Original Article

Comprehensive analysis of differentially expressed serum microRNAs in humans responding to *Brucella* infection

Cuiping Zhang[1,2#], Quan Fu[3,4#], Meng Ding[1,2], Tingting Chen[1,2], Xiaolan Lu[1,2], Yujie Zhong[1,2], Yuying Bian[1,2], Fengmin Zhang[3], Chen-Yu Zhang[2], Chunni Zhang[1,2], Cheng Wang[1,2]

[1]Department of Clinical Laboratory, Jinling Hospital, State Key Laboratory of Analytical Chemistry for Life Science, Nanjing University School of Medicine, Nanjing University, Nanjing 210002, China; [2]Jiangsu Engineering Research Center for microRNA Biology and Biotechnology, Advance Research Institute of Life Sciences, School of Life Sciences, Nanjing University, Nanjing 210046, China; [3]Department of Microbiology, Harbin Medical University, Harbin 150081, China; [4]Department of Clinical Laboratory, Affiliated Hospital of Inner Mongolia Medical University, Hohhot 010050, China

Contributions: (I) Conception and design: C Zhang, C Wang; (II) Administrative support: C Zhang, C Wang, CY Zhang; (III) Provision of study materials or patients: Q Fu, F Zhang; (IV) Collection and assembly of data: M Ding, T Chen, X Lu; (V) Data analysis and interpretation: CP Zhang, Q Fu, Y Zhong, Y Bian; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

[#]These authors contributed equally to this work.

Correspondence to: Dr. Cheng Wang. Department of Clinical Laboratory, Jinling Hospital, State Key Laboratory of Analytical Chemistry for Life Science, Nanjing University School of Medicine, Nanjing University, Nanjing 210002, China. Email: wangcheng919@smail.nju.edu.cn; Prof. Chunni Zhang. Department of Clinical Laboratory, Jinling Hospital, State Key Laboratory of Analytical Chemistry for Life Science, Nanjing University School of Medicine, Nanjing University, Nanjing 210002, China. Email: zchunni27@hotmail.com; Prof. Chen-Yu Zhang. Jiangsu Engineering Research Center for microRNA Biology and Biotechnology, Advance Research Institute of Life Sciences, School of Life Sciences, Nanjing University, Nanjing 210046, China. Email: cyzhang@nju.edu.cn.

Background: MicroRNAs (miRNAs), a subset of small non-coding RNA molecules, play crucial roles in various pathophysiological processes. Studies increasingly indicate that dysregulated miRNAs are associated with bacterial infection. Nevertheless, little is known about miRNAs that respond to *Brucella* infection and their potential clinical value. Our research aimed to identify the serum miRNAs altered during *Brucella* infection.

Methods: We enrolled serum samples from 73 patients diagnosed with brucellosis and 65 age- and sex-matched control individuals. Illumina sequencing via synthesis (SBS) technology was performed for an initial screen of miRNAs expression profile in serum samples pooled from 29 patients and 29 controls, respectively. A quantitative real-time polymerase chain reaction (qRT-PCR) assay was conducted in the training and validation sets to confirm the concentrations of differentially expressed miRNAs in individual serum samples from 73 patients and 65 controls.

Results: The Illumina SBS technology identified 1,372 known miRNAs and 1,893 novel miRNAs in brucellosis patients. The three markedly upregulated miRNAs (miR-15a-3p, miR-7-2-3p, miR-103b) in brucellosis patients were subsequently validated by qRT-PCR assay, of which miR-103b was confirmed to be significantly and steadily increased in the brucellosis patients compared with the controls (>2-fold, P<0.001). The area under the receiver operating characteristic (ROC) curve (AUC) for miR-103b was 0.714 (95% CI, 0.624–0.804). Bioinformatics analysis predicted that some putative target genes of miR-103b are involved in immune regulation or the processes of apoptosis and autophagy in humans.

Conclusions: The serum miR-103b level markedly increases after *Brucella* infection and has the potential to serve as an auxiliary diagnostic indicator for *Brucella* infection that deserves further investigation.

Keywords: *Brucella* infection; brucellosis; serum; miR-103b; immune response

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Introduction

MicroRNAs (miRNAs), a subset of non-coding RNA molecules of approximately 22 nucleotides in length, are believed to regulate gene expression by targeting mRNAs for posttranscriptional repression or mRNAs destabilization (1). Our group and others have recently highlighted that serum miRNAs can exist stably and that the special expression profiles of altered miRNAs are associated with many diseases by its special expression profiles (2-6). miRNAs, as an emerging area of investigation, are being paid increasing attention due to their altered expression profiles in numerous diseases, and the vital roles of miRNAs in microbial infection are gradually being uncovered. Accumulating evidence has demonstrated the functions of miRNAs in pathogens transmission and pathogenesis (7,8). The expression of host miRNAs can be regulated by pathogens, and the differentially expressed miRNAs can function as auxiliary indicators for diagnosing bacterial diseases (9,10). Herein, we speculated that the altered profile of serum miRNAs may furnish biomarkers for *Brucella* infection and may represent a promising new field for brucellosis research.

Brucellosis, a zoonotic infectious disease, is caused by a group of microorganisms belonging to the genus *Brucella* that are facultative intracellular Gram-negative bacteria (11). *Brucella* affects multiple systems throughout the body with a variety of clinical symptoms, including undulant fever, arthritis, endocarditis, osteoarthritis and meningitis in humans, as well as miscarriage or stillbirth in livestock (12). Approximately 500,000 individuals around the world are diagnosed with brucellosis annually, and the morbidity rate in some prevalent districts is as high as ten cases per 100,000 population, especially in Mediterranean countries, the Middle East and Latin America (13-15). In China, Inner Mongolia, Xinjiang, Qinghai, Ningxia and Henan Provinces are the main regions with severe epidemics (16). Additionally, *Brucella* infection not only causes serious public health problems in humans that mainly affect developing countries, but also results in significant economic losses in livestock (13,14,17,18). Although there are many studies on *Brucella* infection, few studies have been devoted to exploring the effects of brucellosis on serum miRNA expression. Since serum and plasma are accessed with relative ease, circulating biomarkers are one of the most promising means of diagnosis. Therefore, clarifying miRNA expression profile as a novel diagnostic indicator could provide some valuable information for the diagnosis of brucellosis.

In our study, to investigate the differentially expressed miRNA profile in brucellosis patients, we performed a comprehensive analysis of miRNA expression with Illumina SBS technology and subsequently confirmed candidates by qRT-PCR assay. The objective of our research was to identify the miRNAs with altered serum profiles and to explore their diagnostic value for *Brucella* infection.

Methods

Ethics approval

The present study was conducted in accordance with the principles of the World Medical Association Declaration of Helsinki. Meanwhile, we obtained written informed consent from all participants to allow the use of samples and clinical data for investigation. All collected samples were manipulated according to the protocols approved by the ethics committee of each participating institution.

Participants and processing of serum samples

For this case-control retrospective study, we enrolled 73 brucellosis patients, 64 of whom had acute infections and 19 of whom had chronic infections, at the Affiliated Hospital of Inner Mongolia Medical University or Zhungeer Qi Center for Disease Control and Prevention, Inner Mongolia, China, between 2016 and 2017. There were 48 males and 25 females, with average ages of 47.55±12.88 years and 45.51±9.01 years, respectively. Among them, 18 (75%) patients were positive on blood culture, and the others were diagnosed via serological tests. In addition, most of brucellosis patients (68/73, 94%) were Han Chinese. Meanwhile, we recruited 65 age- and gender-matched healthy Han Chinese (41 males and 24 females) as the parallel control group; these healthy controls underwent routine medical examinations at the Healthy Physical Examination Center of Jinling Hospital, Nanjing, China. The mean age of the control group was 48.00±14.18 years and 42.46±16.62 years in males and females, respectively. There was no significant difference in age (P=0.172) or gender (P=0.743) between the two groups.

All blood samples were collected in separating glue/coagulant tubes according to the standard operating procedure. All the collected samples were processed by centrifugation at 1,500 ×g for 10 min at room temperature, and the supernatants were subsequently centrifuged at

2,000 ×g for 10 min at 4 ℃ to remove the debris (19). The obtained serum samples were stored at –80 ℃ pending RNA extraction.

RNA extraction

For Illumina SBS technology, equal volumes of sera (20 mL) from 29 patients with brucellosis and 29 normal controls (0.69 mL each) were pooled separately to form case and control sample pools. Total RNAs were extracted from two pools using TRIzol reagent (Invitrogen, MA, USA) as previously described (3). The aqueous phase was subjected to 3 steps of acid phenol/chloroform purification to eliminate protein residues before isopropyl alcohol precipitation. The resulting RNA pellet was dissolved in 20 μL diethylpyrocarbonate (DEPC) water and stored at –80 ℃ until further analysis.

For the qRT-PCR assay, total RNA was extracted from 100 μL serum with a 1-step phenol/chloroform purification protocol as previously described (20); the serum was mixed with 300 μL deionized water, 200 μL acid phenol, and 200 μL chloroform. The mixture was vortex-mixed vigorously and incubated at room temperature for 15 min. After phase separation, the aqueous layer was blended with 1.5 volumes of isopropyl alcohol and 0.1 volumes of 3 mol/L sodium acetate (pH 5.3). This mixed solution was stored at –20 ℃ for at least 1 h. The RNA pellet was collected by centrifugation at 16,000 ×g for 20 min at 4 ℃. We discarded the supernatant and washed the precipitate with 750 mL/L ethanol. The RNA pellet was dried for 10 min at room temperature. Finally, the pellet was dissolved in 30 μL DEPC water and stored at –80 ℃ until further analysis.

Illumina SBS technology

Illumina SBS technology (BGI Genomics Co., Shenzhen, China) was applied to RNA separately extracted from two pooled samples as previously described (21). Briefly, after PAGE purification of small RNA molecules (<50 nucleotides) and ligation of a pair of adaptors to the RNA 5' and 3' ends, RNA molecules were amplified using primers for the adaptor regions for 17 cycles. Fragments that were approximately 90 bp (small RNA + adaptors) in length were isolated from the agarose gel. The purified DNA was used for cluster generation and sequencing analysis on the Illumina sequencing system according to the manufacturer's instructions. Image files were generated by the sequencer and processed to produce digital-quality data. The subsequent procedures performed with Illumina HiSeq 2000 were summarizing data production, evaluating sequencing quality, calculating length distribution of small RNA reads and filtrating reads contaminated by rRNA, tRNA, mRNA, snRNA, and snoRNA. Finally, clean reads were compared with miRBase 21.0. The experimental procedure of small RNA library construction was shown in Supplementary materials. After analysis, miRNAs with $P<0.05$ and fold change values >2 and <–2 were determined to be statistically significant between the groups.

Quantification of miRNAs by qRT-PCR analysis

To normalize serum miRNAs, we added synthetic plant exogenous small non-coding RNA MIR2911 (5'-GGCCGG-GGGACGGGCUGGGA-3') during the RNA isolation process as an external reference to normalize the expression levels of the miRNAs (22). MIR2911 was added to each serum sample at an ultimate concentration of 10^6 fmol/L during RNA extraction, as perviously reported (20). There was no obvious difference between the two groups with regard to the CT values of MIR2911 ($P>0.05$) (*Figure S1*).

We performed a qRT-PCR assay according to the manufacturer's instructions (Roche Light Cycler® 480 II, Roche Diagnostics Ltd., Rotkreuz, Switzerland) (3). Briefly, the reverse transcription reaction was conducted in a 10 μL volume: 2 μL of total RNA, 1 μL of 10 mmol/L dNTPs, 0.5 μL of AMV reverse transcriptase (TaKaRa, Dalian, China), 1 μL of a stem-loop RT primer (Applied Biosystems), 2 μL of 5× reverse transcription buffer and 3.5 μL of DEPC water. For the synthesis of cDNA, the reaction mixtures were incubated at 16 ℃ for 30 min, at 42 ℃ for 30 min, and at 85 ℃ for 5 min, and then they were stored at 4 ℃. Real-time PCR was performed (1 cycle of 95 ℃ for 5 min, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min) with the Lightcycler® 480 II system (Roche). The reaction was performed with a final volume of 20 μL containing 1 μL of cDNA, 0.3 μL of Taq, 0.33 μL of hydrolysis probe (Applied Biosystems), 1.2 μL of 25 mmol/L $MgCl_2$, 0.4 μL of 10 mmol/L dNTPs, 2 μL of 10× PCR buffer, and 14.77 μL of DEPC water. To ensure the reliability of the results, all reactions, including those involving non-template controls, were carried out in triplicate. The relative contents of the miRNAs were normalized to the level of MIR2911 and were calculated using the comparative cycle threshold (CT) method ($2^{-\Delta CT}$). ΔCT was calculated by subtracting the CT values of MIR2911 from the CT values of the miRNAs. The

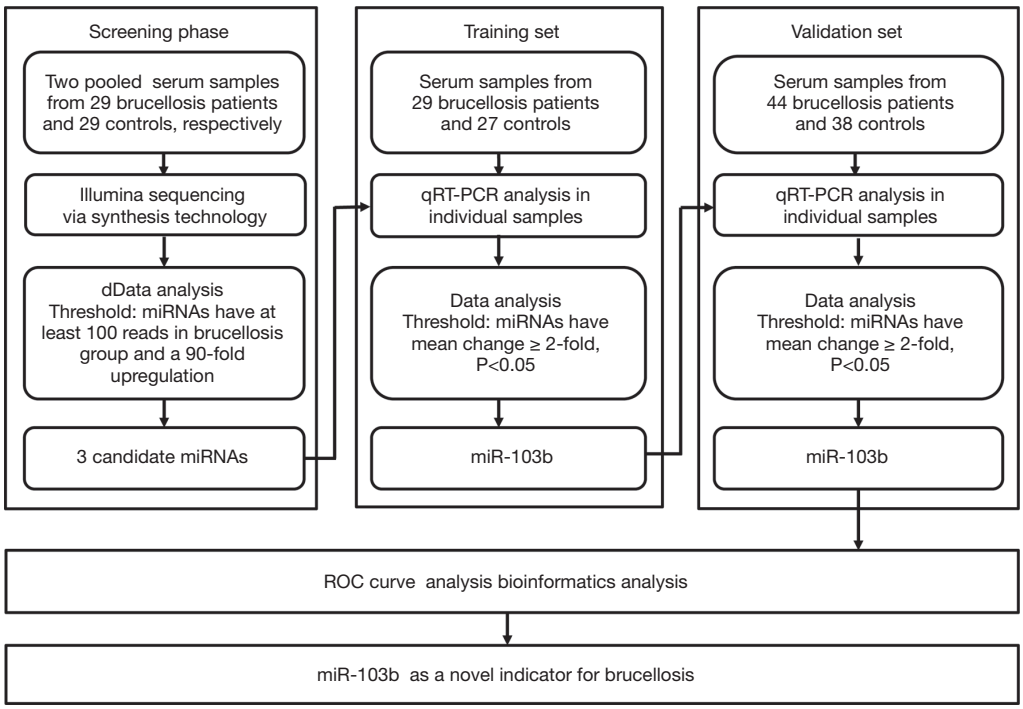

Figure 1 Flow chart of the experimental design. qRT-PCR, quantitative real-time polymerase chain reaction; ROC, receiver operating characteristic.

concentrations of miRNAs relative to the concentration of MIR2911 were calculated using the equation $2^{-\Delta CT}$ (23).

Data analysis

SPSS software 23.0 (IBM, NY, USA) and GraphPad Prism 6.0 (GraphPad Software, CA, USA) were used for the statistical analysis. Data are displayed as the mean ± SEM for miRNAs. The nonparametric Mann-Whitney U-test was selected to compare differences in variables between the two groups. For the differentially expressed miRNAs, we constructed ROC curves and calculated the area under the ROC curve (AUC) to assess their ability to distinguish brucellosis patients from control individuals. Binary logistic regression analysis was carried out to evaluate the risk of miR-103b on brucellosis. P<0.05 was considered statistically significant.

Results

Serum miRNAs profile analyzed by Illumina SBS technology

A multiphase, case-control study was designed to identify differentially expressed serum miRNAs (*Figure 1*). We first performed Illumina SBS technology to identify differentially expressed serum miRNAs between brucellosis patients and controls. The results indicated that the serum contained multiple and heterogeneous small RNA species (<50 nucleotides), as shown in *Table 1*. Among them, miRNA content was the most abundant (more than 40%) in the pooled serum samples (*Figure S2*). Pearson's correlation scatter plots were used to compare the serum miRNA profile in brucellosis patients relative to healthy controls, in which the square of Pearson's correlation coefficient (R^2) value was 0.651 (P<0.0001) (*Figure 2*). In total, 3,265 miRNAs (1,372 known miRNAs and 1,893 novel miRNAs) were identified. Of the 1,372 known miRNAs, 1136 miRNAs were expressed differentially with more than a 2-fold change: 445 upregulated miRNAs and 691 downregulated miRNAs in the brucellosis sample compared with the control sample. A miRNA was regarded as markedly altered if the Illumina SBS technology detected more than 100 reads in either the patient or control group, and if the miRNA showed at least a 2-fold difference in expression level between the brucellosis and control groups. According to the above criteria, 354 known miRNAs

Table 1 The categories of small RNAs in pooled serum respectively from controls and brucellosis patients by Illumina SBS technology

Variable	Controls (20 mL)	Brucellosis patients (20 mL)
Total (match genome)	15,625,447	19,248,782
intergenic	9.05%	4.34%
sRNA	3.09%	5.91%
snRNA	0	0.01%
unmap	13.1%	2.47%
intron	18.54%	16.61%
piRNA	2.2%	1.15%
rRNA	0.62%	0.14%
snoRNA	0.11%	0.03%
precursor	0.15%	0.15%
repeat	4.04%	10.52%
exon	7.25%	2.33%
miRNA	41.81%	56.33%
tRNA	0.04%	0.01%

exhibited a significant discrepancy between the two groups, with 106 upregulated (*Table S1*) and 248 downregulated (*Table S2*) miRNAs in brucellosis patients, respectively. To narrow down the candidates after global screening of the upregulated miRNAs, we therefore selected 3 miRNAs upregulated with the highest fold change values to validate the miRNA sequencing accuracy by qRT-PCR assay.

Confirmation of Illumina SBS technology results by qRT-PCR analysis

Our previous studies have proven that circulating miRNAs can be efficiently extracted from serum and quantitatively detected by qRT-PCR assay which is reliable and reproducible (3,5). Therefore, we used qRT-PCR assay to verify the contents of the 3 markedly upregulated miRNAs (miR-15a-3p, miR-7-2-3p, miR-103b) in brucellosis patients. We first measured the expression levels of the 3 miRNAs in the training set composed of individual serum samples from 29 brucellosis patients and 27 healthy controls. Comparing the concentrations between the two groups, only the expression of miR-103b was significantly increased, with 2.4-fold upregulation and a P value <0.05 in the brucellosis group (*Table 2*). Therefore, miR-103b was selected for further verification.

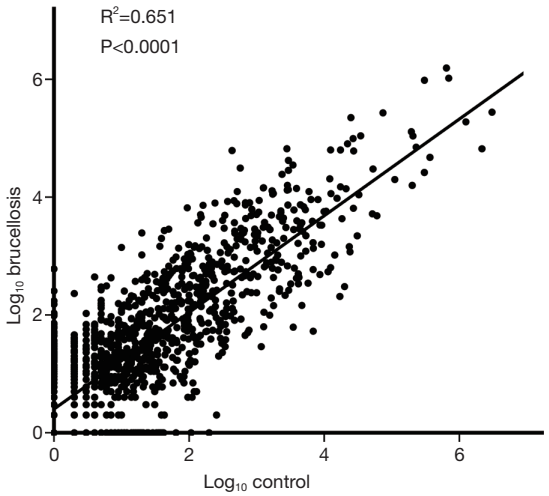

Figure 2 Pearson correlation scatter plot of serum miRNA levels. Pearson correlation scatter plot of serum miRNA levels in the brucellosis group and control group as determined by Illumina SBS technology, P<0.0001.

We examined the serum level of miR-103b in a larger cohort consisting of an additional 44 brucellosis patients and 38 matched controls (validation set). Consequently, consistent with the findings in the training set, the level of miR-103b also exhibited a significant increase (>3-fold, P<0.001) in the brucellosis patients in the validation set (*Table 2*). *Figure S3* summarizes the differences in concentrations of miR-103b in all the patients and control individuals enrolled in the training and validation sets.

ROC curve and binary logistic regression analysis

To distinguish brucellosis patients from healthy controls, ROC curve analysis was performed with miR-103b to assess the diagnostic value according to the AUC. We obtained an AUC of 0.714 (95% CI, 0.624–0.804) for the total serum samples (*Figure 3*). In addition, we performed univariate logistic regression analysis to further weigh the usefulness of miR-103b for the diagnosis of brucellosis. We defined brucellosis status as the dependent variable and controlled for other variables, including age and sex. The regression coefficient of miR-103 was 2.807, and the odds ratio was 16.557 (95% CI, 5.451–50.288), suggesting that miR-103b

Table 2 Relative concentrations of candidate miRNAs to MIR2911 in the serum samples from brucellosis group and control group determined by individual qRT-PCR assay in the training set and the validation set[a]

miRNA	Controls	Brucellosis	Fold change	P value[b]
Training set				
Samples, n	27	29		
miR-15a-3p	52.53 (6.14)	99.48 (18.27)	1.89	0.1011
miR-7-2-3p	14.39 (1.70)	27.91 (6.35)	1.94	0.1378
miR-103b	58.65 (7.04)	145.15 (30.27)	2.47	0.0195
Validation set				
Samples, n	38	44		
miR-103b	43.66 (3.99)	132.90 (19.85)	3.04	0.0003

[a], miRNA data is presented as the mean ± SEM ($\times 10^{-2}$). [b], Brucellosis *vs.* Controls.

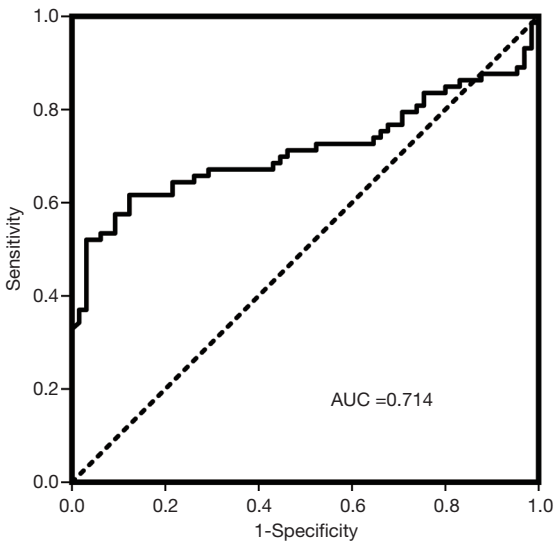

Figure 3 ROC curve analysis of miR-103b. ROC curve for the ability of miR-103b to distinguish brucellosis patients from normal controls in all subjects.

Table 3 The target genes of miR-103b predicted by bioinformatics analysis

Target gene	Description	Validated or not
CTLA4	Cytotoxic T-lymphocyte-associated protein 4 (24)	Yes
ATG7	Autophagy related 7	No
CXCL9	Chemokine (C-X-C motif) ligand 9	No
CASP7	Caspase-7, apoptosis-related cysteine peptidase	No

is a potential risk factor for *Brucella* infection.

Target analysis of miR-103b

To explore the possible biological role of the upregulation of miR-103b in response to *Brucella* infection, we predicted the potential target genes associated with infection and immunity for miR-103b by bioinformatics analysis through several widely used target prediction databases, including TargetScan, miRDB and miRPathDB. The bioinformatics analysis revealed 72 potential target genes associated with miR-103b (*Figure S4*), of which cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), autophagy related 7 (ATG7), chemokine (C-X-C motif) ligand 9 (CXCL9) and caspase-7, apoptosis-related cysteine peptidase (CASP7), are involved in immune regulation or the regulation of apoptosis and autophagy in humans (*Table 3*).

Discussion

Human brucellosis is a multisystem disease with a large array of clinical symptoms but a lack of specific manifestations, which still presents a diagnostic challenge for clinicians owing to the limitations of detection methods (25,26). Recently, it has become promising to explore the pathogenesis and progress of infectious diseases from the molecular level with the application of high-throughput sequencing and PCR assays (27,28). In addition, a study has demonstrated that the optimal clinical specimen for brucellosis detection by PCR is serum and not whole blood, in that the sensitivity of the experiment is higher in the serum than that in the whole blood (29). Therefore, we attempted to investigate brucellosis from the perspective of

serum small non-coding RNAs perspective. Some altered small non-coding RNAs might provide serological indicator candidates underlying *Brucella* infection.

It is well known that miRNAs are involved in numerous physiological and pathologic processes. Aberrantly expressed miRNAs have also been associated with various diseases, including cancers, infertility, aging and others (3,30,31). Meanwhile, mounting evidence indicates that miRNAs play a key role in bacterial infections by regulating inflammatory reactions (32-34). In the course of infection, *Brucella* mainly attacks the body's immune systems, modulating the innate and adaptive immunity, autophagy, apoptosis and possibly the expression of small non-coding RNAs in the serum (35). Considering its great potential in the regulation of the immune system, herein, we focused on the characteristic miRNA expression profile related to *Brucella* infection.

In this study, we first utilized Illumina SBS technology to comprehensively analyze the serum miRNA expression profile of brucellosis patients. We found that the miRNA expression profile in brucellosis patients was different from that in normal controls, with 1,136 dysregulated known miRNAs. After further validation of 3 miRNAs with highest fold upregulation by qRT-PCR in individual serum samples arranged in two independent cohorts, miR-103b was confirmed to be significantly and steadily upregulated in serum samples from brucellosis patients. In addition, ROC curve analysis showed that the corresponding AUC was 0.714, and the odds ratio was 16.557 larger than 1, indicating that miR-103b is a promising auxiliary serological index for brucellosis detection. We suspect that if serum miR-103b is detected in conjunction with other existing test indicators, it will be possible to improve diagnostic accuracy for brucellosis.

Until now, most existing studies have mainly concentrated on the levels of miRNAs in cells, mainly in RAW264.7 cells, CD8+ T cells and peripheral blood mononuclear cells (PBMCs) (11,12,36). A research group conducted a comprehensive analysis of miRNA expression profile in RAW264.7 cells with or without *Brucella* infection using high-throughput sequencing technology and found 57 differentially expressed miRNAs, of which eight altered miRNAs with high levels of abundance had putative target genes involved in the processes of apoptosis, autophagy and the immune response, indicating that *Brucella* may establish a stable infection by regulating miRNA expression (12). Furthermore, Budak *et al*. confirmed that differentially expressed miRNAs (miR-1238-3p, miR-494, miR-6069, and miR-139-3p) in PBMCs participate in the formation and progression of brucellosis (36). Previous studies have also shown that *Brucella* inhibits TNF-α to affect immune responses via Omp25 regulation of different miRNAs in macrophages (37). These findings fully demonstrate that miRNAs are closely related to human brucellosis, and they are crucial in pathogen-host interactions; however, no study to date has characterized the miRNA expression patterns in the sera of brucellosis patients, which we identified as being upregulated in the serum of brucellosis patients, may provide insight into the molecular mechanisms underlying *Brucella* infection.

Through bioinformatics analysis, we found that CTLA-4 was a potential target gene of miR-103b; CTLA-4 is a main component of the immune system and is expressed exclusively on activated CD4+ and CD8+ T cells and binds the same ligands (38). Eskandari-Nasab *et al*. demonstrated that CTLA-4 variants (CTLA-4 – 318C/T polymorphisms) act as risk factors for developing *Brucella* infection, facilitating the pathogenesis of disorders characterized by aberrant T-cell responses (24). Other predicted genes, such as ATG7, CXCL9 and CASP7, have also been linked to immune processes such as autophagy and apoptosis (39-41). The target genes associated with miR-103b and their functional linkage deserves further exploration.

In summary, for the first time, we investigated the serum miRNA expression profile in patients with brucellosis by Illumina SBS technology screening and confirmed it with qRT-PCR assays, and demonstrated that miR-103b is significantly upregulated in brucellosis patients. Further analysis suggests that miR-103b has potential to serve as an auxiliary detection indicator, combined with other existing laboratory tests such as blood culture and serological tests may improve the diagnostic accuracy for brucellosis. Further exploration and validation are required to evaluate the potential target genes of miR-103b and their relationship with the occurrence and development of brucellosis.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

Ethical Statement: The present study was conducted in accordance with the principles of the World Medical Association Declaration of Helsinki. Meanwhile, we obtained written informed consent from all participants to allow the use of samples and clinical data for investigation. All collected samples were manipulated according to the protocols approved by the ethics committee of each participating institution.

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Supplementary methods—small RNA library construction

Total RNAs were extracted from the two serum pools using TRIzol reagent (Invitrogen, MA, USA), and the RNA samples were then sent to BGI Genomics Co. in Shenzhen for further analysis. The detail information about library preparation are as follows:

First, the concentrations and purity of the total RNAs were measured using Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit) and NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) to guarantee elimination of inorganic ions or polycarbonate contamination, and the qualified RNA samples were prepared for library construction. Subsequently, 0.2–1 μg of total RNA sample was used to separate RNA segment of different size by PAGE gel. The molecules of 18–50 nt RNA stripe were then recycled and ligated to adapters on the 5'- and 3'-terminals of the small RNAs. The reaction condition of adaptor ligation was performed according to the manufacture's recommendation. Briefly, for 3′ adaptor system (TruSeq Small RNA Sample Pre Kit, Illumina), the reaction was performed under following cycling conditions: 70 ℃ for 2 min, 28 ℃ for 1 h and 28 ℃ for 15 min; For 5' adaptor mix system, the reaction was performed at 70 ℃ for 2 min and 28 ℃ for 1 h. Furthermore, the samples were subjected to RT-PCR for synthesis of cDNAs and amplification of cDNA fragments. In brief, reverse transcription was performed using First-Strand Master Mix and Super Script II (Invitrogen) for small RNAs at 70 ℃ for 2 min, followed by 50 ℃ for 1 h; PCR amplification with PCR Primer Cocktail and PCR Master Mix were performed to enrich the cDNA fragments under the reaction condition that initial denaturation for 30 s at 98 ℃ followed by 11 cycles of denaturation for 10 s at 98 ℃, 30 s annealing at 60 ℃, 15 s extension at 72 ℃, and a final extension for 10 min at 72 ℃. Finally, the PCR products were purified with 1% agarose gels and dissolved the recycled products in EB solution for Illumina SBS sequencing using Illumina HiSeq 2000.

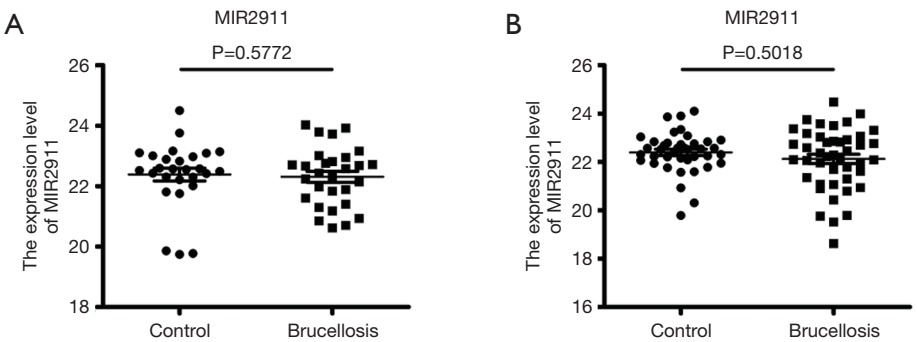

Figure S1 The serum levels of MIR2911 in the training set (A) and the validation set (B) measured by qRT-PCR assay.

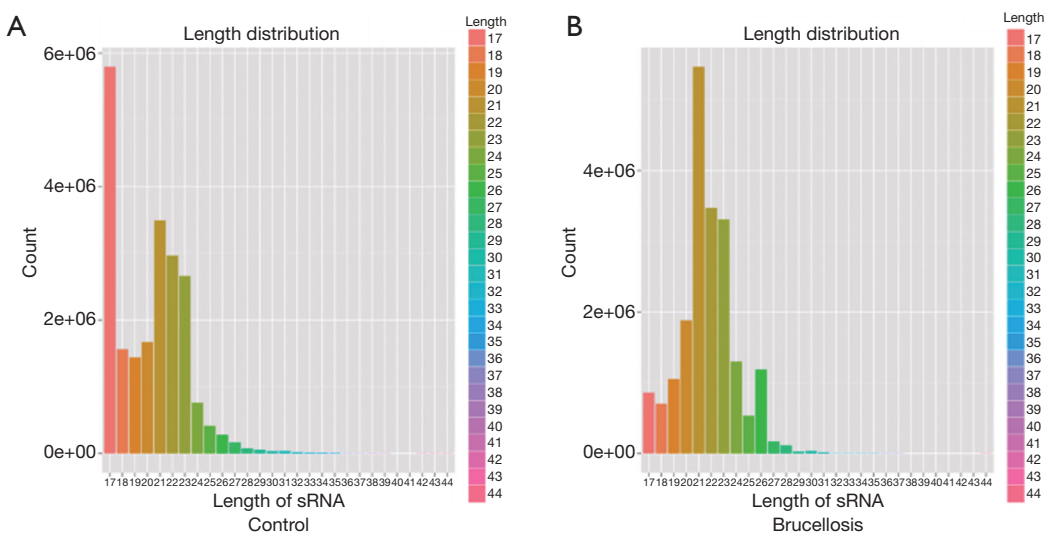

Figure S2 The length distribution of small non-coding RNAs screened by Illumina sequencing via synthesis (SBS) technology in control group (A) and brucellosis group (B).

Table S1 Upregulated miRNAs in the serum sample from brucellosis determined by Illumina SBS technology

miRNA	Count (control)	Count (brucellosis)	Fold change (brucellosis/control)
miR-15a-3p	0	198	10,500
miR-7-2-3p	2	255	96.57143
miR-103b	53	6,873	95.6168
miR-642b-5p	1	107	81
let-7c-5p	206	17,056	60.96156
let-7i-5p	303	20,046	48.71802
miR-4732-5p	62	3,483	41.39462
miR-503-3p	29	1,171	29.69856
miR-1246	66,243	2,162,570	24.04372
miR-142-5p	145	4,673	23.7251
miR-660-5p	4	111	20.27586
miR-98-3p	5	129	19
miR-1284	5	119	17.52778
let-7f-5p	542	11,398	15.48705
miR-26a-2-3p	1,178	24,099	15.06688
miR-26b-3p	63	1,272	14.883
let-7g-5p	636	12,445	14.41228
let-7a-3p	485	8,654	13.13979
miR-7641	346	5,766	12.2743
miR-331-5p	7	114	12.08
let-7a-2-3p	1,440	23,519	12.02846
miR-589-3p	99	1,563	11.63624
miR-3613-3p	45	674	11.02778
miR-194-3p	50	738	10.86667
miR-15b-5p	55	771	10.32071
miR-629-3p	2,204	30,825	10.30059
let-7f-1-3p	1,039	14,329	10.15659
miR-345-5p	20	274	10.08333
miR-3613-5p	23	311	9.927711
let-7b-3p	15,806	200,789	9.356014
let-7a-5p	4,824	60,335	9.211533
miR-16-1-3p	144	1,781	9.111969
miR-183-3p	337	4,153	9.077526
miR-16-5p	215	2,641	9.049127
miR-96-3p	29	342	8.674641
let-7f-2-3p	589	6,860	8.577967
let-7b-5p	26,214	304,148	8.545214
miR-4732-3p	111	1,285	8.524406
let-7g-3p	262	2,979	8.372216
miR-3184-3p	276,762	3,048,029	8.111181
miR-1290	5,205	51,656	7.30921
miR-532-5p	435	4,242	7.181412
miR-101-3p	336	3,197	7.008271
miR-98-5p	33	305	6.822785
miR-182-3p	420	3,884	6.810122
miR-451b	38	347	6.736264
miR-502-3p	252	2,169	6.337927
miR-15b-3p	194	1,642	6.234241
miR-3074-5p	500	4,135	6.091718
miR-183-5p	277	2,190	5.821464
miR-92a-3p	47,316	367,235	5.716203
miR-6866-3p	16	122	5.626087
miR-130b-3p	40	292	5.375
miR-4525	14	101	5.29703
miR-3605-3p	52	360	5.101604
miR-361-5p	85	586	5.075163
miR-486-3p	189,535	1,250,919	4.860838
miR-197-5p	17	105	4.565574
miR-3529-3p	81	471	4.283019
let-7d-5p	624	3,615	4.266533
miR-16-2-3p	1,581	9,097	4.237915
miR-25-3p	19,904	110,993	4.107032
miR-6723-5p	39	210	3.960854
miR-505-5p	53	276	3.839895
miR-128-3p	1,354	6,914	3.760698
miR-6815-3p	24	122	3.739884
miR-30c-5p	69	344	3.668008
miR-323a-3p	32	149	3.434783
miR-224-5p	22	101	3.386076
miR-942-5p	64	294	3.37961
miR-363-3p	1,003	4,494	3.299626
miR-21-5p	1,210	5,342	3.251608
miR-1304-3p	96	411	3.153401
miR-501-3p	4,303	18,339	3.138918
miR-421	32	136	3.134783
miR-10b-5p	680	2,855	3.092154
miR-1294	238	999	3.091068
miR-4488	6,475	27,090	3.081352
miR-181a-5p	432	1,773	3.022837
miR-3138	113	460	2.99877
miR-550a-3-5p	55	224	2.997475
miR-27b-3p	1,687	6,844	2.987975
miR-93-5p	33	129	2.886076
miR-3605-5p	94	348	2.725258
miR-503-5p	75	277	2.718519
miR-199b-3p	81	289	2.627787
miR-99a-3p	582	2,075	2.625686
miR-664a-3p	239	832	2.563953
miR-107	53	184	2.559055
miR-450b-3p	52	177	2.508021
miR-6805-5p	129	436	2.490302
miR-20a-3p	42	138	2.42053
miR-340-3p	45	147	2.404321
miR-451a	70,407	229,653	2.402303
miR-550a-3p	108	342	2.333333
miR-4489	40	125	2.302083
miR-3679-3p	40	123	2.263889
miR-103a-3p	784	2,332	2.19089
miR-30d-3p	10,984	32,574	2.184147
miR-5010-5p	107	317	2.181818
miR-877-3p	249	719	2.126674
miR-22-5p	128	361	2.07709
miR-181b-5p	219	616	2.071701
miR-7-1-3p	90	252	2.061728
miR-92b-3p	618	1,717	2.046088
let-7d-3p	3,341	9,192	2.026284

Table S2 Downregulated miRNAs in the serum sample from brucellosis determined by Illumina SBS technology

miRNA	Count (controls)	Count (brucellosis)	Fold change (brucellosis/control)
miR-1249-3p	604	0	-43.470
miR-4726-5p	105	0	-7,560
miR-3653-3p	255	1	-367
miR-1275	173	1	-249
miR-127-5p	152	1	-218.8
miR-4430b-3p	61,728	433	-190.573
miR-339-5p	1,401	10	-190.245
miR-6852-5p	2,464	20	-167.292
miR-4785	107	1	-154
miR-5187-3p	441	4	-151.143
miR-2277-5p	231	2	-151.091
miR-431-3p	101	1	-145.4
miR-769-3p	6,578	94	-95.0602
miR-4665-5p	2,705	43	-85.386
miR-4665-3p	427	7	-83.0541
miR-4668-5p	298	5	-79.4444
miR-199a-5p	2,860	50	-77.6717
miR-4433a-3p	31,155	572	-73.9512
miR-1250-5p	267	5	-71.1852
miR-378d	1,045	20	-70.9528
miR-6763-5p	311	6	-69.9375
miR-4433b-5p	1,991	39	-69.2232
miR-134-3p	7,264	161	-63.2028
miR-1260b	1,462	37	-53.6837
miR-1181	106	3	-47.6875
miR-3150a	177	5	-47.1852
miR-193b-5p	5,048	148	-46.3393
miR-370-3p	7,843	242	-44.5557
miR-758-3p	157	5	-41.8519
miR-326	4,557	151	-40.995
miR-2355-3p	145	5	-38.6667
miR-766-5p	3,657	135	-36.7584
miR-765	131	5	-34.9259
miR-127-3p	12,420	516	-32.6823
miR-1307-3p	66,275	2,805	-32.0806
miR-4532	254	11	-31.5172
miR-3150a	371	16	-31.4148
miR-483-5p	8,452	372	-30.8458
miR-1307-5p	504	24	-28.5591
miR-450a-5p	104	5	-27.7037
miR-1260a	396	20	-26.8868
miR-4443	432	22	-26.5726
miR-769-5p	1,357	70	-26.3235
miR-1908-5p	8,685	463	-25.4707
miR-129-2-3p	156	9	-23.3958
miR-1268b	384	24	-21.7838
miR-485-5p	1,568	99	-21.4952
miR-3614-5p	669	31	-21.4273
miR-939-5p	3,525	226	-21.1761
miR-378	7,741	508	-20.6873
miR-4709-3p	211	14	-20.527
miR-30c-1-3p	965	64	-20.4867
miR-204-3p	854	58	-20.0195
miR-139-3p	4,900	338	-19.6791
miR-3656	41,971	2,955	-19.2851
miR-4792	177	13	-18.4638
miR-6740-3p	205	15	-18.4375
miR-4479	148	11	-18.3621
miR-450a-2-3p	672	50	-18.2491
miR-642b-3p	146	11	-18.1207
miR-409-3p	4,028	313	-17.4738
miR-873-3p	542	43	-17.1096
miR-4533	122	10	-16.566
miR-23a-5p	1,487	123	-16.4141
miR-300-3p	1,413	119	-16.1157
miR-619-3p	8,032	682	-15.9906
miR-3188	141	12	-15.8594
miR-193a-3p	14,450	1,265	-15.5101
miR-185-3p	14,332	1,256	-15.4944
miR-328-3p	2,026	181	-15.2044
miR-1228-3p	1,069	96	-15.1159
miR-4726-3p	111	10	-15.0755
miR-3928-3p	366	33	-15.0514
miR-129-1-3p	278	25	-15.0451
miR-7704	230	21	-14.9099
miR-26a-5p	7,354	682	-14.6407
miR-485-3p	1,115	104	-14.5644
miR-181c-3p	137	13	-14.2899
miR-6741-5p	892	85	-14.235
miR-133a-3p	420	41	-13.9309
miR-483-3p	24,344	2,377	-13.906
miR-1273g-3p	8,019	788	-13.8166
miR-4419a	35,057	3,447	-13.8089
miR-5187-5p	1,067	106	-13.6637
miR-4748	178	18	-13.4842
miR-6515-5p	401	41	-13.2995
miR-6515-3p	555	57	-13.2252
miR-483-5p	28,431	2,952	-13.0769
miR-1259-1-3p	241	25	-13.0376
miR-654-3p	352	37	-12.9235
miR-27a-5p	502	53	-12.8577
miR-6741-5p	614	65	-12.8087
miR-296-3p	1,340	142	-12.8074
miR-874-3p	1,437	154	-12.674
miR-939-3p	459	50	-12.4642
miR-5585-3p	201	22	-12.3675
miR-151a-3p	223,478	24,848	-12.2116
miR-3916	144	16	-12.1882
miR-1908-3p	2,968	331	-12.1779
miR-345-3p	412	47	-11.9076
miR-8485	847	100	-11.5019
miR-382-5p	718	87	-11.2082
miR-6763-3p	164	20	-11.1321
miR-3130-3p	611	75	-11.0477
miR-193a-5p	8,782	1,090	-10.8366
miR-6852-3p	893	112	-10.8199
miR-1273d	752	95	-10.7381
miR-4508	3,681	469	-10.6565
miR-7847-3p	258	34	-10.5167
miR-574-5p	12,816	1,687	-10.3149
miR-3184-5p	12,792	1,699	-10.2224
miR-6884-3p	339	45	-10.2092
miR-9-3p	214	29	-10
miR-215-5p	293	40	-9.94811
miR-6087	615	84	-9.94807
miR-382-3p	6,201	849	-9.91733
miR-3158-3p	554	79	-9.51551
miR-576-3p	1,250	186	-9.13373
miR-3198	287	43	-8.6614
miR-1273f	11,226	1,726	-8.8308
miR-134-5p	11,504	1,770	-8.82466
miR-619-5p	4,327	688	-8.5388
miR-671-3p	611	98	-8.47206
miR-4750-5p	266	45	-8.00837
miR-375	4,846	825	-7.9753
miR-126b	278	48	-7.87795
miR-4326	190	33	-7.81143
miR-148a-5p	443	78	-7.71913
miR-4647	239	42	-7.713
miR-378c	2,645	474	-7.57803
miR-744-5p	15,854	2,858	-7.53185
miR-296-5p	105	19	-7.48515
miR-3180-5p	100	19	-7.12871
miR-7851-3p	5,549	1,073	-7.02233
miR-744-3p	5,083	997	-6.92185
miR-4734-3p	2,431	477	-6.92089
miR-122-5p	63,192	12,440	-6.89719
miR-3158-5p	1674	332	-6.84545
miR-1273e	2947	587	-6.81742
miR-422a	112	23	-6.60656
miR-184	933	195	-6.4942
miR-28-3p	13,167	2,835	-6.30812
miR-186-3p	13,386	2,941	-6.17987
miR-493-3p	309	69	-6.0765
miR-1273g-5p	343	78	-5.97821
miR-4741	118	27	-5.93706
miR-24-2-5p	249	58	-5.83713
miR-1273h-3p	672	160	-5.70283
miR-760	2,735	659	-5.63527
miR-625-3p	464	113	-5.57429
miR-125a-3p	661	163	-5.50579
miR-566	105	26	-5.47826
miR-320e	469	118	-5.4
miR-766-3p	1,438	363	-5.3789
miR-4459	1,145	290	-5.36109
miR-4734-5p	2,339	599	-5.30205
miR-3929	2,485	640	-5.27241
miR-99a-5p	1,954	514	-5.18263
miR-1273a	98,482	26,768	-4.9954
miR-589-5p	730	199	-4.98009
miR-3648	220	60	-4.97799
miR-378f	128	35	-4.90161
miR-146a-3p	80,324	22,172	-4.93115
miR-3591-3p	269,441	74,203	-4.93027
miR-378a-3p	63,109	17,406	-4.92288
miR-5010-3p	166	46	-4.89754
miR-221-3p	14,041	3,896	-4.89332
miR-145-3p	791	220	-4.8825
miR-6842-3p	588	171	-4.67108
miR-339-3p	1,920	588	-4.43311
miR-3180	328	102	-4.36414
miR-181b-3p	302	94	-4.36345
miR-1254	1,291	407	-4.30737
miR-3922-3p	219	69	-4.30601
miR-320b	109,865	34,667	-4.303
miR-423-3p	963,085	304,710	-4.29148
miR-197-3p	271	86	-4.27832
miR-4429	2,865	906	-4.15622
miR-6640-5p	128	42	-4.13004
miR-15a-5p	1,496	497	-4.0877
miR-186-5p	4,956	1,656	-4.06334
miR-1306-3p	414	139	-4.04342
miR-92b-5p	1,135	378	-4.04042
miR-3187-3p	435	147	-4.01926
miR-1273h-5p	626	214	-3.97266
miR-3691-3p	159	55	-3.91781
miR-6729-3p	104	36	-3.91623
miR-654-5p	293	102	-3.89834
miR-106-3p	3,720	1,309	-3.85889
miR-6740-5p	158	56	-3.82828
miR-1299	589	209	-3.82581
miR-330-3p	723	259	-3.78951
miR-3913-3p	493	178	-3.76246
miR-193b-3p	823	306	-3.65166
miR-3665	400	150	-3.60138
miR-1538	161	62	-3.5228
miR-142-3p	1,738	673	-3.50659
miR-342-3p	5,847	2,283	-3.4774
miR-194-5p	127	50	-3.44906
miR-143-3p	7,336	2,901	-3.4335
miR-5095	181	72	-3.41099
miR-4516	2,313	926	-3.39161
miR-24-3p	3,066	1,253	-3.32219
miR-146b-5p	5,521	2,286	-3.27919
miR-320a	1,554,360	644,710	-3.27353
miR-425-5p	2,488	1,039	-3.2515
miR-4646-3p	514	216	-3.23057
miR-150-5p	298	127	-3.18722
miR-320c	60,757	27,280	-3.02399
miR-3960	472	212	-3.02224
miR-4466	123	56	-2.9798
miR-423-3p	348	159	-2.97153
miR-3180-3p	216	100	-2.93396
miR-5096	663	307	-2.93301
miR-625-5p	168	78	-2.90736
miR-425-3p	4,662	2,168	-2.9196
miR-1259-2-3p	294	137	-2.9146
miR-223-3p	2,185	1,022	-2.9029
miR-30c-2-3p	372	177	-2.89394
miR-532-3p	3,089	1,472	-2.8494
miR-222-3p	12,434	5,958	-2.83063
miR-100-3p	957	467	-2.78263
miR-1292-5p	129	63	-2.77844
miR-1291	155	77	-2.75839
miR-139-5p	445	224	-2.6984
miR-324-3p	282	148	-2.58929
miR-99b-5p	1,793	953	-2.55474
miR-155-3p	328	178	-2.50371
miR-424-3p	670	369	-2.49524
miR-941	2,817	1,571	-2.43473
miR-1224-5p	313	175	-2.4278
miR-129-5p	173	97	-2.42218
miR-146b-3p	2,817	1,580	-2.42078
miR-4689	232	131	-2.40624
miR-181a-2-3p	4,421	2,497	-2.40408
miR-10a-3p	1,402	808	-2.35583
miR-30d-5p	6,051	3,570	-2.30138
miR-2110	20,462	12,102	-2.29571
miR-3656	7,432	4,451	-2.26707
miR-4446-3p	388	235	-2.24077
miR-1-3p	170	104	-2.2196
miR-1285-3p	3,899	2,425	-2.18306
miR-93-3p	541	339	-2.16694
miR-432-3p	256	163	-2.13194
miR-6654-5p	574	373	-2.08953
miR-7110-3p	1,436	934	-2.08746
miR-423-5p	1,044,294	696,892	-2.03463
miR-652-3p	1,799	1,208	-2.02202

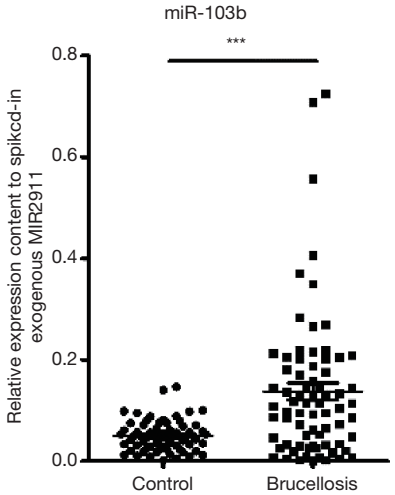

Figure S3 The relative levels of miR-103b in the combined two sets. The relative content of miR-103b was calculated using the $2^{-\Delta CT}$ method. Each P Value was derived from a nonparametric Mann-Whitney U-test. ***, P<0.001.

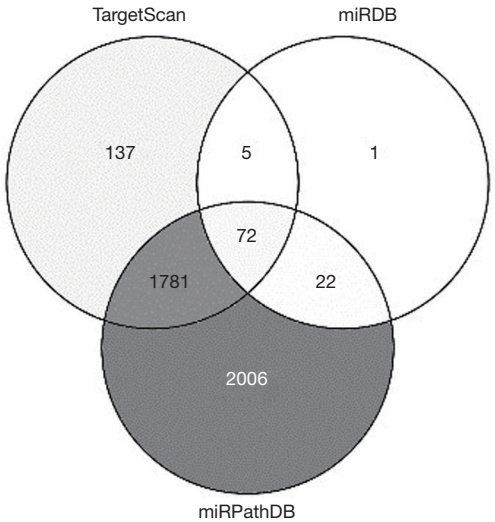

Figure S4 The predicted miR-103b target genes with online miRNA target prediction databases (TargetScan, miRDB, and miRPathDB).