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Original Article

RNA expression profiling from the liquid fraction of synovial fluid in knee joint osteoarthritis patients

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Abstract: Objective: To investigate the RNA profile of synovial fluid (SF) from osteoarthritis (OA) patients and carry out cluster analysis of OA-related genes. Methods: RNA of SF from OA patients was isolated using RNA-specific Trizol. A cDNA library was built and subjected to the second-generation sequencing using HisSeq4000 with a data size of 8G. The sequencing reads were aligned to the UCSC human reference genome (hg38) using Tophat with default parameters. Gene function enrichment was generated using DAVID. Results: The minimum weight 0.096 μg RNA of SF sample was used for sequencing analysis, which produced 66,154,562 clean reads, 91.28% of which were matched to the reference with 2,682 genes identified. Some of the unmatchable reads matched RNAs of bacteria, mainly *Pseudomonas*. The detected human RNAs in samples fell into different categories of genes, including protein-coding ones, processed and unprocessed pseudogenes, and long noncoding, antisense and miscellaneous RNAs that mediate various biological functions. Interestingly, 80% of the expressed genes belonged to the mitochondrial genome. Conclusion: These results suggest that less than 0.1 μg RNA is sufficient for establishing a cDNA library and deep sequencing, and that the liquid fraction of SF contains a whole RNA repertoire that may reflect a history of previous microorganism infections.

Keywords: Osteoarthritis, knee joint, synovial fluid, transcriptome, second-generation sequencing

Introduction

Osteoarthritis (OA) is the most common arthritis and the leading cause of disability in adults. In worldwide, about 250 million people are affected, which causes a relatively large social and economic burden [1]. Partly due to increased aging and obese populations, the incidence of OA is increasing worldwide, with 83% occurring in the knee joints (KOA) [2]. The incidences of KOA and associated disability are probably higher in China than those of many developed countries, because China has its largest population in rural areas where heavy carrying is a routine part of their labor.

While OA is quite common, its definition is actually not strict but, instead, utilizes a combination of symptoms and radiographic criteria [3]. That often makes it difficult to differentiate OA from other joint diseases such as rheumatoid arthritis and undifferentiated arthritis [4]. The definition of OA is opaque in part because the etiology of OA is still impenetrable. Germane to the lack of lucid criteria for diagnosis of the different types of arthritis, the treatment and management of these diseases sometimes also becomes unspecific and ineffective, making it difficult to evaluate the treatment outcome.

There have been many different attempts to delve into the etiology and mechanisms of different joint diseases in order to differentiate one joint disease from another and to improve the diagnosis and treatment, with synovial fluid (SF) as the main sample source [5]. Actually, SF is also commonly used in evaluation of efficacy or prognosis of various treatments or manage-

ment of joint diseases [6]. For example, genetic influences in various types of arthritis have been explored, and some genetic backgrounds have been found to predispose certain individuals to OA or rheumatoid arthritis [7]. There are also some studies on metabolic profile and gene expression profile of different types of arthritis [8, 9], including mRNA profile, microRNA profile, matrix metalloproteinase profile, proteomic profile, and cytokine profile. Some of these studies involve a huge number of protein-specific primary antibodies. There are also some bioinformatic analyses with various databases or published data as the information sources [10]. As expected, these studies resulted in very heterogeneous data and conclusions.

Most of the above-mentioned profiling studies utilized joint tissues or synovial tissues or utilized a specific cell type of synovial tissues, such as chondrocytes, or of a SF, such as fibroblasts, whereas few studies utilized the liquid component of SF [11]. For example, few, if any, studies investigated the transcriptome of the liquid component of SF. While these published studies provide valuable information on gene expression profiles of certain synovial cell types or tissues, they do not supply an overall picture of the SF with regard of its RNA repertoire. Having a global picture of RNA in cell-free SF has its unique merits, including the possibility of a better diagnosis of joint diseases, since it is a trend in today's medical research to use the transcriptome in diagnosis of different highly heterogeneous diseases. The liquid fraction of SF is more comparable between one situation and another, as it rules out the differences in cell type. For instance, when contrasting a healthy status to an infectious one, the SF from the patient with infection may be rife with bacteria and inflammatory cells, making the comparison reflect mainly a difference in cell types.

Since there are various types of RNAs in cell-free serum or plasma of blood samples from humans and animals, it needs to be determined whether there are some types of RNA in the liquid component of SF and whether they are at a sufficient quantity for the establishment of a cDNA library and the ensuing deep RNA sequencing to profile the RNAs.

Materials and methods

Collection, cell removal, and storage of synovial fluid

Synovial fluid (SF) was aspirated using a syringe, from the cavity of the affected knee joints of OA patients for diagnostic purposes. The procedure is a routine clinical practice and was approved by the institutional ethical committee (Approval No. 2022-028). The patients signed informed consent forms. After sending a portion of the SF sample to the hospital's clinical laboratory for various examinations, an aliquot of the sample was transferred into a 15-mL Falcon tube (Becton, Dickinson and Company, USA) and centrifuged at 4,000 rpm for 5 min to spin down the cells. Some of cell-free portion at the top was aspirated, transferred into a new tube, and stored at -20°C for RNA extraction later.

Isolation of RNA from the liquid fraction of SF

4 mL of the cell-free SF was added to 4 mL of RNA-specific Trizol (pH 4.8) (Invitrogen, USA) until they were mixed well. After storage at room temperature for 10 min, 1 mL of the mixture was transferred to a 1.5-mL Eppendorf tube (Eppendorf AG, Germany) and 200 μL of chloroform (Sigma-aldrich Company, USA) was added, followed by mixing and storage at room temperature for 15 min. After centrifugation at 12,000 rpm for 15 min at 4°C, the aqueous fraction was collected and the protein extraction with chloroform was repeated once. The aqueous fraction (about 800 μL) was transferred into a new Eppendorf tube and 800 μL of 2-isopropanol was added to precipitate RNA at room temperate for 20 min. After centrifugation at 12,000 rpm at 4°C for 15 min and removal of the supernatant, 1 mL of 75% ethanol was added to wash the RNA precipitate, followed by centrifugation at 12,000 rpm at 4°C for 10 min. After the ethanol was discarded, the RNA precipitate was dissolved in 20 μL of water pre-treated with diethylpyrocarbonate (DEPC) (Sigma-Aldrich Company, USA). The RNA samples extracted from the same SF specimen were pooled and concentrated by precipitation with ethanol, and then suspended in DEPC water and stored at -80°C.

Table 1. Quality of RNA samples from liquid fraction of synovial fluid

Sample	Concen. (μg/μL)	Vol. (μL)	Total RNA (μg)	OD260/280	OD260/230	28S-18S	RIN
Li	4	24	0.096	0.8	0.106	0	0
Niu	10	24	0.24	1.172	0.23	0	1.2

Note: "0", "Concen.", "Vol.", and "RIN" mean undetectable, concentration, total volume, and RNA integrity number, respectively.

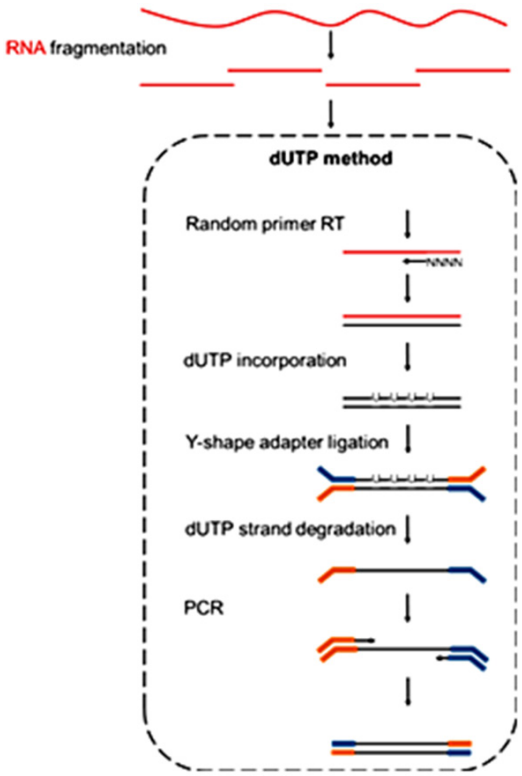

Figure 1. Procedure of establishment of cDNA library.

Establishment of cDNA library

Since this study was to test whether the liquid fraction of SF contained enough RNA for sequencing, one sample with the smallest amount of RNA was selected and designated as "sample Li", and another sample with a relatively larger amount of RNA, designated as "sample Niu". Both patients did not manifest any obvious systemic infection or infection of the affected knee joint, according to the results of various clinical or laboratory examinations. As shown in **Table 1**, both the quantity and the quality of the RNA samples were poor, far worse than what was routinely used for RNA sequencing.

The RNA samples were mixed with a fragmentation buffer (AM8740, Ambion, Inc., An Applied Biosystems Business; www.ambion.com) to fragment RNAs, followed by reverse transcription to the first strand cDNA with random hexamers as routine. The second strand of the cDNAs was synthesized in the presence of RNase H, using DNA polymerase I (Sigma-aldrich Company, USA) with the addition of dATP, dCTP, dGTP and dUTP (Promega Corporation, USA), followed by purification of double-stranded cDNAs using Agencourt AMPure XP beads (www.beckmancoulter.com). The purified cDNA fragments were appended with dATP, dCTP, dGTP and dUTP, using Taq DNA polymerase (Promega Corporation, USA), followed by ligation to adapters and then fragment selection using Agencourt AMPure XP beads again. After degradation of the dU-containing single-stranded cDNAs using an USER enzyme (M5505, NEB, www.neb.com), the DNA fragments were amplified with 8 cycles of polymerase chain reaction (PCR). The PCR products were purified again using Agencourt AMPure XP beads (BeckmanCoulter Company, USA) to establish the cDNA library. The key steps of the library establishment are depicted in **Figure 1**.

cDNA sequencing, sequence alignment, and gene clustering

The cDNA library was quantified with Qubit 2.0 (Invitrogen, USA) and diluted to 1.5 ng/μL, followed by determination of the insert sizes using Agilent 2100 (Agilent Technologies, USA). Quantitative-PCR was used to further quantify it to ensure proper cDNA quality and quantity for sequencing. The cDNA library was subjected to the second-generation sequencing using HisSeq4000 at Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, China, with a data size of 8G.

The sequencing reads were aligned to the UCSC human reference genome (hg38) using Tophat v.2.0.9 software program with default parameters (Johns Hopkins University, Baltimore, MD, USA). Tophat uses the Bowtie2 algorithm to perform the alignment by removing

Table 2. Matched reads and genes identified from sequencing

Sample	Clean reads*	Reads matched	Mapped ratio	Genes
Li	66,154,562 (33,077,281 pairs)	60,383,101	91.28%	2,682
Niu	64,463,162 (32,231,581 pairs)	14,441,399	22.40%	5,081

Note: *Those reads that had five nucleotides unmatchable to the reference were excluded.

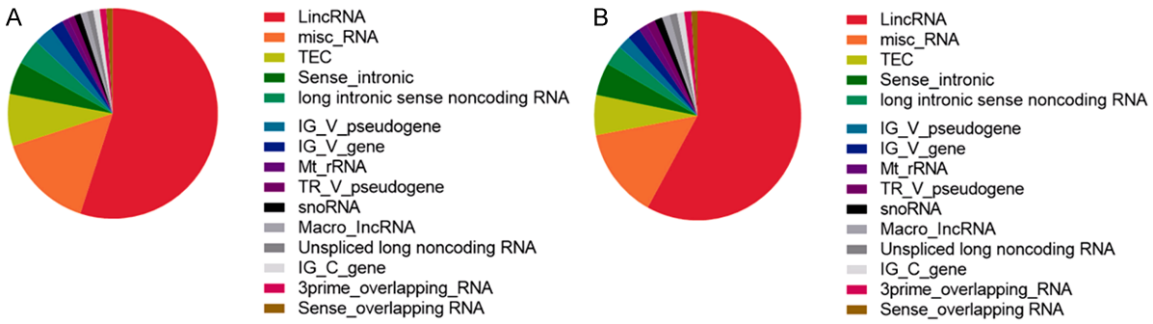

Figure 2. Distribution of detected RNA genes into different gene categories in samples. A: Different RNA gene categories in Sample Li; B: Distribution of RNA genes in Sample Niu. Note: LincRNA: long intervening noncoding RNA; misc_RNA: miscellaneous RNA; TEC: to be experimentally confirmed; IG_V_pseudogene: immunoglobulin_V_pseudogene; IG_V_gene: immunoglobulin_V_gene; Mt_rRNA: mitochondrial rRNA; TR_V_pseudogene: T cell receptor_V_pseudogene; snoRNA: small nuclear RNA; IG_C_gene: immunoglobulin_C_gene.

the parts of the reads that fail to pass the quality control while mapping the qualified reads to the reference genome. Normalized read counts were calculated to fragments per kilobase of transcript per million mapped reads (FPKM). Gene function enrichment was generated by DAVID (https://david.ncifcrf.gov/), which is a web-based tool designed for gene annotation and functional clustering.

Statistical analysis

The measured data were represented by mean ± standard deviation. The enumerated data were expressed as percentage or rate. SPSS 23.0 software (Shanghai Yuchuang Network Technology, China) was used for all statistical analyses. Differences between the sample Niu and Li were analyzed by Student's t-test or chi square test. $P<0.05$ indicated statistical significance.

Results

Sequencing data

As shown in **Table 1**, cDNA library from 0.096 μg (sample Li) and 0.24 μg (sample Niu) RNA were established. In the simple Li, the second-generation sequencing produced 66,154,562 clean reads, 91.28% of which were matched to the human genome with a total of 2,682 genes identified, as shown in **Table 2**. Since these identified "genes" included different types of non-coding RNAs, including microRNAs and antisense RNAs, as seen in **Figure 2A**, all detected genes were referred to as "RNA genes", which covered both protein-coding and noncoding RNAs, to distinguish them from classically annotated genes.

The sample Niu was much better in quality and quantity, as shown in **Table 1**, and 64,463,162 clean reads were obtained from the sample Niu. Interestingly, only 22.40%, i.e. 14,441,399 of the reads were matched to the human genome, although 5,081 genes were identified, many more than the 2,682 genes identified in sample Li, as shown in **Table 2**. Since the match rate was surprisingly low, whether the RNA sample contained alien RNAs from microorganisms was analyzed (**Figure 2B**). We thus aligned the unmatched 50,021,763 reads to the nucleotide collections of the NCBI (National Center for Biotechnology Information, USA), and obtained 40,082 matched reads, 25,205 of which had a coverage of over 60%. Interestingly, 20,109 (79.78%) of these 25,205 reads were matched to *pseudomonas*, a genus of Gram-negative, aerobic Gamma-proteobacteria. This result confirmed that the SF sample

Table 3. Ten most abundantly expressed genes

Li	Niu
NEDD4	MT-ND6
BLOC1S6	NEDD4
MT-ND6	MT-ND5
MT-ND4	MT-ND4
MT-ND5	BLOC1S6
MT-CYB	MT-CYB
MT-ND2	MT-ND2
MT-ATP8	MT-ND4L
MT-CO2	MT-CO2
MT-ATP6	MT-CO1

contained alien RNAs, which was one of the reasons for the low match rate to the human RNA transcripts.

Bioinformatic analyses of identified genes

According to the number of the genes in each category, distribution of all identified RNA genes to different categories revealed that most detected genes were protein-coding ones, processed pseudogenes, long noncoding RNAs (lincRNAs), antisense RNAs, miscellaneous RNAs, and unprocessed pseudogenes. However, many other types of RNAs were also detected at a lower abundance in both Li and Niu samples, as shown in **Figure 2**.

Arrangement of the identified RNA genes in the order of their expression abundance showed that the ten most abundantly expressed genes were protein coding ones. These were was similar between the two samples. Interestingly, 80% of genes were encoded by the mitochondrial genome, which was highly similar between the two samples as well, as shown in **Table 3**. This indicated that these mitochondrial RNAs were either highly expressed or more stable in the viscous SF.

As shown in **Figure 3**, the bioinformatic analyses also showed that the two samples were similar in the subcellular localizations of the detected RNA genes, which included different organelles, macromolecular complexes, and even extracellular regions. As expected, cellular components, metabolic processes, and multicellular processes were among the main biologic functions with the most RNA genes detected. Consistent with these results, most detected RNA genes were found to be related to sort of binding activity or catalytic activity among various molecular functions.

The analysis of molecular pathways revealed that cancer-related pathways were the most prominent in both samples. However, the two samples were distinguished from each other by many other pathways. As shown in **Table 4**, for example, the PI3K-Akt and the focal adhesion signaling pathways were identified in sample Niu but not in sample Li. Fewer molecular signaling pathways were identified in sample Li, in part because fewer genes were identified than in sample Niu.

Discussion

Although synovial fluid (SF) is very viscous, a routine procedure including removal of cells by a low speed centrifugation and removal of proteins with Trizol and chloroform, was able to supply a sufficient amount, that is less than 0.1 μg, of RNA, for establishment of a cDNA library and the ensuing second generation DNA sequencing. This tiny amount of RNA is the smallest known quantity sufficient for this purpose, but whether an even lesser quantity of RNA is also sufficient for this purpose is unknown. A pretreatment of the SF with a hyaluronidase may decrease the viscosity and increase the yield of RNA, especially small RNAs that may be hard to precipitate from the viscous SF [12, 13]. However, this additional step may also degrade some long RNAs. Peers may consider this step by balancing its strength against its weakness.

One ethical constraint for study of the transcriptome in SF is the difficulty in collecting SF from healthy individuals with comparable gender and age [14]. There were also reasons that we were not able to include normal SF samples for comparison. As introduced earlier, most relevant studies analyzed the transcriptome by comparing different subtypes of arthritis, such as comparison of OA with rheumatoid arthritis, using cells from SF or directly using different joint tissues or synovial tissues [15, 16]. While these studies on tissues or cells may provide us with clues for how a particular synovial tissue or cell type may be involved in joint diseases, they do not provide us with a general picture of the gene expression profile in SF in general and in the liquid fraction of SF in particular [17,

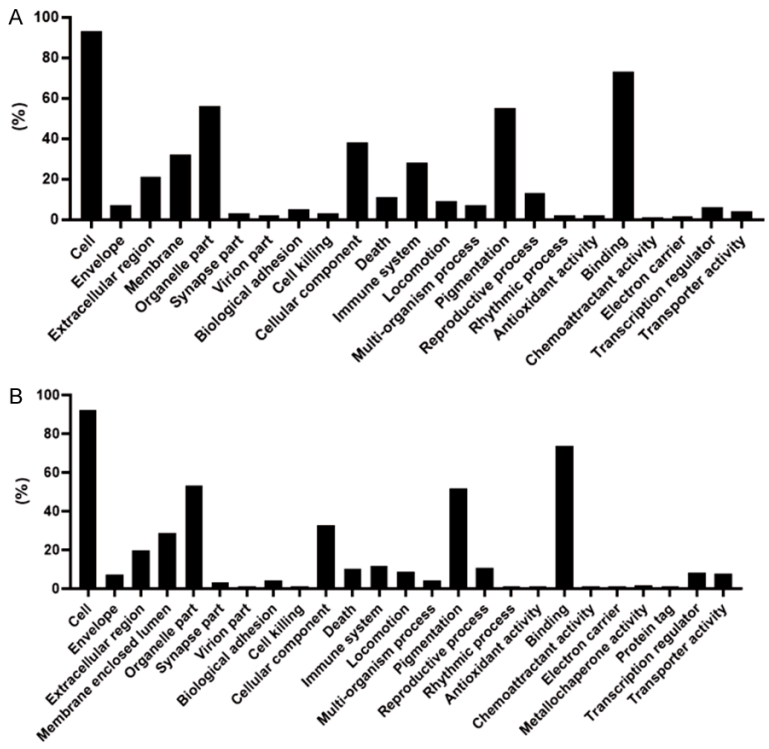

Figure 3. Distribution of detected RNA genes for different subcellular or functional categories, arranging in the percentage occupied by this number in the total identified genes from samples. A: Sample Li; B: Sample Niu.

18]. This study showed that the liquid fraction of SF contains a whole RNA repertoire, of which those derived from the mitochondrial genome were the most abundant in terms of expression level. These RNAs must either be secreted from synovial cells or tissues or be derived from dregs of various cell types in SF, which in turn either be shed off from synovial tissues or be cells in SF per se. Since SF cells may have died a long time ago, the data seem to suggest that SF can maintain RNAs at a relatively high quality for a long time.

Prominent cancer-related pathways were identified to be the most prominent ones in both samples [19], which is somewhat unexpected. Since cancer-related pathways are characterized mainly by promotion of cell proliferation and/or inhibition of cell differentiation and cell death, it seems SF cells or synovial tissues have a high cell turnover, i.e. quick cell proliferation and differentiation in association with quick cell death [20, 21]. This is reasonable because knee joints have much activity every day, which may cause death of many cells.

The two samples studied had disparities in the RNA repertoire. The disparities mainly occurs in the terms for the molecular pathways. Compared with sample Li, sample Niu displayed more-diversified and more-complex signaling pathways. Interestingly, some pathways or cellular processes related to viral infection showed activity in both samples. It is possible that OA may be related to abnormal immune activity or that knee joints are actually easily infected by viruses, although some of the infections may not be virulent and cause disease [22-24]. These possibilities await verification with more samples and with a proper control of SF from healthy individuals.

An unexpected finding in our study is that a large portion of clean reads were unmatchable to the human genome, especially in sample Niu that had 77.60% of the clean reads being unmatchable. Even in sample Li there still were 8.72% of the reads being unmatchable, which is higher than most of other RNA samples from cells or tissues in our long-term practice in RNA deep sequencing. Some of these unmatchable reads may be technical spuriousness derived from RT or PCR, as one of us has described previously [25-29]. Another possibility may be a contribution from alien RNAs, i.e. those from microorganisms such as virus, bacteria, or fungi [30-32]. This is indeed one of the reasons for sample Niu from which we found that many of the clean but unmatchable reads were matched to bacteria, mainly *Pseudomonas*, some strains of which are known human pathogens [33, 34]. Considering that the two patients did not have any obvious systemic infection or infection of the affected knee joint at the time of sample collection, as shown by all clinical and laboratory examinations, these foreign RNAs should be remains of microorganisms from previous infections, some of which may not even be virulent enough to cause noticeable infection, especially considering that

Table 4. KEGG analyses of gene distribution to various functional signaling pathways according to their read counts

Sample		Li				Niu	
Term	Count	%	*P* value	Term	Count	%	*P* value
hsa05200: Pathways in cancer	44	3.049203	0.023812	hsa05200: Pathways in cancer	70	2.659574	0.04867
hsa03013: RNA transport	34	2.356202	1.96E-06	hsa04151: PI3K-Akt signaling pathway	66	2.507599	0.014145
hsa05169: Epstein-Barr virus infection	27	1.871102	0.005192	hsa04510: Focal adhesion	46	1.74772	0.002661
hsa05202: Transcriptional misregulation in cancer	23	1.593902	0.015794	hsa04810: Regulation of action cytoskeleton	42	1.595745	0.028737
hsa03040: Spliceosome	21	1.455301	0.004734	hsa05205: Proteoglycans in cancer	41	1.557751	0.019779
hsa04068: FoxO signaling pathway	19	1.316701	0.02169	hsa05203: Viral Carcinogenesis	41	1.557751	0.028682
hsa04611: Platelet activation	18	1.247401	0.03177	hsa03013: RNA transport	35	1.329787	0.034929
hsa04110: Cell cycle	17	1.178101	0.040597	hsa03040: Spliceosome	31	1.177812	0.008055
hsa03015: mRNA surveillance pathway	16	1.108801	0.00582	hsa04068: FoxO signaling pathway	30	1.139818	0.016021
hsa04919: Thyroid hormone signaling pathway	16	1.108801	0.04007	hsa03010: Ribosome	29	1.101824	0.031774
hsa00650: Butanoate metabolism	7	0.4851	0.018468	hsa04919: Thyroid hormone signaling pathway	26	0.987842	0.020687
hsa03060: Protein export	6	0.4158	0.033538	hsa04512: ECM-receptor interaction	23	0.87386	0.005493
				hsa05100: Bacterial invasion of epithelial cells	22	0.835866	0.00298
				hsa04666: Fc gamma R-mediated phagocytosis	20	0.759878	0.030033
				hsa05222: Small cell lung cancer	20	0.759878	0.033609
				hsa05213: Endometrial cancer	14	0.53915	0.031693
				hsa04621: NOD-like receptor signaling pathway	14	0.53915	0.048042
				hsa00650: Butanoate metabolism	10	0.379939	0.01114

the analyses of molecular pathways suggest a higher immune activity in the SF. The observation that these alien RNAs became long-lasting in SF may indicate that SF has little nuclease activity and its viscous nature helps to stabilize RNAs. If so, the transcriptome of the liquid fraction of SF may provide us with clues for not only the current, but also previous infection(s) by microorganisms, including those that are less virulent. This point, and whether these foreign RNAs have any beneficial or detrimental effects on the joint, deserve further investigations [35, 36].

In conclusion, this study showed that an amount of RNA less than 0.1 μg could be used to establish a cDNA library for sequencing. The cell-free liquid fraction of SF contains a whole RNA repertoire consisting of most RNA categories, including protein-coding ones and different non-coding regulatory ones, with the most abundantly expressed ones being derived from the mitochondrial genome. These RNAs participate in most biological functions and cellular processes, including cancer-related pathways and transcriptional regulations. Interestingly, the liquid component of SF also contains a significant amount of alien RNAs that likely belong microorganisms that previously infected the joint, which suggests that SF may have little nuclease activity and is capable of sustaining the integrity of RNAs for a long time.

This study has some limitations that should be noted. First, the sample size was inadequate. Second, this research was performed in a single center. Third, the mechanisms of signal transduction system for RNAs expression were not further detected. Therefore, conducting a multicenter, randomized controlled and large-sample clinical study to confirm the roles of RNAs in biologic functions and cellular processes during development of KOA is warranted to more precisely guidelines in prevention and treatment of KOA.

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Disclosure of conflict of interest

None.

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