The present study was performed to investigate the molecular mechanism of ankylosing spondylitis (AS). The interaction between micro (mi)RNA-130a and its target tumor necrosis factor (TNF)-1α and histone deactylase (HDAC)3 was assessed in peripheral blood mononuclear cells (PBMCs) from AS patients. Increased HDAC3 and decreased miRNA-130a levels were observed in PBMCs from AS patients. HDAC3 knockdown or HDAC3 inhibition promoted the expression of miRNA-130a, and HDAC3 was recruited to the promoter region of the gene encoding miRNA-130a, and HDAC3 was recruited to the promoter region of the gene encoding miRNA-130a in PBMCs. In addition, miR-130a overexpression led to a decrease, whereas miR-130a inhibition led to an increase of TNF-1α expression in PBMCs. Furthermore, HDAC3 knockdown or HDAC3 inhibition was associated with simultaneous upregulation of the expression of miR-130a and downregulation of the expression of TNF-1α in PBMCs. These results indicated that HDAC3 was involved in the regulation of the underlying molecular mechanism of AS by forming a negative feedback loop with miR-130a and enhancement of TNF-1α expression.

Introduction

Acetylation or deacetylation of histone proteins is regulated by histone acetyltransferase (HAT) or histone deactylase (HDAC), respectively. The equilibrium between HAT and HDAC acts as a switch controlling the level of gene transcription, including that of genes coding for inflammatory cytokines (1). HAT coordinates the recruitment and activation of transcription factors by inducing conformational changes in histones, allowing for access to gene promoters. HDAC counteracts HAT activity by targeting of histones as well as non-histone signal transduction proteins which have roles in inflammation (2). Conditional deletion of HDAC1 in T cells leads to enhanced airway inflammation and increases in the synthesis of T-helper type 2 cell cytokine production (3). The finding that HDAC activity was depressed in synovial tissues from patients with rheumatoid arthritis indicated that strategies restoring HDAC function may have a therapeutic value in this disease (2).

Conversely, inhibition of HDAC with HDAC inhibitors was demonstrated to limit the production of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-1α (4), and the expression of the sirtuin 1 gene is regulated by nuclear factor (NF)-κB, which is activated by TNF-1α (5). Of note, pharmacological inhibitors of HDAC activity have demonstrated potent therapeutic effects in animal models of arthritis and other chronic inflammatory diseases (6,7). A recent study reported a markedly elevated HAT/HDAC ratio in rheumatoid arthritis (RA) and ankylosing spondylitis (AS) during anti-TNF-α therapy, while rituximab increased HAT as well as HDAC (8). Previous studies have reported an imbalance between HAT and HDAC in peripheral blood mononuclear cells (PBMCs) or synovial tissues from patients with RA and AS (9,10).

AS is a chronic inflammatory type of arthritis affecting the axial as well as peripheral skeletons and soft tissues. Changes in the expression of microRNA (miRNA) have been demonstrated to be involved in the pathogenesis of various types of arthritis, including RA and osteoarthritis (OA) (11,12). A number of studies have shown that altered miRNA expression in synovia, PBMCs or T cells from patients with RA or OA is linked with innate immunity and inflammation (13-15). It was recently demonstrated that miR-16, miR-221 and let-7i are overexpressed in T cells from patients with AS, and mechanistic studies showed that the increased let-7i expression facilitates the T helper type 1, interferon (IFN)-γ-associated immune response in T cells (16). Bioinformatics analyses
are widely used to identify potential targets of miR-130a in endothelial progenitor cells (17), hepatitis C virus (18) and cardiomyocytes (19,20). To date, the underlying mechanisms of miR-130a regulation in PBMCs from patients with AS have largely remained elusive.

Advances in the development of effective therapies for AS have been limited as the underlying mechanisms of AS causing immune and inflammatory responses have not yet been elucidated. Therefore, revealing the molecular mechanisms of AS is indispensable for developing effective treatments. In the present study, PBMCs were used investigate the pathogenesis of AS through miR-130a via HDAC-associated pathways.

Materials and methods

Peripheral blood samples and cell culture. Human peripheral blood samples were obtained with written informed patient consent from the Department of Orthopedics, The Third People’s Hospital of Hefei (Hefei, China). The present study was approved by the Ethics Committee of the Department of Orthopedics, The Third People’s Hospital of Hefei. Peripheral blood samples from 20 AS patients and 20 normal healthy control subjects were collected between February 2013 and December 2014.

The human PBMCs were separated from the peripheral blood samples at the Cell Resource Center, Shanghai Institutes for Biological Sciences (Shanghai, China), and maintained in RPMI-1640 (Invitrogen Life Technologies, Inc., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies) at 37°C in a humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA), in an atmosphere of 5% CO₂ and 95% air. The medium was replenished every day.

Hierarchical cluster analysis. Microarray date were obtained from the Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/), using the GEO accession number GSE25101. Observations with adjusted P-values ≥0.05 were removed, and thus excluded from further analysis. The hierarchical cluster analysis was created using a method of hierarchical clustering by GeneSpring GX, version 7.3 (Agilent Technologies, Santa Clara, CA, USA).

RNA interference. The small interfering (si) RNA for human HDAC3 and scramble siRNA were obtained from Dharmacon (Lafayette, CO, USA). The following primers were used: HDAC3 forward, 5′-CAGUGCAUUGGACAAGCCUCUCA-3′ and reverse, 5′-UGAAGACGAGUGCCACUCAGAGG-3′; miRNA-130a forward, 5′-GCUAUCGACUCAGUGCUCUGGU-3′ and reverse, 5′-ACCAACGAGACAGUGCCACUCUCA-3′; scramble forward, 5′-CACGAGUGGGUAAACUCUCA-3′ and reverse, 5′-UGAAGACGAGGCUAACCCACUGUG-3′. The siRNA oligonucleotides (at a final concentration of 100 nM) were transfected into human PBMCs using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. Trichostatin A (TSA) and dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (cat nos. V900931 and D2650; Sigma-Aldrich, St. Louis, MO, USA). The HDAC3 inhibitor TSA and negative control DMSO were used at a concentration of 25 μM for 5 h when the cells had reached 60-80% confluence.

miRNA mimic and transfection. The human miR-130a duplex mimic (miR-130a mimic) and negative control oligonucleotide duplex mimic (miR-NC) were designed and synthesized by Guangzhou Ribobio Co., Ltd. (Guangzhou, China). miR-130a mimic sequence: 5′-CAGUGCAUUGUAAAGGG-3′; and miR-NC sequence: 5′-UUUCUCGAAGGUGUCAGUTT-3′. Once cells reached 30-50% confluence they were transfected with 1 nM miRNAs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Total RNA was extracted 24 h post-transfection, and total cell protein was extracted 48 or 72 h post-transfection.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNA extraction from PBMCs was performed by TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions. Synthesis of cDNA was performed by reverse transcription reaction with 2 μg total RNA using moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) with oligo dT (15) primers (Fermentas; Thermo Fisher Scientific) according to the manufacturer's instructions. The first-strand cDNAs served as the template for the regular PCR performed using TransScript® II Green Two-Step qRT-PCR SuperMix (Transgen Biotech Co., Ltd., Beijing, China), which included EasyTaq® DNA polymerase, dNTPs and buffer. The cycling conditions were as follows: 2 min of polymerase activation at 95°C followed by 40 cycles at 95°C for 15 sec, 55°C for 60 sec and 72°C for 20 sec. PCR was performed using the following primers: HDAC3 forward, 5′-TTGCGATTCTGTTTTGTGCT-3′ and reverse, 5′-GTGGGGTCTCAGTGGG-3′; miRNA-130a forward, 5′-TTGCGATTCTGTTTTGTGCT-3′ and reverse, 5′-GTGGGGTCTCAGTGGG-3′; β-actin forward, 5′-ACAGGGGAGGTGATAGCATT-3′ and reverse, 5′-GACCAAAAGCCTTCATACATCTC-3′. All primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). β-actin as an internal control was used to normalize the data to determine the relative expression of the target genes. RT-qPCR was carried out on a LightCycler 480 (Roche Diagnostics GmbH, Penzberg, Germany). After completion of the reaction, the amplification curve and melting curve were analyzed. Gene expression values were determined using the 2^ΔΔCt method.

Western blot analysis. The PBMCs were homogenized and extracted with NP-40 buffer (Beyotime Institute of Biotechnology, Haimen, China), followed by 5-10 min boiling and centrifugation (10,500 g x 15 min, 4°C) to obtain the supernatant. Samples containing 50 μg protein were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride Transfer Membrane (Millipore, Billerica, MA, USA). After saturation with 5% (w/v) non-fat dry milk in Tris-buffered saline and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the rabbit anti-human polyclonal HDAC3 (cat. no. ab16047) and mouse anti-human monoclonal TNF-1 (cat. no. ab10204) antibodies (Abcam, Cambridge, MA, USA) at dilutions of 1:500-1:2,000 at 4°C overnight. After three washes with TBST, membranes were
incubated with secondary immunoglobulins (Igs) conjugated to horseradish peroxidase, including goat anti-mouse IgG (cat. no. ab6789) and goat anti-rabbit IgG (cat. no. ab6721) (Abcam) at a dilution of 1:10,000 and 1:20,000. After 1 h of incubation at 37°C, membranes were washed three times with TBST. Blots were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences). Signals were densitometrically assessed (Odyssey Application Software version 3.0; LI-COR Biosciences) and normalized to the β-actin signals to correct for unequal loading using the mouse monoclonal anti-β-actin antibody (1:10,000 dilution; cat. no. ab6276; Abcam).

Chromatin immunoprecipitation (ChIP) assay. PBMCs were cross-linked with 0.5% formaldehyde (Beyotime Institute of Biotechnology) for 10 min at room temperature. Cross-linking was stopped by adding 125 mM glycine (Beyotime Institute of Biotechnology) at a dilution of 1:10,000 and 1:20,000. After 1 h of incubation at 37°C, membranes were washed three times with TBST. Blots were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences). Signals were densitometrically assessed (Odyssey Application Software version 3.0; LI-COR Biosciences) and normalized to the β-actin signals to correct for unequal loading using the mouse monoclonal anti-β-actin antibody (1:10,000 dilution; cat. no. ab6276; Abcam).

Statistical analysis. Values are expressed as the mean ± standard error of the mean for each group. All statistical analyses were performed by using GraphPad Prism version 4.0 (GraphPad Inc., La Jolla, CA, USA). One-way analysis of variance or Student's t-tests were applied, as well as regression analysis to inhibitor cocktail (all Beyotime Institute of Biotechnology) for 10 min at 4°C. Pellets obtained by centrifugation at 10,006 x g for 5 min were suspended in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) and sonicated using a BioruptorSonicator (Diagenode, Seraing, Belgium) to shear chromatin into 500-bp fragments. The immunoprecipitated protein-DNA complexes were collected using protein A agarose beads (Upstate Biotechnology, Inc., Lake Placid, NY, USA). Proteinase K (1 mg/ml, Sigma-Aldrich) digestion and DNA purification (Wizard Plus Minipreps DNA Purification system; Promega) were performed after blocking with BSA and salmon sperm DNA. The resultant product was then subjected to PCR amplification.

incubated with secondary immunoglobulins (Igs) conjugated to horseradish peroxidase, including goat anti-mouse IgG (cat. no. ab6789) and goat anti-rabbit IgG (cat. no. ab6721) (Abcam) at a dilution of 1:10,000 and 1:20,000. After 1 h of incubation at 37°C, membranes were washed three times with TBST. Blots were visualized using the Odyssey Infrared Imaging System (LI-COR Biosciences). Signals were densitometrically assessed (Odyssey Application Software version 3.0; LI-COR Biosciences) and normalized to the β-actin signals to correct for unequal loading using the mouse monoclonal anti-β-actin antibody (1:10,000 dilution; cat. no. ab6276; Abcam).

Chromatin immunoprecipitation (ChIP) assay. PBMCs were cross-linked with 0.5% formaldehyde (Beyotime Institute of Biotechnology) for 10 min at room temperature. Cross-linking was stopped by adding 125 mM glycine (Beyotime Institute of Biotechnology). Cells were solubilized in a buffer containing 10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 1% sodium deoxycholate, 1 mM phenylmethanesulfonylfluoride and protease
analyze data. Differences with a P-value of <0.05 were considered statistically significant.

**Results**

**HDAC3 expression is increased and miRNA-130a expression is decreased in PBMCs from AS patients.** A recent study has shown that in PBMC nuclear extracts from AS patients, the levels of HAT were decreased, while HDAC tended to be increased compared with that in healthy control subjects (8). Consistent with this previous study, the results of the present study showed a >3-fold increase in HDAC3 mRNA and protein expression in PBMCs from patients with AS compared with that in healthy control subjects (Fig. 1A-C). The present study then investigated the possible link between miRNA expression and AS. A hierarchical cluster analysis of differentially expressed miRNAs in the PBMCs from AS patients was performed. The results showed that the down-regulation of miRNA-130a was associated with AS, the expression of which was lowest among all miRNAs. As shown in Fig. 1D, miRNA-130a expression was markedly decreased in PBMCs from AS patients compared with that in PBMCs from normal controls.

**miRNA-130a is regulated by HDAC3.** The deregulation of miRNAs has been frequently identified in immunopathogenesis and has been linked to AS (16,21). The expression of miRNA-15a/miRNA-16-1 is repressed through recruitment of HDAC3 in mantle cells and other non-Hodgkin B-cell lymphomas (21). Based on these data, the present study investigated whether expression of miRNA-130a in PBMCs from AS patients was affected by HDAC3 knockdown. Increased miRNA-130a expression was observed in the HDAC3 siRNA group and in the HDAC3 inhibitor [trichostatin A (TSA)] group (Fig. 2A). Furthermore, a ChIP assay was performed using HDAC3 antibody to detect binding at the miRNA-130a promoter. This approach revealed that HDAC3 was able to bind to miRNA-130a promoters in PBMCs and the percentage input was significantly increased for AS patients compared with that for normal controls (Fig. 2B). These findings suggested that HDAC3-mediated histone hypoacetylation contributed to the regulation of the expression of miRNA-130a.

**TNF-1α is regulated by HDAC3 via miRNA-130a.** miR-130a overexpression led to a decrease, whereas miR-130a inhibition caused an increase in the mRNA and protein expression of TNF-1α in PBMCs (Fig. 3A and B). Furthermore, HDAC3 knockdown or HDAC3 inhibition caused an increased in the expression of miR-130a and a decrease of the mRNA and protein expression of TNF-1α in PBMCs (Fig. 3C and D). These observations suggested that HDAC3 has an important
role in the underlying mechanism of AS by regulating miR-130a expression via its target TNF-1α.

Discussion

Among the numerous HDACs, HDAC3 is widely expressed and conserved in a wide range of species (22). HDAC3 regulates the c-Jun N-terminal kinase pathway (23), NF-κB activity (24), mitogen-activated protein kinase activation (25) and nuclear translocation of regulator of calcineurin 1 (26). The present study reported several important observations regarding the involvement of HDAC3 in the molecular mechanisms of AS. First, increased mRNA and protein levels of HDAC3 and decreased levels of miRNA-130a were observed in PBMCs from AS patients. Furthermore, HDAC3 knockdown or HDAC3 inhibition promoted the expression of miRNA-130a and HDAC3 could be recruited to the promoter region of the gene encoding for miRNA-130a in AS patients. From these results, it can be deduced that HDAC3 is involved in the regulation of a distinct underlying molecular mechanism of AS by forming a negative feedback loop with miR-130a. In addition, miR-130a overexpression led to a decrease, whereas miR-130a inhibition caused an increase in TNF-1α expression in PBMCs. Furthermore, HDAC3 knockdown or HDAC3 inhibition caused an increase in the expression of miR-130a and a decrease in the expression of TNF-1α in PBMCs. All of these observations suggested that HDAC3 has an important role in the underlying molecular mechanism of AS by regulating miR-130a expression via its target TNF-1α.

A recent study demonstrated that miRNA-130a is required for normal immune function. miRNA-130a was demonstrated to markedly inhibit hepatitis virus C (HCV) replication in the HCV replicon as well as in J6/IFN-α cells. Overexpression of miRNA-130a led to increases in the expression of type I IFN (IFN-α/β), interferon-induced 17 kDa protein, ubiquitin-specific peptidase 18 and MX dynamin-like guanosine triphosphatase A, which are involved in the innate immune response; furthermore, miR-130A overexpression led to decreases in the expression of miR-122, a well-defined miRNA promoting HCV production (18). miRNAs are potentially differentially expressed in the T cells of AS patients, which may alter the expression of the downstream target molecules that may contribute to the pathogenesis of AS (16). In the present study, miRNA-130a inhibition increased the expression of TNF-1α, while miRNA-130a knockdown decreased the expression of TNF-1α. These results indicated that TNF-1α may be a candidate target of miRNA-130a, as it appeared to have an important regulatory role in the underlying mechanisms of AS.

AS therapy has been revolutionized by the increasing knowledge of the pathogenetic mechanisms of the disease, involving dysfunction and excessive secretion of multiple pro-inflammatory molecules, in particular TNF-1α (16,27). Among five biological agents currently used in AS therapies, anti-TNF-1α monoclonal antibody adalimumab, TNF receptor fusion protein etanercept and anti-TNF-1α monoclonal antibody infliximab have been largely demonstrated to be effective at reducing AS activity and controlling joint damage and various aspects of the diseases as well as to be reasonably safe (27-29). Clinical studies have indicated that AS patients respond to the above treatments. Upon TNF-1α inhibition, HAT activity increases considerably in patients with AS (by 198%), leading to a marked increase of the HAT/HDAC ratio in AS patients during anti-TNF-1α therapy. The HDAC inhibitor TSA induces a decline in HDAC (by 43.6%) (8). In the present study, the HDAC3-inhibitory function of TSA appeared to directly regulate the expression of miRNA-130a as well as TNF-1α. Therefore, the present study identified the HDAC3/miRNA-130a/TNF-1α axis as a novel regulator of the pathogenetic mechanisms of AS.

In conclusion, the present study identified that HDAC3 had an important role in the underlying molecular mechanism of AS by regulating miR-130a expression via its target TNF-1α. These results suggested that HDAC3, as an inflammatory mediator which was shown to be elevated in PBMCs of patients with AS, may, at least in part, contribute to the pathogenetic mechanism of AS. Furthermore, the present study indicated that HDAC3 may represent a therapeutic target for the treatment of AS.

References

1. Kim N, Sun HY, Youn MY and Yoo JY: IL-1β-specific recruitment of GCN5 histone acetyltransferase induces the release of PAF1 from chromatin for the de-repression of inflammatory response genes. Nucleic Acids Res 41: 4495-4506, 2013.
2. Grabiec AM, Tak PP and Reedquist KA: Targeting histone deacetylase activity in rheumatoid arthritis and asthma as prototypes of inflammatory disease. Should we keep our HATs on? Arthritis Res Ther 10: 226, 2008.
3. Grausenburger R, Bilic I, Boucheron N, Zupkovitz G, El-Housseiny R, Tschischarov R, Zhang Y, Rembold M, Gaissberger M, Hartl A, et al: Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection. Blood 117: 1205-1217, 2011.
4. Katto J, Engel N, Abbas W, Herbein G and Mahlknucht U: Transcription factor NFκB regulates the expression of the histone deacetylase SIRT1. Clin Epigenetics 5: 11, 2013.
5. Blanchard F and Chipoy C: Histone deacetylase inhibitors: New drugs for the treatment of inflammatory diseases? Drug Discov Today 10: 197-204, 2005.
6. Choi JH, Oh SW, Kang MS, Kwon HJ, Oh GT and Kim DY: Trichostatin A attenuates airway inflammation in mouse asthma model. Clin Exp Allergy 35: 89-96, 2005.
7. Toussirot E, Wendling D, Herbein G and CIC-1431: Biological treatments given in patients with rheumatoid arthritis or ankylosing spondylitis modify HAT/HDAC (histone acetyltransferase/histone deacetylase) balance. Joint Bone Spine 81: 544-545, 2014.
8. Gillespie J, Savic S, Wong C, Hemphall A, Inman M, Emery P, Grigg R and McDermott MF: Histone deacetylases are dysregulated in rheumatoid arthritis and a novel histone deacetylase 3-selective inhibitor reduces interleukin-6 production by peripheral blood mononuclear cells from rheumatoid arthritis patients. Arthritis Rheum 64: 418-422, 2012.
9. Strietholt S, Maurer B, Peters MA, Pap T and Gay S: Epigenetic modifications in rheumatoid arthritis. Arthritis Res Ther 10: 219, 2008.
10. Yang CR, Shih KS, Liou JP, WuYW, Hsieh IN, Lee HY, Lin TC and Wang JH: Denbinobin upregulates miR-146a expression and attenuates IL-1β-induced upregulation of ICAM-1 and VCAM-1 expressions in osteoarthritis fibroblast-like synovocytes. J Mol Med (Berl) 2014 [Epub ahead of print].
11. Lu MC, YuCL, Chen HC, Yu HC, Huang HB and Lai NS: Increased mRNA expression of pro-inflammatory cytokines from cells with rheumatoid arthritis leads to decreased insulin-like growth factor-I-mediated interleukin-10 production. Clin Exp Immunol 177: 641-651, 2014.
13. Fulci V, Scappucci G, Sebastiani GD, Giannitti C, Franceschini D, Meloni F, Colombo T, Citarella F, Barnaba V, Minisola G, et al: miR-223 is overexpressed in T-lymphocytes of patients affected by rheumatoid arthritis. Hum Immunol 71: 206-211, 2010.

14. Pauley KM, Satoh M, Chan AL, Bubb MR, Reeves WH and Chan EK: Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. Arthritis Res Ther 10: R101, 2008.

15. Zhu S, Pan W, Song X, Liu Y, Shao X, Tang Y, Liang D, He D, Wang H, Liu W, et al: The microRNA miR-23b suppresses IL-17-associated autoimmune inflammation by targeting TAB2, TAB3 and IKK-α. Nat Med 18: 1077-1086, 2012.

16. Lai NS, Yu HC, Chen HC, Yu CL, Huang HB and Lu MC: Aberrant expression of microRNAs in T cells from patients with ankylosing spondylitis contributes to the immunopathogenesis. Clin Exp Immunol 173: 47-57, 2013.

17. Osbourne A, Calway T, Bromam M, McSharry S, Earley J and Kim GH: Downregulation of connexin43 by microRNA-130a results in cardiac arrhythmias. J Mol Cell Cardiol 74: 53-63, 2014.

18. Fabbroni M, Cantarini L, Caso F, Costa L, Pagano VA, Frediani B, Manganelli S and Galeazzi M: Drug retention rates and treatment discontinuation among anti-TNF-α agents in psoriatic arthritis and ankylosing spondylitis in clinical practice. 2014: 862969, 2014.

19. Fenix-Caballero S, Alegre-del Rey EJ, Castillo-Lara R, Puigventós-Latorre F, Borroto-Rubio JM and López-Velarde JF: Direct and indirect comparison of the efficacy and safety of adalimumab, etanercept, infliximab and golimumab in psoriatic arthritis. J Clin Pharm Ther 38: 286-293, 2013.

20. Costa L, Caso P, Atteno M, Del Puente A, Darda MA, Caso P, Ortolan A, Fiocco U, Ramonda R, Punzi L and Scarp A: Impact of 24-month treatment with etanercept, adalimumab, or methotrexate on metabolic syndrome components in a cohort of 210 psoriatic arthritis patients. Clin Rheumatol 33: 833-839, 2014.