Expression of the genes facilitating methotrexate action within subcutaneous rheumatoid nodules

E.L. Houlder[1], M.J. Millier[1], J. Highton[1], D. Gwynne-Jones[2], L.K. Stamp[3], P.A. Hessian[1]

[1]Leukocyte and Inflammation Research Laboratory, Department of Medicine;
[2]Department of Surgical Sciences, University of Otago, Dunedin, New Zealand;
[3]Department of Medicine, University of Otago, Christchurch, New Zealand.

Abstract

Objective

We sought further understanding of the association between methotrexate (MTX) therapy and accelerated development of subcutaneous rheumatoid nodules. The objective was to establish expression of genes involved in the transport, metabolism, and mechanism of action of MTX within nodule tissue. We also examined for differences in gene expression between nodules from patients actively receiving MTX compared to those not receiving MTX.

Methods

Subcutaneous nodule tissues (n=23) were obtained from 21 patients with RA, undergoing elective surgery. Expression of genes important to the transport (SLC19A1, ABCB1, ABCC1, ABCG2), metabolism (FPGS, GGH), and mechanism of action (TYMS, MTR, MTRR) of MTX, including for the adenosine receptors $ADORA_1$, $ADORA_{2A}$, $ADORA_{2B}$, $ADORA_3$ and $ADORA_{3variant}$ were quantitated by real-time PCR in each nodule sample.

Results

Transcripts for all genes were found in all nodules. Expression of MTR was significantly reduced in nodules from patients receiving MTX therapy. Patterns of gene expression differed, with those metabolising MTX more prominent in nodules from patients receiving MTX when compared to nodules from those not receiving MTX, where genes involved in MTX transport were more prominent.

Conclusion

Genes involved in MTX handling are expressed in rheumatoid nodules, providing further evidence that metabolism of MTX within nodules could exert a local effect. Furthermore the profile of gene expression in nodules differed from that previously observed in rheumatoid synovial membrane. The significant reduction of MTR expression in nodules has implications for MTR- and MTRR-mediated re-methylation reactions. Our data suggest that in contrast to synovium, downstream methylation reactions involving methionine and the biosynthesis of S-adenosylmethionine (SAM) could be reduced in nodule tissue. This could help explain differing responses to MTX in rheumatoid nodules and synovium and warrants further investigation.

Key words

rheumatoid arthritis, nodules, methotrexate, gene expression

Emma L. Houlder, BA
Melanie J. Millier, MSc
John Highton, MBChB, FRACP
David Gwynne-Jones, BM BCh, FRCS, FRACS
Lisa K. Stamp, MBChB, FRACP, PhD
Paul A. Hessian, PhD

Please address correspondence to:
Dr Paul Hessian,
Department of Medicine,
University of Otago,
P.O. Box 56,
Dunedin, New Zealand.
E-mail: paul.hessian@otago.ac.nz

Received on November 23, 2016; accepted in revised form on March 28, 2017.

Funding: this study received grant funding from the Health Research Council of New Zealand. E. Houlder was supported by a summer student research grant provided by the University of Otago Arthritis Research Theme.

Competing interests: none declared.

Introduction

Rheumatoid nodules occur in approximately 30% of patients with rheumatoid arthritis (RA) (1), are generally associated with more severe disease and commonly situated at subcutaneous points of friction. Nodules are more frequent in patients that are rheumatoid factor (RF) positive, as well as being associated with smoking and homozygosity for HLA-DRB1 risk alleles for RA. Accelerated nodule development may be associated with the use of methotrexate (MTX) even though joint disease improves, suggesting different effects of MTX in nodule and synovial tissues (1). The mechanism of MTX action is still not completely understood. As is shown in Figure 1, reduced folate carrier 1 (RFC-1; SLC19A1) and the folate receptor are responsible for MTX entry into cells. Once inside the cell, additional glutamate moieties are added by folylpolyglutamate synthetase (FPGS), resulting in production of MTX polyglutamates ($MTXGlu_n$). Terminal polyglutamates can be removed by γ-glutamyl hydrolase (GGH), allowing $MTXGlu_1$ (parent drug) and $MTXGlu_2$ to be transported out of the cell by members of the ATP-binding cassette (ABC) transporter family, including ABCC1, ABCB1, and ABCG2. MTX polyglutamates are responsible for inhibiting a number of enzymes in the folate pathway, including thymidylate synthase (TYMS), 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase (ATIC) and dihydrofolate reductase (DHFR). This inhibition results in an increase in adenosine synthesis and an inhibition of pyrimidine and polyamine synthesis. The 5-methyltetrahydrofolate-homocysteine methyltransferase (MTR; also known as (MS) methionine synthase) and 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) are involved in catalysing homocysteine to methionine, a step in the polyamine synthetic pathway.

The efficacy of MTX treatment for RA reflects anti-inflammatory and immunosuppressive modes of action. MTX reduces pyrimidine and purine synthesis through folate antagonism, although the importance of this mechanism to the anti-inflammatory effects of MTX has not been fully resolved. In inflamed rheumatoid joints, a reduction in polyamine synthesis accounts for part of the anti-inflammatory action attributable to MTX (2). There is further evidence that links MTX treatment with increased extracellular adenosine. In joint synovial tissue, increased extracellular adenosine is associated with the inhibition of pro-inflammatory cytokine production (3), while the propensity for adenosine to promote multinucleated giant cell formation by human monocytes has been proposed as a possible causative mechanism for MTX in the development of rheumatoid nodules, working through $ADORA_1$ (4, 5). Thus there appears to be a dichotomy of action for MTX between inflamed rheumatoid synovial and nodule tissues. We have previously described expression of these key genes involved in the transport and action of MTX within rheumatoid joint synovia, revealing increases in the expression of a number in those patients taking MTX, including GGH, FPGS and MTRR (6), as well as the adenosine receptor genes, $ADORA_{2A}$ and $ADORA_{2B}$ (3). The extent to which these genes are expressed in the rheumatoid nodule is unknown. Whether MTX can be metabolised locally in nodule tissue and whether there is any difference in gene expression between rheumatoid synovial and nodule tissues are fundamental questions that might help explain the different responses to MTX in the two tissues as well as providing further insight into the link between MTX therapy and the development of rheumatoid nodules. We therefore firstly sought to establish that genes for the MTX transporters (SLC19A1, ABCB1, ABCC1, ABCG2), MTX metabolising enzymes (FPGS, GGH), others involved in MTX's mechanism of action (TYMS, MTR, MTRR) and those encoding four adenosine receptors, $ADORA_1$, A_{2A}, A_3 and $ADORA_{3var}$ are expressed in rheumatoid nodules and then to determine if the profile of expression was different to that previously found in rheumatoid synovial tissues. We also examined for differences in the genes between nodules from patients actively receiving MTX compared to those not receiving MTX.

Patients and methods

Tissue samples

Subcutaneous nodule tissues were obtained from patients with RA as defined by 1987 American Rheumatism Association Classification criteria (7) undergoing elective surgery for nodule removal. Nodules were stored in liquid nitrogen until analysis. Twenty-three nodules, obtained from 21 different patients were analysed including 2 separate nodules, obtained from 2 patients at different time points (21 and 10 months apart, respectively). The study was approved by the New Zealand Health and Disability Ethics Multi-Regional Ethics Committee (MEC/06/02/003). All patients provided written informed consent.

Patient demographics

Of the 21 patients, five were male; mean age (±SE) was 67.5±2 years and RA disease duration was 26.0±2.8 years. All 21 patients had radiographic evidence of erosions. Eighteen patients (86%) were RF positive (mean titre 205±34.9I U/ml). Of 14 patients where anti-cyclic citrullinated peptide (CCP) antibodies were measured, 11 (79%) were positive with mean titre 105.5±27.5 units/ml. For the entire patient cohort, mean ESR was 16.6±3.7mm/hr and CRP 12.5±4 mg/l. Fifteen patients (71%; providing 16 nodules) were taking MTX (7.5–22.5 mg/week) at the time nodule tissue was obtained. The remaining 6 patients (29%; 7 nodules) were grouped as not receiving MTX and included 2 patients who had never received MTX and 6 patients with a history of previous MTX exposure that had been discontinued at least 92 weeks prior to nodule removal. Seven patients were smokers at the time nodule tissue was obtained and 12 patients had a past history of smoking. Remaining patients were never smokers.

RNA extraction, reverse transcription, and quantitative real-time PCR

RNA was extracted from nodule tissue using RNeasy mini kits (Qiagen) and reverse transcribed to cDNA as previously described (3). Quantitative real-time PCR was undertaken using commercial TaqMan assays (Applied Biosystems) for the expression of MTR (Assay ID: Hs00165188_m1), MTRR (Hs00985015_m1), GGH (Hs00914163_m1), ABCB1 (Hs00184500_m1), TYMS (Hs00426568), ABCC1 (Hs00219905_m1), GAPDH (Hs99999905_m1), SLC19A1 (Hs00953345_m1), FPGS (Hs00191956), ABCG2 (Hs01053790_m1), $ADORA_1$ (Hs0037972), $ADORA_{2A}$ (Hs00169123) and $ADORA_{2B}$ (Hs00386497). We also measured three alternative transcripts produced from the $ADORA_3$ gene – the $ADORA_3$ assay (Hs00252933_m1) detects transcripts 1 and 3, while the $ADORA_{3variant}$ assay (Hs00181233_m1) detects transcript variant 2 only. All gene expression was determined in triplicate with values normalised to mean GAPDH expression (Hs999999905_m1) for individual samples.

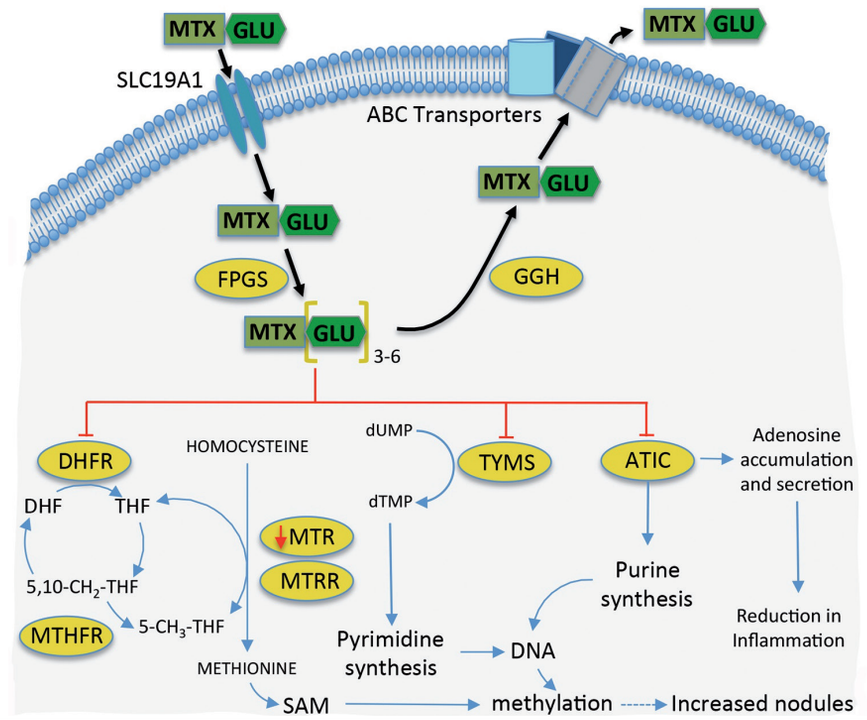

Fig. 1. Methotrexate metabolism and impact.
A schematic representation of key pathways and enzymes known to be involved in the entry, metabolism and mechanism of action for methotrexate (MTX). More details are provided in the main text. Results herein suggest an effect from MTX on the expression of MTR in rheumatoid nodules, involved in the conversion of homocysteine to methionine and the subsequent formation of SAM, utilised for intracellular methylation of DNA, proteins and lipids.
5-CH_3-THF: 5-methyl tetrahydrofolate; 5,10-CH_2-THF: 5,10-methylene tetrahydrofolate; ATIC: 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase; ABC: ATP-binding cassette; DHF: dihydrofolic acid; DHFR: dihydrofolate reductase; FPGS: folylpolyglutamate synthase; GGH: γ-glutamylhydolase; MTHFR: methylenetetrahydrofolate reductase; MTR: 5-metyltetrahydrofolate reductase/MS, methionine synthase; MTRR: 5-methyltetrahydrofolate-homocysteine methyltransferase reductase; MTX: methotrexate; SAM: S-adenosyl methionine; THF: tetrahydrofolate; TYMS: thymidylate synthase.

Statistical analysis

Gene expression data was analysed using GraphPad Prism 6 (GraphPad Software). Mann-Whitney U-tests were performed to assess the difference in gene expression between patients receiving MTX therapy or not. Spearman's correlation tests were used to determine the relationship between gene expression levels. Connectivity between genes was determined based on the number of correlations of $p \leq 0.05$ and strength of the Spearman correlation (r_s) for each individual gene, independent of direction.

Results

Gene expression and the impact of MTX therapy

All the principal genes important in MTX metabolism were expressed in subcutaneous rheumatoid nodule tissue. Current smoking or a history of smoking had no impact on quantitative gene expression (data not shown). With the exception of MTR, the expression of most genes within nodule tissue was unaffected by MTX therapy. The expression of MTR was significantly reduced

(p=0.023) in nodules from patients actively receiving MTX (MTX^+) when compared to nodules from patients not actively receiving MTX (MTX- ; Table I). Although not statistically significant, there was also a trend towards reduced $ADORA_{2B}$ expression in MTX^+ nodules. To investigate the potential for coordinated gene expression within nodule tissue and any effect from MTX therapy, we considered correlations between the expression levels for individual genes. A number of such correlations were identified independent of MTX therapy, revealing a series of increasingly connected genes with expression positively or negatively associated with other genes expressed within nodule tissue (Fig. 2A). TYMS is the most connected gene amongst those evaluated. When subdivided based on MTX therapy, we observed a significant negative correlation between MTR and TYMS expression in MTX^+ nodules that was not observed in MTX^- nodules. In general, significant correlations between genes expressed within MTX^+ nodules involved genes influencing MTX metabolism (Fig. 2B), whereas the correlations in MTX^- nodules were more between genes involved in MTX transport (Fig. 2C). Notably, there was no single gene pairing with significant correlation in both MTX^+ and MTX^- nodules.

Discussion

The appearance or accelerated formation of rheumatoid nodules in patients receiving MTX, with resolution of nodules after drug withdrawal, strongly suggests a causal link. The mechanism by which this accelerated nodulosis occurs is not fully understood but has been postulated to involve increased extracellular adenosine, acting primarily via $ADORA_1$ to promote giant cell formation in macrophages (4). This current study examined the expression of genes involved in the transport, metabolism and mechanism of action of MTX, and of genes encoding adenosine receptors in rheumatoid subcutaneous nodules and compared expression in patients receiving and not receiving MTX therapy. While our analysis is of tissue from a relatively small number of patients, in the context of studies that focus on subcutaneous nodule tissues, our work

Table I. Gene expression in subcutaneous rheumatoid nodules.

Gene	MTX^+ (n=16)	MTX^- (n=7)	p-value
ABCG2	1.273	2.343	0.198
MTRR	0.498	0.696	0.118
MTR	1.682	3.625	**0.023***
GGH	0.146	0.115	0.148
ABCB1	0.563	0.977	0.451
TYMS	0.211	0.135	0.114
ABCC1	0.399	0.460	0.103
SLC19A1	0.019	0.022	0.969
FPGS	0.468	0.394	0.739
$ADORA_1$	0.102	0.264	0.277
$ADORA_{2A}$	0.067	0.078	0.368
$ADORA_{2B}$	2.475	3.481	0.065
$ADORA_3$	0.289	0.339	0.622
$ADORA_{3var}$	0.373	0.366	0.491

Shown are median gene expressions for nodules. Values (ngRNA) are normalised to GAPDH then compared between patients receiving MTX therapy (MTX^+) or without MTX therapy (MTX^-). All data is expressed relative to tonsil cDNA, normalised to GAPDH *p<0.05.

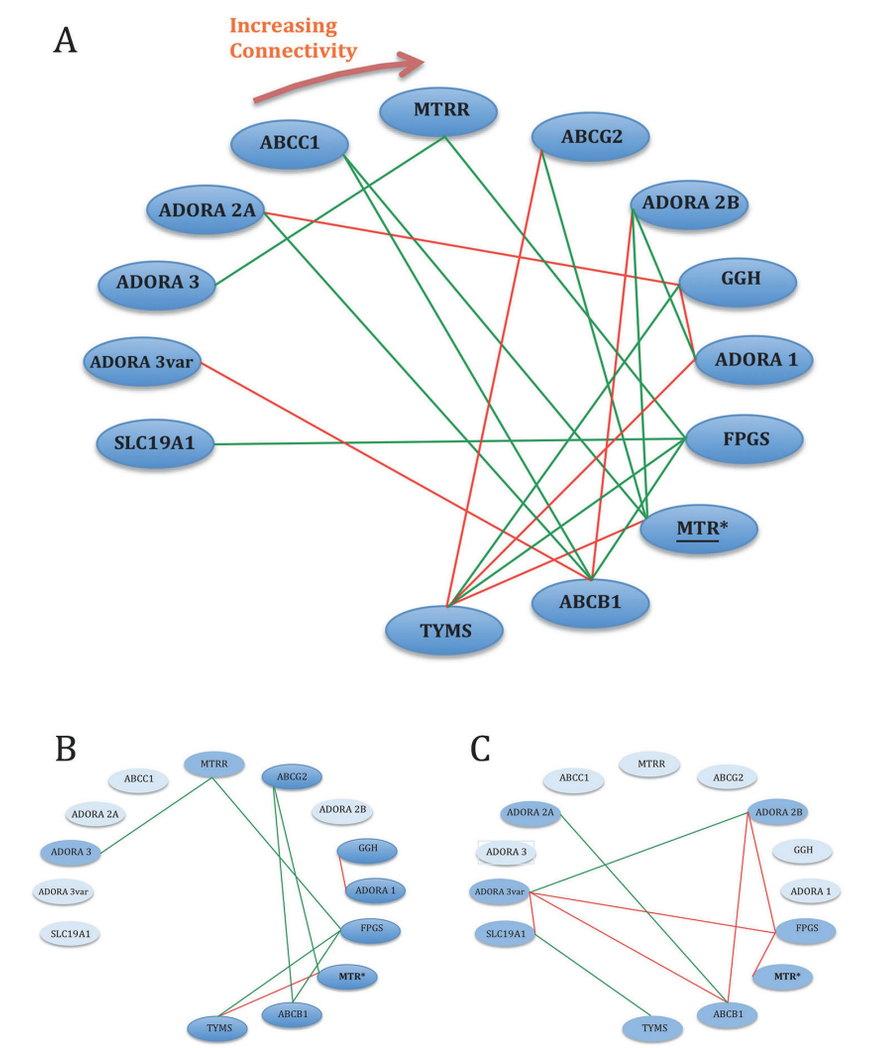

Fig. 2. Gene expression correlations within rheumatoid subcutaneous nodules.
A: Correlations between expressions of all genes are depicted for 23 rheumatoid subcutaneous nodules. Genes are ordered clockwise according to increasing number and strength of significant correlations ($p \leq 0.05$), reflecting increased connectivity. Red lines depict negative correlations, and green lines represent positive correlations. Retaining the same order of genes revealed in "**A**", correlations between expression for (**B**) genes in MTX^+ nodules compared to those for (**C**) genes in MTX^- nodules reveal distinctive patterns respectively incorporating MTX metabolising genes or MTX transport genes.

includes a relatively large sample size.

Transcripts for ABCB1, ABCC1, ABCG2, FPGS, GGH, MTR, MTRR, SLC19A1 and TYMS genes were found in all nodules examined. Transcripts for the adenosine receptor group of G-protein coupled proteins, including $ADORA_1$, A_{2A}, A_{2B}, A_3 and the $ADORA_{3variant}$ were also expressed in nodules. Moreover, smoking did not influence expression of the genes important to MTX handling, consistent with our previous work (8), and further highlighting that smoking seems unlikely to influence gene expression *per se* within established nodule tissue. The expression of the genes involved in MTX transport along with ADORA genes within nodules as we have shown, adds further evidence that MTX can be metabolised and exert a local effect within the nodule that might contribute to nodulosis.

Consistent with the granulomatous nature of subcutaneous rheumatoid nodules, monocyte/macrophages are the principal cellular components of the inflammatory infiltrate (9). Previous work linking MTX metabolism and accelerated nodule formation has focused on any effect from MTX on these cells. It was proposed that increased levels of adenosine, acting primarily through the $ADORA_1$ receptor, promotes syncytium formation and hence production of giant cells (4). In nodules, we found no difference in $ADORA_1$ expression associated with MTX therapy, although there was a trend to reduced expression of $ADORA_{2B}$ that could prove proinflammatory. More recent data concerning monocyte/macrophages suggests that their response to MTX varies, with TYMS expression and activity of the encoded thymidylate synthase (TS) dictating outcome (10-12). In nodule tissue, TYMS was the most connected gene amongst those involved in MTX handling. However, we found no significant difference in TYMS expression when comparing MTX^+ and MTX^- nodules that would implicate TYMS expression as an important component of nodule formation or maintenance or the response to MTX. Previously we have observed increased expression of ABCC1, FPGS, GGH, MTRR, SLC19A1, ADORA2A and ADORA2B in inflamed synovium from RA patients receiving MTX compared to those not receiving MTX (3, 7). Compared to these differences in synovium, we found that in nodules only MTR expression was altered in association with MTX treatment, with significantly less expression of MTR in nodules from patients on MTX therapy, when compared with patients not receiving MTX. These results highlight a seemingly opposing effect from MTX between nodules and synovium that impacts on MTR/MTRR-mediated re-methylation. The methionine synthase (MS) encoded by MTR catalyses re-methylation of homocysteine to methionine, with consequences for S-adenosylmethionine (SAM) concentrations, DNA, lipid and protein methylation and for adenosine availability (Fig. 1) (13). The methionine synthase reductase (MSR) encoded by MTRR is responsible for maintaining levels of activated cobalamin, a necessary cofactor for homocysteine re-methylation. Combined our data suggest re-methylation should be inhibited by reduced MTR/MS expression in MTX^+ nodules but facilitated by the increased MTRR/MSR expression observed in MTX^+ synovium. Consistent with this possibility, an MTR gene polymorphism (A2756G), which impacts negatively on MS function, may be related to the development of MTX-induced accelerated nodulosis (13, 14).

In conclusion we have shown gene expression for the enzymes involved in MTX transport metabolism and mechanism of action are expressed in rheumatoid nodules. This provides evidence that MTX could exert effects locally in nodules. Gene expression in nodules from patients taking MTX showed differences to our previous findings in synovial membrane, and indicate that MTX has a seemingly opposing effect between nodules and synovium, potentially impacting on MTR-MTRR-mediated re-methylation. That this could be a mechanism for MTX-induced nodulosis is supported by knowledge that genetic changes, leading to reduced MS action, are associated with MTX-induced nodulosis. Our results suggest that further investigation of differing MTX metabolism, and particularly methylation status, in nodules and synovia could help to elucidate the mechanism of rheumatoid nodulosis.

Acknowledgments

We are grateful to Jill Drake, Jan Ipenburg, Janine Haslett and Debra Mc Namara for assistance with sample and clinical data collection.

References

1. PRETE M, RACANELLI V, DIGIGLIO L, VACCA A, DAMMACCO F, PEROSA F: Extra-articular manifestations of rheumatoid arthritis: An update. *Autoimmun Rev* 2011; 11: 123-31.
2. CHAN E, CRONSTEIN B: Methotrexate--how does it really work?. *Nat Rev Rheumatol* 2010; 6: 175-8.
3. STAMP L, HAZLETT J, ROBERTS R, FRAMPTON C, HIGHTON J, HESSIAN P: 2012 R. Adenosine receptor expression in rheumatoid synovium: a basis for methotrexate action. *Arthritis Res Ther* 2012; 14: R138.
4. MERRILL J, SHEN C, SCHREIBMAN D *et al*.: Adenosine A1 receptor promotion of multinucleated giant cell formation by human monocytes. *Arthritis Rheum* 1997; 40: 1308-15.
5. PALMER D, HOGG N, HIGHTON J, HESSIAN P, DENHOLM I: Macrophage migration and maturation within rheumatoid nodules. *Arthritis Rheum* 1987; 30: 729-36.
6. STAMP L, HAZLETT J, HIGHTON J, HESSIAN PJR, 40: 1519-22. Expression of methotrexate transporters and metabolising enzymes in rheumatoid synovial tissue. *J Rheumatol* 2013; 40: 1519-22.
7. ARNETT F, EDWORTHY S M, BLOCH D *et al*.: The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 1988; 31: 315-24.
8. KAZANTSEVA M, HIGHTON J, STAMP L, HESSIAN P: Dendritic cells provide a potential link between smoking and inflammation in rheumatoid arthritis. *Arthritis Res Ther* 2012; 14: R208.
9. HIGHTON J, HUNG N, HESSIAN P, STAMP L: Pulmonaruy rheumatoid nodules demonstrating features usually associated with rheumatoid synovial membrane. *Rheumatology* 2007; 46: 811-4.
10. MUNICIO C, SOLER PALACIOS B, ESTRADA-CAPETILLO L *et al*.: Methotrexate selectively targets human proinflammatory macrophages through a thymidylate synthase/p53 axis. *Ann Rheum Dis* 2016; 75: 2157-65.
11. XUE J, SCHMIDT S, SANDER J *et al*.: Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. *Immunity* 2014; 40: 274-88.
12. SOLER PALACIOS B, ESTRADA-CAPETILLO L, IZQUIERDO E *et al*.: Macrophages from the synovium of active rheumatoid arthritis exhibit an activin-dependent pro-inflammatory profile. *J Pathol* 2015; 235: 515-26.
13. BERKUN Y, ATTA I, RUBINOW A *et al*.: 2756GG Genotype of methionine synthase reductase gene is more prevalent in rheumatoid arthritis patients treated with methotrexate and is associated with methotrexate-induced nodulosis. *J Rheumatol* 2007; 34: 1664-9.
14. KARIMIAN M, COLAGAR A: Methionine synthase A2756G transition might be a risk factor for male fertility: Evidence from seven case-control studies. *Endocrinology* 2016; 425: 1-10.