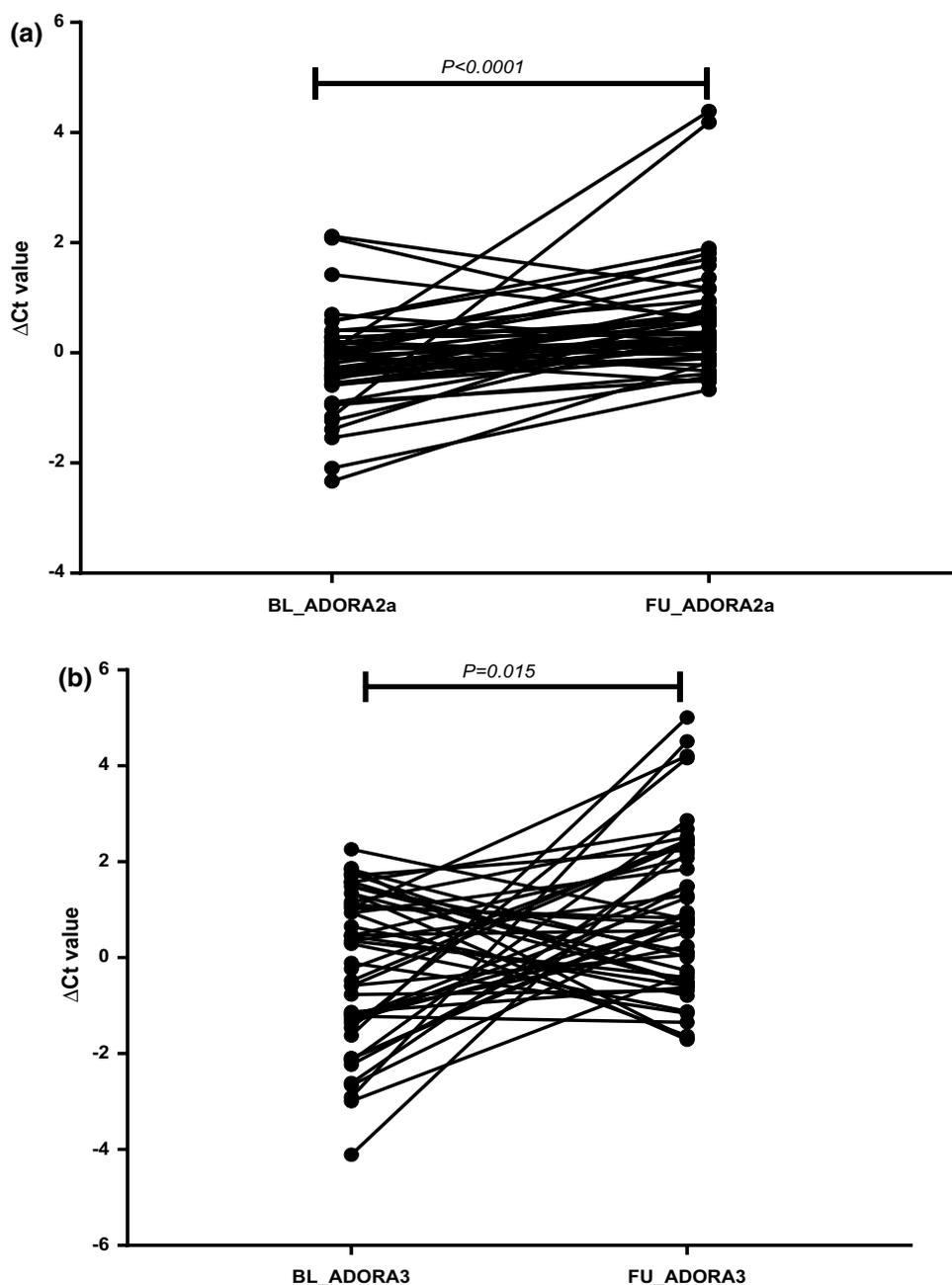
Low levels of ADORA3 mRNA levels in whole blood are associated with non-response to MTX. In addition, MTX administration leads to increase in mRNA levels of ADORA3 and ADORA2a.

MTX suppresses inflammation by increasing the extracellular concentration of adenosine which subsequently acts via adenosine receptors [23]. The protein level expression of ADORA3 has been reported in PBMCs by western blot analysis [24, 25]. At local site mRNA levels of ADORA1 and ADORA2b can easily be detected in fibroblast-like synoviocytes [26]. Both ADORA2a and ADORA3 protein levels are upregulated on lymphocytes and neutrophils of RA patients as compared to that of healthy controls [21]. No previous study has examined the mRNA expression of adenosine receptors in whole blood sample. Whole blood mRNA is easy to obtain, as you need small volume of blood and requires less time. Herein, no expression of ADORA1 and ADORA2b was detected in whole blood mRNA of RA patients. This could be related to poor expression of these receptors on lymphocytes and neutrophils [21]. The expression of ADORA2a and ADORA3 was comparable and moderate correlation was observed between expressions of these two receptors.

Majority of clinical effects mediated by adenosine are thought to be mediated via ADORA2a. In neutrophils, ADORA2a inhibits adhesion, recruitment and superoxide generation [6]. In macrophages, it inhibits pro-inflammatory cytokine production, induces the transition of M1 to M2 macrophages (pro to anti-inflammatory response) and prevents bone destruction by inhibiting osteoclast generation [6]. Adenosine is known to downregulate T cell proliferation, activation and Fas–Fas ligand mediated cell death [27, 28]. The signalling through ADORA2a might be responsible for the development of T regulatory cells (Treg) expressing CD39 and CD73 and hence downregulating T cell activation [29]. The expression of CD39 on Tregs of RA patients was found to be lower in non-responders to MTX [30]. Thus, in general ADORA2a signalling is beneficial in reducing inflammation and this could be the signalling pathway through which MTX works in RA patients. ADORA3 signalling increases the chemotaxis of neutrophils to the site of inflammation and the expression of certain cytokines are inhibited in macrophages [6].

Lack of correlation of ADORA2a and ADORA3 mRNA levels with DAS28 score is at variance with a study showing negative correlation between them, however, they had used PBMC samples and correlated density of ADORA2a and ADORA3 with DAS28 [20]. We next examined the potential of baseline ADORA2a and ADORA3 expression to predict clinical response at 4 months. Patients with non-response to MTX had lower levels of ADORA3 as compared to good and moderate response patients. In lymphocytes, both ADORA2a and ADORA3 activation inhibits NF- κ B signalling and induces anti-inflammatory response [20]. Therefore, patients having lower expression of these receptors will have less of anti-inflammatory response mediated by MTX. In combination with the duration of disease, levels

Fig. 3 Paired analysis of ADORA2a **a** and ADORA3 **b** at baseline ($n=50$) and 4 months after MTX therapy ($n=50$). * $P<0.05$, as determined by Wilcoxon matched-pairs signed rank test. *BL* baseline, *FU* follow-up, ΔCt delta threshold cycle



of ADORA3 contributed significantly to predicting response to MTX.

MTX therapy in patients with RA upregulated whole blood mRNA expression of ADORA2a and ADORA3. In RA patients, MTX therapy has been shown to result in upregulation of ADORA2a and ADORA3 on lymphocytes and neutrophils as compared to that of healthy control [21]. Whereas, on PBMCs the expression of only ADORA3 was increased significantly after MTX therapy [31]. Thus, our results validate the previous observations and support the use of whole blood mRNA.

Low expression of ADORA3 in whole blood mRNA may serve as a potential biomarker for distinguishing RA patients response to MTX therapy. However, the result needs to be validated in another prospective cohort to assess the utility of the proposed cut-off value as there is some overlap between responders and non-responders. For applicability across laboratories, absolute quantification of ADORA3 mRNA using a calibration curve of known standard sample may be better. ADORA3 whole blood mRNA level had modest sensitivity for prediction thus alone it may not be useful in clinic but in combination with other biomarkers it may

provide better sensitivity and specificity as has been shown for pharmacogenetic biomarkers [32].

To conclude, baseline whole blood mRNA expression of ADORA3 appears to be a fair predictor of response to MTX therapy in RA patients.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests regarding the publication of this paper.

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