CEOG Clinical and Experimental Obstetrics & Gynecology

miRNA profiling reveals the upregulation of osteogenesis-associated miRNAs in ovariectomy osteoporosis mice

He Hu[1], Guowei Zhang[2], Geng Tian[1], Guang Lv[1], Yongli Jin[3]

[1]Medical College, Inner Mongolia University for the Nationalities, Tongliao City, Inner Mongolia
[2]College of Nursing, Inner Mongolia University for the Nationalities, Tongliao City, Inner Mongolia
[3]The Affiliated Hospital to Inner Mongolia University for the Nationalities, Tongliao City, Inner Mongolia (China)

Summary

Backgrounds: Osteoporosis is a common aging-related degenerative bone disease, particularly in postmenopausal women, that have a reduced estrogen level due to aging. Various factors have been recognized to promote osteoporosis development, such as microRNAs (miRNAs), which regulate the balance between osteogenesis and osteoclasis. Aim: This study was to profile miRNAs in the ovariectomy osteoporosis mice with miRNA profiling array, and to associate the deregulated miRNAs with osteogenesis. *Materials and Methods:* The authors firstly profiled, with miRNAs microarray analysis, the serum miRNAs in a mouse model with postmenopausal osteoporosis, post-ovary excision. Then they validated the dysregulated miRNAs via quantitative real-time polymerase chain reaction (qRT-PCR) method. In addition, these deregulated miRNAs were subjected to a pathway analysis. Results: It was indicated by the present results that in the ovariectomized mice, ten miRNAs were upregulated, such as miR-27a-5p, miR-26a-5p, miR-106a-5p, and miR-133a-5p and the upregulation of miR-26a-5p, miR-133a-1-5, miR-141-5p, miR-200a-5p, and miR-205-5p were validated by the followed qRT-PCR method. In addition, the pathway analysis demonstrated that these miRNAs might inhibit the bone formation via regulating osteogenesis. Conclusions: In conclusion, the authors found the upregulation of miRNAs, which downregulates osteogenesis in a mice model of postmenopausal osteoporosis. The present findings suggest the potential inhibition by miRNAs in osteogenesis in postmenopausal osteoporosis.

Key words: miRNA profiling; Postmenopausal osteoporosis; Osteogenesis; Mice model.

Introduction

Osteoporosis is a common disease characterized by bone loss and bone fragility leading to an increased risk of fracture at the wrist, spine, hip, and proximal humerus [1, 2]. The incidence of osteoporosis in postmenopausal women is high, that have a reduced estrogen level due to aging and continuous calcium loss, and causes an imbalance between bone resorption by osteoclasts and bone formation by osteoblasts [3]. Millions of females over the age of 50 are afflicted by osteoporosis worldwide, especially in China with large aging population, and the incidence of osteoporosis increases over time gradually [4]; therefore the prevention and diagnosis of osteoporosis is very important. It has been reported that the occurrence of osteoporosis is associated with many factors, such as SMAD4, CACNG1, and TRIM63, and miR-331 could be a potential biomarker for osteoporosis [5-8]. Because osteoporosis does not have any clinical symptoms until bone fracture, it requires more attention on the molecular mechanisms, and more potential biomarkers need to be found for the prediction and prevention of it.

MicroRNA (miRNA) is a kind of small, non-coding, single-stranded RNA, commonly contains 18 to 23 nucleotides. miRNAs are present in the genome in the form of single copy, multi copy or gene cluster, and most of them are located in the intergenic spacer. In the past 20 years, there are ,many studies about miRNAs, and researchers found that miRNAs expression profiling has proven useful in diagnosing and understanding the development and progression of many diseases, such like cancer [9, 10], endometriosis [11], Alzheimer's disease [12], and chronic hepatitis B [13]. It also has been reported that miRNAs are potential biomarkers for osteogenesis and osteoclasis [14, 15]. The expression level of miR-503 is reduced in postmenopausal osteoporosis patients; otherwise, if the miR-503 is silenced in vivo, the level of RANK is increased, therefore miR-503 could regulate osteoporosis by targeting RANK [16]. Moreover, the miR-27a plays an important role in bone formation [17]. Most of the studies are about the single molecular miRNA in osteoporosis, and they need to screen more miRNAs which are dysregulated in osteoporosis. miRNA microarray analysis is a multiplex lab-on-a-chip; there are amounts of nucleic acid probe on the chip, which could high-throughput miRNA molecular screening [18-20]. In this study, the authors screened the serum miRNAs in postmenopausal osteoporosis patients by miRNA

Revised manuscript accepted for publication October 10, 2016

Table 1. — *Information about miRNA probes utilized in the present study*

All_Transcript ID#	Alignments	Sequence Length	Sequence	Probe target
miR-27a-5p	chr8:84208685-84208706 (+)	22	AGGGCUUAGCUGCUUGUGAGCA	MIMAT0004633
miR-26a-5p	chr10:126995543-126995564 (+) / chr9:119031811-119031832 (+)	22	UUCAAGUAAUCCAGGAUAGGCU	MIMAT0000533
miR-106a-5p	chrX:52742541-52742563 (-)	23	CAAAGUGCUAACAGUGCAGGUAG	MIMAT0000385
miR-133a-5p	chr18:10782950-10782970 (-) / chr2:180398401-180398421 (+)	21	GCUGGUAAAAUGGAACCAAAU	MIMAT0003473
miR-137-5p	chr3:118433865-118433887 (+)	23	ACGGGUAUUCUUGGGUGGAUAAU	MIMAT0016986
miR-141-5p	chr6:124717959-124717980 (-)	22	CAUCUUCCAGUGCAGUGUUGGA	MIMAT0004533
miR-182-5p	chr6:30165962-30165986 (-)	25	UUUGGCAAUGGUAGAACUCACACCG	MIMAT0000211
miR-200a-5p	chr4:156054949-156054970 (-)	22	CAUCUUACCGGACAGUGCUGGA	MIMAT0004619
miR-205-5p	chr1:193507503-193507524 (-)	22	UCCUUCAUUCCACCGGAGUCUG	MIMAT0000238
miR-214-5p	chr1:162223397-162223418 (+)	22	UGCCUGUCUACACUUGCUGUGC	MIMAT0004664

Table 2. — *Up-regulated serum miRNAs in the ovariectomized or control mice*

All_Transcript ID #	All_Accession $	Control *	Osteoporosis	*t* test p-value	FDR p-value & (All Conditions)
miR-27a-5p	MIMAT0004633	3.96	4.54	0.034	0.990
miR-26a-5p	MIMAT0000533	7.30	8.78	0.005	0.910
miR-106a-5p	MIMAT0000385	8.53	9.39	0.024	0.810
miR-133a-5p	MIMAT0003473	3.12	3.50	0.030	0.905
miR-137-5p	MIMAT0016986	7.68	8.45	0.018	0.809
miR-141-5p	MIMAT0004533	2.46	3.44	0.005	0.809
miR-182-5p	MIMAT0000211	3.27	3.92	0.032	0.924
miR-200a-5p	MIMAT0004619	2.49	3.37	0.005	0.912
miR-205-5p	MIMAT0000238	6.20	7.04	0.008	0.681
miR-214-5p	MIMAT0004664	6.98	8.29	0.003	0.655

#: all_Transcript ID(Array Design): miRNA name in miRBase; $: all_Accession: accession number of miRNA in miRBase

**: geometric average of probe signal value (log2); &: False Discovery Rate*

microarray analysis, to find the dysregulated miRNA, and to further validate it through the quantitative real-time polymerase chain reaction (qRT-PCR) method. This study has provided new osteoporotic biomarkers to identify postmenopausal women who are at high risk for developing osteoporosis.

Materials and Methods

Mice experiments were performed according to the Principles of Laboratory Animal Care (National Institutes of Health publication number 85-23, revised 1985). The osteoporotic mice model with ovary excision was utilized to mimic the postmenopausal women with osteoporosis. Eight-week-old female Balb/C mice underwent ovariectomy surgery under general anesthesia. The control group of mice also underwent similar surgery under general anesthesia without ovariectomy. Twelve weeks post-ovariectomy surgery, osteoporosis was confirmed by pathological examination.

To investigate the serum level of miRNAs, the serum samples were collected from five ovariectomized mice with osteogenesis and five from normal mice. The total miRNAs were extracted from the serum with Trizol according to the manufacturer's specification. The extraction of miRNA samples were kept at -80 °C before utilization.

Total miRNAs from the serum were analyzed by miRNA microarray. A spectrophotometer was used to examine the concentration and purity of the miRNAs, the optical density of 1.8 to 2.0 under OD260/280 were accepted to microarray. Total miRNAs were labeled with cyanine 3-CTP by the miRNA labeling kit, according to the manufacturer's instruction. Each miRNAs sample was mixed into the miRNA hybridization buffer, after purification, the miRNAs were competitively hybridized to a affymetrix microRNA 4.0 array containing 30,424 mature miRNA (all species), according to the manufacturer's protocol manual.

The hybridization signal was scanned, and the signal intensity were analyzed by ScanArray 3.0 software, and the final data were calculated by log2 transformation of the normalized data. Target genes for miRNAs were respectively predicted by Targetscan and Miranda databases and were selected from the intersected target genes from the two databases.

One Step SYBR primescript RT-PCT kit was used to quantity the relative serum level of miR-27a- 5p, miR-26a-5p, miR-106a-5p, miR-133a-5p, miR-137-5p, miR- 141-5p, miR-182-5p, miR-200a-5p, miR-205-5p, and miR-214-5p in ovariectomized mice and in normal mice, following the product's manual. The probe of each miRNA is shown Table 1. The results were calculated and presented as relative level by ΔΔ Ct method and all these miRNAs were normalized to U6 (as internal control). All the experiments were performed three times inde-pendently.

The geometric average of probe signal value from the microar-

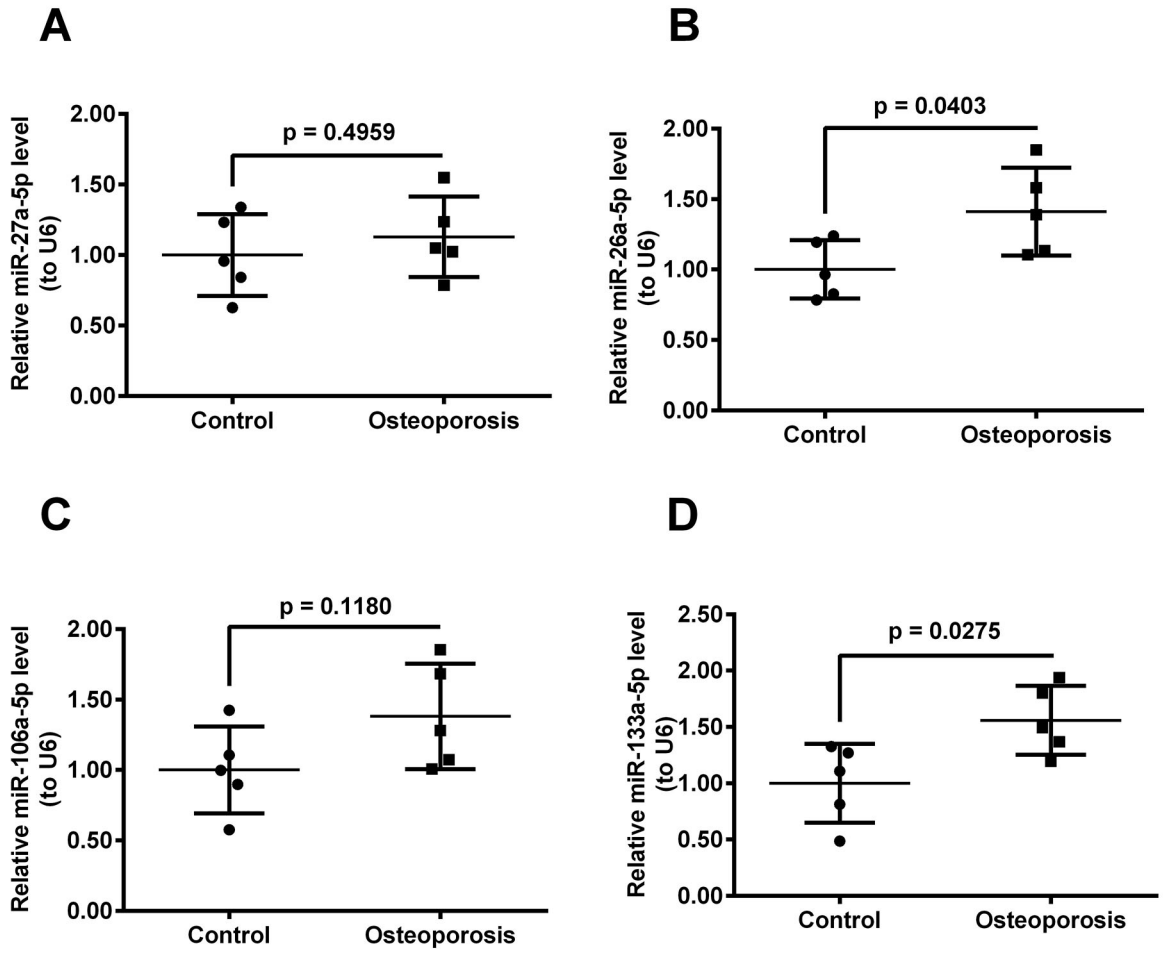

Figure 1. — Relative serum levels of miR-27a-5p, miR-26a-5p, miR-106a-5p and miR-133a-5p in ovariectomized mice.
The relative levels of miR-27a-5p (A), miR-26a-5p (B), miR-106a-5p (C) and miR-133a-5p (D) were examined by RT-qPCR in the serum of ovariectomized mice (n = 5), The level of each miRNA was presented as a relative value to the control sample (normal mice serum, n = 5). Statistical significance were shown respectively. $p<0.05$(*), $p<0.01$(**) or no significance (ns).

ray were estimated using the Student's t-test, otherwise, the False Discovery Rate (FDR) of all conditions were also analyzed. GraphPad Prism v6.0 was used to analyze the qRT-PCR data. The correlation between two groups was analyzed by Student's t-test. The results were considered statistically significant at the level of $p < 0.05$ or less.

Results

In order to investigate the dysregulated serum miRNAs in the ovariectomized mice, the authors compared the serum miRNA level between the ovariectomized mice and the normal mice by miRNAs microarray analysis. According to the hybridization signal intensity, they found ten miRNAs' serum level that were up-regulated in the ovariectomized mice, compared to the control mice. The transcript ID of these ten miRNAs were miR-27a-5p, miR-26a-5p, miR-106a-5p, miR-133a-5p, miR-137-5p, miR-141-5p, miR-182-5p, miR-200a-5p, miR-205-5p, and miR-214-5p (in miRBase database). The t-test p-value of each miRNA was lower than 0.05, meanwhile, the FDR p-value of all the conditions was much higher than 0.05. The detailed data are shown in Table 2.

Based on the result of the miRNA microarray analysis, ten miRNAs serum level were abnormal in ovariectomized mice; to further confirm the up-regulated miRNAs in osteoporosis mice, the authors used qRT-PCR assay to verify the ten miRNAs again, and with U6 as internal control. The detail information about these miRNAs are displayed in Table 2, containing the sequence and the probes. The miRNAs were extracted from the osteoporotic mice post-ovary excision (n=5) and from normal mice (n=5). The relative serum levels of miR-27a-5p, miR-26a-5p, miR-106a-5p,

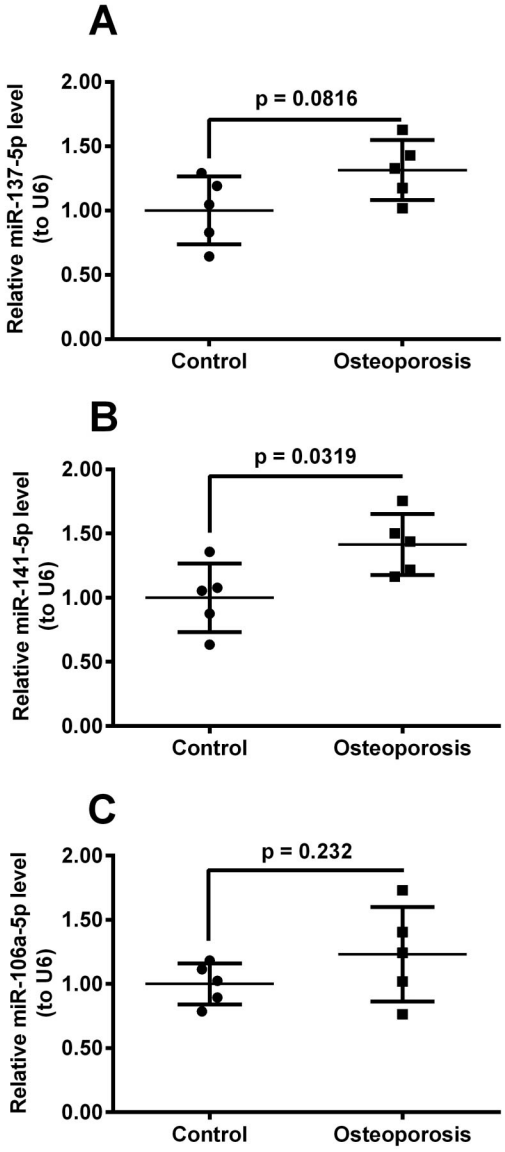

Figure 2. — Relative serum levels of miR-137-5p, miR-141-5p and miR-182-5p, in ovariectomized mice.
The relative levels of miR-137-5p, (A), miR-141-5p (B) and miR-182-5p (C) were examined by RT-qPCR in the serum of ovariectomized mice (n = 5), The level of each miRNA was presented as a relative value to the control sample (normal mice serum, n = 5). Statistical significance were shown respectively. p<0.01(**), p<0.001(***) or no significance (ns).

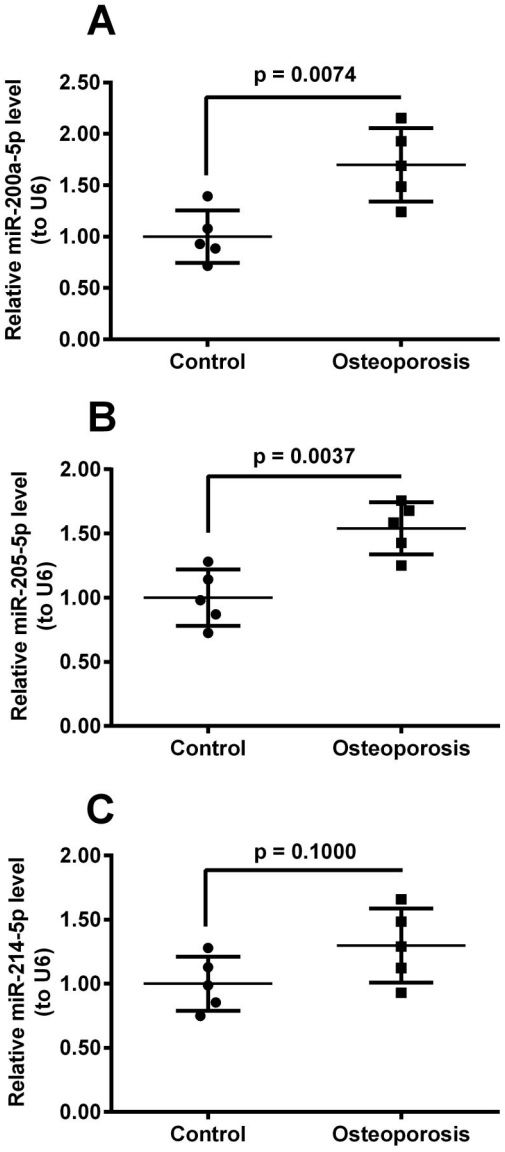

Figure 3. — Relative serum levels of miR-200a-5p, miR-205-5p and miR-214-5p in ovariectomized mice.
The relative levels of miR-200a-5p (A), miR-205-5p (B) and miR-214-5p (C) were examined by RT-qPCR in the serum of ovariectomized mice (n = 5), The level of each miRNA was presented as a relative value to the control sample (normal mice serum, n = 5). Statistical significance were shown respectively. p<0.01(**) or no significance (ns).

and miR-133a-5p were confirmed by the qRT-PCR assay. The results are shown in Figure 1. The relative serum level of miR-26a-5p was up-regulated in osteoporotic mice postovary excision (Figure 1B, p = 0.0403). Figure 1D demonstrates that the relative level of miR-133a-5p in ovariectomized mice was much higher than in control mice (p = 0.0275). However, the relative level of miR-27a-5p (p = 0.4959) and miR-106a-5p (p = 0.1180) showed no significant difference between the ovariectomized mice and control (Figures 1A and 1C).

As Figure 2B shows, the level of miR-141-5p was significantly increased in osteoporotic mice (p = 0.0319); nevertheless, miR-137-5p and miR-182-5p, the expression levels were not statistically significant between the osteo-

porotic and normal mice (Figure 2A: p = 0.0816; Figure 2C: p = 0.232). The serum level of miR-200a-5p (p = 0.0074) and miR-205-5p (p = 0.0037) were remarkably up-regulated in osteoporotic mice (Figures 3A and 3B); moreover, the change of miR-214-5p were not significant (Figure 3D: p = 0.1000).

Discussion

Osteoporosis increases the risk of fracture in postmenopausal women, usually with decreased bone strength and low bone mineral density (BMD) [21]. MicroRNAs are important post-transcriptional molecules that regulate gene expression in various of physiological process. There are many studies that have reported that miRNAs are critical pathological factor in bone-related diseases; they could regulate the process of bone formation, remodeling, and degeneration [22, 23]. miRNAs microarray analysis could detect hundreds of miRNAs in a high-throughput way and has been confirmed widely [24, 25]. In the present study, the osteoporotic mice model with ovary excision was used to investigate the abnormally expressed miRNAs in serum by miRNAs microarray analysis. The analysis of the microarray results prove that there were ten miRNAs up-regulated in the serum of osteoporosis mice. The ten miRNAs, respectively, are miR-27a-5p, miR-26a-5p, miR-106a-5p, miR-133a-5p, miR-137-5p, miR-141-5p, miR-182-5p, miR-200a-5p, miR-205-5p, and miR-214-5p (Table 1). This result demonstrated that the expression serum level of these miRNAs are dysregulated in osteoporosis serum, compared to the normal mice, and they might be potential biomarkers for postmenopausal osteoporosis. However, the shortness of microarray analysis has a high false positive rate. Therefore, the present authors chose qRT-PCR method to further validate the serum level of the ten miRNAs identified by microarray. In the qRT-PCR, there were five miRNAs' serum level that indeed increased, meanwhile the change of the serum level of the other miRNAs was not significance. The up-regulated miRNAs were miR-26a-5p, miR-133a-1-5, miR-141-5p, miR-200a-5p, and miR-205-5p. A previous study has reported that the miR-133 can induce postmenopausal osteoporosis by repressing SLC39A1 expression and weaken osteogenic differentiation of hMSCs [7]. miR-26a-5p could inhibit the proliferation, migration, and invasion of tumor cells [26]. Therefore, the present authors suspect that the up-regulation of the miR-26a-5p, miR-133a-1-5, miR-141-5p, miR-200a-5p, and miR-205-5p, may inhibit osteogenesis and result in weakening bone formation and low bone density, and finally causes osteoporosis.

The current results showed that the change of serum level (miR-26a-5p, miR-133a-1-5, miR-141-5p, miR-200a-5p, and miR-205-5p) could predict the occurrence of osteoporosis, but the detailed molecular mechanisms of these miRNAs are still unclear. The present findings suggest the potential inhibition by miRNAs in osteogenesis in postmenopausal osteoporosis.

Conclusion

In conclusion, the present study detected the dysregulated miRNAs in osteoporotic mice model by microarray analysis, and followed by qRT-PCR validation. Through identification of these two methods, the authors found five miRNAs that were indeed up-regulated in osteoporosis mice model with ovary excision: miR-26a-5p, miR-133a-1-5, miR-141-5p, miR-200a-5p, and miR-205-5p. The discovery of the regulatory molecules is profound in the prevention and treatment of osteoporosis.

Acknowledgement

The present study was supported by the grant from the Youth Project in Inner Mongolia University for the Nationalities (INUN_2015_03).

References

[1] Huang C., Li S.: "Association of blood neutrophil lymphocyte ratio in the patients with postmenopausal osteoporosis". *Pak. J. Med. Sci.*, 2016, *32*, 762.
[2] Tella S.H., Gallagher J.C.: "Prevention and treatment of postmenopausal osteoporosis". *J. Steroid Biochem. Mol. Biol.*, 2014, *142*, 155.
[3] Meng J., Zhang D., Pan N., Sun N., Wang Q., Fan J., et al.: "Identification of miR-194-5p as a potential biomarker for postmenopausal osteoporosis". *Peer J.*, 2015, *3*, e971.
[4] Baccaro L.F., Conde D.M., Costa-Paiva L., Pinto-Neto A.M.: "The epidemiology and management of postmenopausal osteoporosis: a viewpoint from Brazil". *Clin. Interv. Aging*, 2015, *10*, 583.
[5] Liu Y., Wang Y., Yang N., Wu S., Lv Y., Xu L.: "In silico analysis of the molecular mechanism of postmenopausal osteoporosis". *Mol. Med. Rep.*, 2015, *12*, 6584.
[6] Ozmen B., Kirmaz C., Aydin K., Kafesciler S.O., Guclu F., Hekimsoy Z.: "Influence of the selective oestrogen receptor modulator (raloxifene hydrochloride) on IL-6, TNF-alpha, TGF-beta1 and bone turnover markers in the treatment of postmenopausal osteoporosis". *Eur. Cytokine Netw.*, 2007, *18*, 148.
[7] Zhang Y.: "MiR-133 is involved in estrogen Deficiency-Induced osteoporosis through modulating osteogenic differentiation of mesenchymal stem cells". *Med. Sci. Monitor*, 2015, *21*, 1527.
[8] Wang Y., Li L., Moore B.T., Peng X.H., Fang X., Lappe J.M., *et al.*: MiR-133a in human circulating monocytes: a potential biomarker associated with postmenopausal osteoporosis". *PLoS One*, 2012, *7*, e34641.
[9] Kadera B.E., Li L., Toste P.A., Wu N., Adams C., Dawson D.W., Donahue T.R.: 'MicroRNA-21 in pancreatic ductal adenocarcinoma tumor-associated fibroblasts promotes metastasis". PLoS One, 2013; 8, e71978.
[10] Bertoli G., Cava C., Castiglioni I.: "MicroRNAs: new biomarkers for diagnosis, prognosis, therapy prediction and therapeutic tools for breast cancer". *Theranostics*, 2015, *5*, 1122.
[11] Cho S., Mutlu L., Grechukhina O., Taylor H.S.: "Circulating microRNAs as potential biomarkers for endometriosis". *Fertil. Steril.*, 2015, *103*, 1252.
[12] Bekris L.M., Leverenz J.B.: 'The biomarker and therapeutic potential of miRNA in Alzheimer's disease". *Neurodegener. Dis. Manag.*, 2015, *5*, 61.

[13] Tan Y., Ge G., Pan T., Wen D., Gan J.: "Serum MiRNA panel as potential biomarkers for chronic hepatitis B with persistently normal alanine aminotransferase". *Clin. Chim. Acta*, 2015, *451*, 232.
[14] Chen J., Qiu M., Dou C., Cao Z., Dong S.: "MicroRNAs in bone balance and osteoporosis". *Drug Dev. Res.*, 2015, *76*, 235.
[15] Dong S., Yang B., Guo H., Kang F.: "MicroRNAs regulate osteogenesis and chondrogenesis". *Biochem. Biophys. Res. Commun.*, 2012, *418*, 587.
[16] Chen C., Cheng P., Xie H., Zhou H., Wu X., Liao E., Luo X.: "MiR-503 regulates osteoclastogenesis via targeting RANK". *J. Bone Miner. Res.*, 2014, *29*, 338.
[17] You L., Pan L., Chen L., Gu W., Chen J.: "MiR-27a is essential for the shift from osteogenic differentiation to adipogenic differentiation of mesenchymal stem cells in postmenopausal osteoporosis". *Cell Physiol. Biochem.*, 2016, *39*, 253.
[18] Nishida N., Nagahara M., Sato T., Mimori K., Sudo T., Tanaka F., *et al.*: "Microarray analysis of colorectal cancer stromal tissue reveals upregulation of two oncogenic miRNA clusters". *Clin. Cancer Res.*, 2012, *18*, 3054.
[19] Murakami Y., Tanahashi T., Okada R., Toyoda H., Kumada T., Enomoto M., *et al.*: "Comparison of hepatocellular carcinoma miRNA expression profiling as evaluated by next generation sequencing and microarray". *PLoS One*, 2014, *9*, e106314.
[20] Wang H.: 'Predicting microRNA biomarkers for cancer using phylogenetic tree and microarray analysis". Int. J. Mol. Sci., 2016, 17, 773.
[21] Stevenson M., Jones M.L., De Nigris E., Brewer N., Davis S., Oakley J.: "A systematic review and economic evaluation of alendronate, etidronate, risedronate, raloxifene and teriparatide for the prevention and treatment of postmenopausal osteoporosis". *Health Technol. Assess.*, 2005, *9*, 1.
[22] van Wijnen A.J., van de Peppel J., van Leeuwen J.P., Lian J.B., Stein G.S., Westendorf J.J., *et al.*: "MicroRNA functions in osteogenesis and dysfunctions in osteoporosis". *Curr. Osteoporos. Rep.*, 2013, *11*, 72.
[23] Khuu C., Utheim T.P., Sehic A.: "The three paralogous microRNA clusters in development and disease, miR-17-92, miR-106a-363, and miR-106b-25". *Scientifica*, 2016, *2016*, 1379643.
[24] Ries J., Vairaktaris E., Agaimy A., Kintopp R., Baran C., Neukam F.W., Nkenke E.: "miR-186, miR-3651 and miR-494: potential biomarkers for oral squamous cell carcinoma extracted from whole blood". *Oncol. Rep.*, 2014, *31*, 1429.
[25] Hausler S.F., Keller A., Chandran P.A., Ziegler K., Zipp K., Heuer S., *et al.*: "Whole blood-derived miRNA profiles as potential new tools for ovarian cancer screening". *Br. J. Cancer,* 2010, *103*, 693.
[26] Guo K., Zheng S., Xu Y., Xu A., Chen B., Wen Y.: "Loss of miR-26a-5p promotes proliferation, migration, and invasion in prostate cancer through negatively regulating SERBP1". *Tumour Biol.*, 2016, *37*, 12843.

Corresponding Author:
HE HU, M.D.
No. 996, West Lamulun Street
Tongliao City
Inner Mongolia, 028041 (China)
e-mail: huhe8080@sohu.com