Expression of AIM2 in rheumatoid arthritis and its role on fibroblast-like synoviocyte

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Abstract

Background: To determine whether any differences of AIM2 inflammasome expression levels between rheumatoid arthritis (RA) and osteoarthritis (OA) and investigate the effects of AIM2 when transferred into RA fibroblast-like synoviocytes (RA-FLS).

Methods: Serum AIM2 levels between OA and RA patients were compared by ELISA. Different expression levels of AIM2, ASC, Caspase-1 and IL-1β between RA and OA synovium were semi-quantified by RT-qPCR and immunohistochemical (IHC) staining. IHC staining were recorded by H scores, and determine the correlation with ESR and CRP levels of RA patients. SiRNA AIM2 was transferred to RA-FLS and observe its effects on proliferation and migration by MTT assay and transwell test respectively.

Results: In RA sera, no significant difference was observed between OA and RA patients. However, in affected knee synovium, AIM2, ASC, Caspase-1 and IL-1β were expressed higher in RA than that of OA. Plus, H score of AIM2, ASC, and IL-1β were positively correlated to ESR and CRP levels in RA patients. After transferred

AIM2 siRNA to FLS and incubation for 48 hours, the proliferation of FLS were significantly inhibited, and the apoptosis rate were significantly increased compared to FLS in control group. However, no effect on migration was detected.

Conclusions: AIM2 participated in the proliferation of FLS, and might be a potential target for therapy.

Key words: absent in melanoma 2; fibroblast-like synoviocyte; metformin; siRNA; proliferation; therapeutic target

Introduction

Rheumatoid arthritis (RA) refers to an autoimmune disease characterized by joint synovial inflammation[1]. Its pathology consists of inflammatory cell infiltration, synovial lining cell proliferation, microvascular regeneration, as well as destruction of cartilage and bone tissue[2]. The researches on genetic architecture of RA has been well characterised through conventional and genome-wide approaches, and more than 100 loci are associated with disease risk and progression[3], among which, absent in melanoma 2 (Aim2) was one of the gene detected[4].

Aim2 involved in innate immune response by recognizing cytosolic double-stranded DNA and inducing caspase-1-activating inflammasome formation in macrophages[5]. Upon binding to DNA, Aim2 is thought to undergo oligomerization and to associate with PYCARD (PYD and CARD domain containing/ASC, apoptosis-associated speck-like protein containing a CARD) initiating the recruitment of caspase-1 precusrsor and processing of interleukin (IL)-1β and IL-18[6-7]. Inappropriate recognition of

cytoplasmic self-DNA by AIM2 contributes to the development of psoriasis, dermatitis, arthritis, and other autoimmune or inflammatory diseases[8]. However, limited researches of Aim2 inflammasome on arthritis were reported, and no investigation of clinical patients had been reported, and the Aim2 inflammasome in pathogenesis of RA is not well demonstrated.

we proposed to investigate the differential expression partial of AIM2 pathway associated proteins including AIM2, ASC, Caspase-1 and IL-1β from mRNA to protein aspects between OA and RA synoviums. RA-fibroblast-like synoviocytes (RA-FLS) is critical to pathogenesis of RA as develop a unique aggressive phenotype that increases invasiveness into the extracellular matrix and further exacerbates joint damage[9]. So in this study, we also try to observe the SiRNA Aim2 on activation of RA-FLS.

Materials and methods

Patients' serum for enzyme linked immunosorbent assay (ELISA)

We enrolled 49 RA and 25 OA patients with comprehensive medical records from the Rheumatology department, Integrated Traditional Chinese and Western Medicine hospital, Southern Medical University, China between Oct. 2017 to Dec. 2018. Exclusion criteria included other autoimmune diseases, acute inflammation, fever, thyroid disease, diabetes, liver and kidney diseases. Patients' sera were collected for ELISA assays of AIM2 (R&D Systems, USA). The ELx808TM absorbance microplate reader was used to measure the absorbance values at 450 nm. Concentration of proteins in the samples were calculated using a standard curve for each protein. General information of patients recruited are given in table 1.

Table 1. General information of patients recruited for ELISA in this study

General information	RA(n=49)	OA(n=25)
Gender (male/female)	11/38	7/18
Age(y/o)	50.87±9.35	58.48±9.69**
disease course (years)	7.27±5.11	6.41±8.88
ESR (mm/h)	56.12±40.36	34.7±24.88*
CRP (mg/L)	12.16±20.36	2.21±2.76*

RA, rheumatoid arthritis; OA, osteoarthritis; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein. *, $p < 0.05$; **, $p < 0.01$.

Patients' synovium for immunohistochemical (IHC) staining

Arthroscopic surgery was applied to 41 RA and 26 OA patients for therapeutics purpose. Patients' general information as table 2.

Patients' synovium for immunohistochemical (IHC) staining

Arthroscopic surgery was applied to 41 RA and 26 OA patients for therapeutics purpose (patients' general information in table 2). Regular streptavidin biotin-based immunoperoxidase staining for Aim2, Aim2, ASC, caspase-1, and IL-1β was performed to formalin fixed, paraffin embedded pathology keen synovium specimens. H score was applied to quantify the staining intensity[10]

Table 2. General information of patients recruited for IHC in this study

General information	RA (n=41)	OA (n=26)
Gender	6/35	6/20

(male/female)		
Age (y/o)	51.6 ± 2.408	60.62 ± 2.296
Disease course (years)	6.538 ± 0.8864	6.417 ± 1.428
ESR (mm/h)	79.68 ± 6.896	38.38 ± 6.636***
CRP (mg/L)	30.26 ± 5.437	7.015 ± 4.216**

RA, rheumatoid arthritis; OA, osteoarthritis; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein. **, $p < 0.01$; ***, $p < 0.001$.

Patients' synovium for quantitative real-time polymerase chain reaction (qPCR)

Synovium specimens from 10 RA patients and 9 OA patients obtained through knee arthroscopy (patients' general information in table 3). Specimens were soaked in TRIzol® Reagent (Thermo Scientific, USA) after remove adipose tissue under sterilized environment, then stored under -20℃ refrigerator for qPCR on mRNA of Aim2, ASC, caspase-1, and IL-1β. qPCR was also applied to evaluate relative expression of mRNA AIM2, ASC, caspase-1, and IL-1β in FLS after transferred with AIM2 siRNA.

Table 3. General information of patients recruited for RT-qPCR in this study

General information	RA (n=10)	OA (n=9)
Male: female	2:8	5:4
Age (years)	54.2 ± 8.65	66.6 ± 8.61**
ESR (mm/h)	86.5 ± 50.5	37.8 ± 29.3**

CRP (mg/L)	19.1 ± 13.63	5.13 ± 7.19*

RA, rheumatoid arthritis; OA, osteoarthritis; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein. *, $p < 0.05$; **, $p < 0.01$.

The primers for amplifying the target mRNAs are listed in table 4.

Table 4. qPCR primers used in this study

Primer name	Sense primer/sequence	Antisense primer
AIM2	AGCAAGATATTATCGGCACAGTG	GTTCAGCGGGACATTAACCTT
ASC	CCTACTGTTCTTTCTGTGGGAAG	CGAGGTCGTCAGCCATCAC
CASPASE-1	TTTCCGCAAGGTTCGATTTTCA	GGCATCTGCGCTCTACCATC
IL-1β	TTCGACACATGGGATAACGAGG	TTTTTGCTGTGAGTCCCGGAG
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

Immunofluorescent staining for AIM2

For immunofluorescent staining, both sections of RA and OA patients were blocked with normal goat serum in 0.01 M phosphate-buffered saline for 1 hour. The primary rabbit anti-rat AIM2 antibody (1:200) was incubated overnight at 4°C together with either mouse anti-human vimentin (1:400); The following day, the sections were incubated for 60 minutes at 37°C with FITC-conjugated goat and anti-mouse (1:1000) and anti-rabbit

(1:1000). The nuclei of cells were stained with DAPI. The results were examined under a fluorescence microscopy.

AIM2 siRNA preparation

AIM2 siRNA were produced by Ribobio Company, China. The following siRNA sequences were used: AIM2 siRNA 1 (5′-GAGCTCTTCACCACTTTCA-3′), AIM2 siRNA 2 (5′-GGAGCGGGTGTATTTACAT-3′), AIM2 siRNA 3 (5′-CGTCGAGTCTTTGTCAGAA-3′).

Isolation and culture of fibroblast-like synoviocytes (FLS)

FLS were derived from synovial tissue specimens harvested from patients by needle arthroscopy. FLS were isolated by enzyme digestion and subsequently cultured in Dulbecco's modified Essential medium (DMEM) containing 10% fetal bovine serum (FBS, Invitrogen) containing antibiotics (penicillin and streptomycin) at 37°C with 5% CO2. Cells cultured between passages 4 and 9 were used for this study. Cells were frozen with cell freezing medium and stored in -80℃ freezer until used.

Methyl thiazolyl terazolium (MTT) assay

MTT (methyl thiazolyl terazolium) assay was used to ascertain the effects of AIM2 siRNA on FLS viability at different concentrations. FLS samples were digested using 0.25% pancreatin were transferred to two 96-well plates with 3-5×10^3 cells/well. After 24 h of culture, the FLS were transferred with AIM2 siRNA (20 nM). Six replicates were used at each time point. At different time intervals (0, 24, 48 and 72 h), MTT solution (5 mg/ml, Sigma-Aldrich, St. Louis, USA) was added, followed by 4 h incubation period. Then the culture medium was aspirated and 150 µl of dimethyl sulfoxide

(DMSO) was added to each well to dissolve the formazan crystals. Optical density (OD) was measured at 490 nm using Universal Microplate Reader (Bio-Tek instruments, Winooski, USA). Data curves were plotted with OD values on the y-axis and time intervals on the x-axis. Each experiment was done in triplicates.

Flow cytometry for apoptosis

Flow cytometry was performed to evaluate the effects of SiRNA Aim2 on FLS apoptosis. 48 hours after FLS treated by SiRNA aim2 and NC in each groups in 6-well plates, the cells were collected (around 3×105/well), washed two times with PBS, and resuspended in 500μl 1×binding buffer, mixed with 5μl of Annexin-V-fluorescein isothiocyanate (FITC) and 5μl of propidium iodide (PI), and eventually detected by flow cytometer (BD LSRFortessaTM, USA). The scatter diagram was distributed as follows: Q3: healthy cells (FITC-/PI-); Q2: apoptotic cells at an advanced stage (FITC+/PI+); Q4: apoptotic cells at an early stage (FITC+/PI-). The apoptosis rate = ratio of apoptotic cells to the total cells in Q4 + ratio of apoptotic cells to the total cells in Q2. Each experiment was conducted three times.

Transwell test

For the matrigel invasion assay, cells at the logarithmic growth phase were digested, collected, re-suspended and diluted into a concentration of 3×104/mL in serum-free medium. The cell suspension (200 μl) was added to the upper chamber coated with Matrigel (BD Bioscience) that were diluted with DMEM media (1:3), while 500 μl DMEM media containing 10% FBS was added in the lower chamber. After incubation for 12 h at 37 °C, the cells in the upper membrane were discarded and cells on the lower membrane

were fixed using 4% Paraformaldehyde for 25 min and stained with 0.1% crystal violet (Beyotime, USA) for 10 min. Next, five random fields were counted.

Automated electrophoresis western blot analysis

FLS were seeded at 1-2×10^5 cells per well in 6-well plates and incubated for adherence. Medium was replaced in the wells with fresh medium containing metformin (5 mM) or saline for 48h. After aspiration of the medium, cell monolayers were rinsed with 1 ml ice-cold PBS and lysed in 80 µl of lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1mM EDTA,1mM EGTA, 1%(v/v) TritonX100, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate) supplemented with fresh 1 mM Na_3VO_4, 1mM dithiothreitol containing 1 X protease inhibitor cocktail (Roche Molecular Biochemicals, Basel, Switzerland). Lysates were pre-cleared by centrifugation at 18000 g for 15 min at 4℃. Supernatants were collected, protein concentrations were measured using Bradford assay (Thermofisher, Massachusetts, USA) and lysates were adjusted to 5 mg/ml protein concentration. Capillary electrophoresis western blot analysis was carried out using manufacturer's reagents provide in the the user manual (ProteinSimple WES, San Francisco, USA). Briefly, 5.6 µl of the cell lysate was mixed with 1.4 µl of fluorescent master mix and heated at 95℃ for 5 min. The samples, blocking reagent, wash buffer, antibody of tublin, AIM2, and TNF-α (1:100 R&D Systems), secondary antibody and chemiluminescent substrate were dispensed into the microplates. The electrophoretic separation and immunodetection was performed automatically using default settings. The data was analyzed using in-built Compass software for SW 4.0. The truncated and full-length AIM2 and TNF-α

intensities (area under the curve) were normalized to that of the tubulin peak (control). In most of the figures, electropherograms are represented as pseudo-blots, generated using Compass software.

Statistical analysis

Statistical analysis was conducted with GraphPad Prism 7.0 software. All the data were denoted as mean ± SD. Differences between two groups were evaluated for statistical significance using Student's t-test. Kruskal-Wallis and Dunn's multiple comparison post-hoc test was used to evaluate the differences among three or more groups. Correlations were evaluated using Spearman's Rank correlation test. $P \leq 0.05$ was considered as statistically significant.

Results

Aim2 expressed higher in RA synovium than that of OA

Through ELISA, no difference of Aim2 expression was observed between serum of OA and RA (figure 1A). However, relative expression levels of mRNA Aim2 and Aim2 pathway related proteins including ASC, and IL-1β were higher in local synovium of RA than that of OA, relative expression of caspase-1 was higher in RA than OA without a statistical significance (figure 1B), and H score in synovium of RA, Aim2 and Aim2 pathway related proteins including ASC, caspase-1 and IL-1β, were higher expressed than of OA (figure 1C). Aim2, ASC, caspase-1, and IL-1β were observed positively correlated to clinical RA disease activity indicated by ESR and CRP (figure 2).

By IHC stianing, AIM2 and Aim2 pathway related proteins was detected in nucleus and cytoplasm, seen in most type of cells including macrophages, lymphocyte and also fibroblast-like synoviocyte (FLS). As FLS produce cytokines that perpetuate inflammation and proteases that contribute to cartilage destruction, play a critical effector role in RA pathogenesis[11], we focused our study on Aim2 pathway in FLS. It was confirmed that Aim2 could be expressed in cytoplasm of FLS (figure 3A), and Aim2 and its mRNA relatively higher in RA-FLS than that of OA (figure 3B, C).

AIM2 siRNA inhibited the proliferation while not migration of RA-FLS

As we designed 3 SiRNAs for Aim2, the examining on efficacy showed all of which successfully silenced the mRNA Aim2 expression and also downstream molecules including ASC, caspase-1, and IL-1β (figure 4A). Western blot confirmed the successfully silencing on Aim2 expression by Si-1, and also inhibited the expression of TNF-α (figure 4B).

Through MTT assay, AIM2 siRNA inhibits RA-FLS in a dose dependant manner (figure 4C). the flow cytometry for apoptosis indicated AIM2 siRNA promoted the apoptosis compare to that of NC (figure 4D). However, there was no difference in migrated cells in the two groups (figure 4E).

Discussion

As one of the most common seen autoimmune diseases, both innate and adaptive immune reaction participated the pathogenesis of

RA[12]. AIM2 as an important inflammasome component that senses potentially dangerous cytoplasmic DNA, leading to activation of the ASC pyroptosome, caspase-1and processing of IL-1β and IL-18[12]. However, the role of AIM2 pathway in the pathogenesis of RA had rarely been investigated. So we try to compare the AIM2 pathway associated proteins including AIM2, ASC, Caspase-1 and IL-1β between synovium of RA and OA in this study. And it turned out that these inflammatory related factors were up-regulated in RA synovium than that of OA. However, recently Mendez-Frausto et al[14]. observed AIM2 was diminished at a systemic level in patients with RA, and monocytes of RA patients are prone to releasing IL-1β in the absence of AIM2 inflammasome signals, which were not quite agree with our results.

AIM2 pathway plays a putative role in tumorigenic reversion and may control cell proliferation[15]. AIM2 expression suppressed the proliferation and tumorigenicity of human breast cancer cells, and that AIM2 gene therapy inhibited mammary tumor growth in an orthotopic tumor model. AIM2 significantly increased sub-G1 phase cell population, indicating that AIM2 could induce tumor cell apoptosis. Moreover, AIM2 expression greatly suppressed nuclear factor-kappa B transcriptional activity and desensitized tumor necrosis factor-alpha-mediated nuclear factor-kappa B activation[16]. Insighted by the role of AIM2 in cancer research, we focused our study on FLS, which mimic cancer cells with bio-behaviors of proliferation and invasion, and currently regarded as a crucial effector in pathogenesis of RA[17]. And it turned out that when AIM2 was silenced in FLS by SiRNA method, the TNF-α, and downstream molecules including ASC, Caspase-1 and IL-1β were all downregulated, which leads to the proliferation of FLS was

inhibited, and apoptosis was increased. However, the migration of FLS showed by tanswell assay was not affected.

In summary, based on the knowledge of AIM2 pathway, it might participated in the pathogenesis of both OA and RA. Our clinical investigation based on synovium obtained by arthroscopic surgery primarily indicated an more aggressive expression of AIM2 pathway associated proteins in RA synovium than that of OA. Furthermore, SiRNA AIM2 inhibited the FLS proliferation and promoted its apoptosis in vitro study. However, whether AIM2 be reliable target for treatment needs further research.

List of abbreviation
RA :rheumatoid arthritis
OA: osteoarthritis
FLS: fibroblast-like synoviocytes
Aim2: absent in melanoma 2
ASC: Apoptosis associated speck like protein containing a CARD
CRP:C reactive protein
ESR: erythrocyte sedimentation rate
PYCARD: Apoptosis-associated speck-like protein containing a CARD
TNF-α: tumor necrosis factor a

Ethics approval This study was approved by the Ethics Committee of Integrated Traditional Chinese and Western Medicine hospital, Southern Medical University (aproval No. NFZXYEC-2017-002).

Patient consent for publication Not Applicable.

Data availability statement Data are available upon request.

Competing interests None declared.

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Author's Contributions YC and CHX designed the study. FJQ, YC performed most of the experiments and collected the data and composed the manuscript, contributed equally to this work. XFZ obtained ethics approval documents for the study. All the authors have read and approved the manuscript for publication.

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Figure 1

The differences of Aim2 pathway related molecules between OA and RA. Aim2 expression was observed without difference between serum of OA (n=25) and RA (n=49) by ELISA (A). However, mRNA Aim2 and Aim2 pathway related proteins including ASC,and IL-1β was expressed higher in RA than that of OA, caspase-1 was higher in RA than OA without a statistical significance (B). H score in synovium of RA, Aim2 and Aim2 pathway related proteins including ASC, caspase-1 and IL-1β, were higher expressed than of OA (C). *p<0.05, **p<0.01, ****p <0.0001; OA, osteoarthritis; RA, rheumatoid arthritis; Aim2, absent in melanoma 2; ASC, Apoptosis-Associated Speck-Like Protein Containing A CARD; IL, interleukin.

Figure 2

AIM2, ASC and IL-1β were positively correlated with ESR and CRP. AIM2 showed positive correlation with ESR (r = 0.74, p = 0.001, 95% CI: 0.38-0.9) and CRP (r = 0.65, P = 0.003, 95% CI: 0.25-0.86) (A, E). The positive correlation between ASC and ESR (r = 0.5, p = 0.005, 95% CI: 0.16-0.74) and CRP (r = 0.42, p = 0.02, 95% CI: 0.05-0.69) were detected (B, F). The positive correlation between IL-1β and ESR (r = 0.62, p = 0.0004, 95% CI: 0.31-0.81) and CRP (r = 0.41, p = 0.02, 95% CI: 0.05-0.67) was detected. The correlation between CASPSE-1 and ESR and CRP was not statistically significant (p > 0.05) (C, D).

Figure 3

Aim2 expressed higher in RA-FLS than that of OA. Aim2 expressed in cytoplasm of FLS, and higher in RA-FLS than OA-FLS (A, vimentin was labelled as green, Aim2 as red and nuclei as blue by Immunofluorescent staining). the relative expression of Aim2 in mRNA and protein levels was higher in RA-FLS than that of OA (B, C). *p<0.05, ****p <0.0001; OA, osteoarthritis; RA, rheumatoid arthritis; Aim2, absent in melanoma 2.

Figure 4

SiRNA of Aim2 inhibited the proliferation while not migration of RA-FLS. Si-1 successfully silenced the mRNA Aim2 expression and also downstream molecules including ASC, caspase-1, and IL-1β (A). Western blot confirmed the successfully silencing on Aim2 expression by Si-1, and also inhibited the expression of TNF-α (B). Through MTT assay, Si-1 inhibits RA-FLS in a dose dependant manner (C). The flow cytometry for apoptosis indicated SiRNA Aim2 promoted the apoptosis compare to that of NC (D). However, there was no difference in migrated cells in the NC and Si-1 groups (E).