Original Article

Urinary miRNAs as biomarkers for idiopathic osteonecrosis of femoral head: A multicentre study

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ABSTRACT

Objectives: Urinary microRNAs (miRNAs) have shown great diagnostic and prognostic values for multiple diseases. The profile of urinary miRNAs in patients with idiopathic osteonecrosis of femoral head (ONFH) is currently unclear.

Methods: We first randomly chose ten patients with each Association Research Circulation Osseous (ARCO) stage (I, II, III and IV) and ten healthy participants from the entire cohorts for initial screening. The miRNA polymerase chain reaction (PCR) array was then performed to identify the differentially abundant miRNAs in urine of these participants. We then verified the findings in the entire cohort. Clinical features including age, gender, bone mass index (BMI), lesion size and stages were recorded. We then analysed the association between the level of urinary miRNAs and clinical features.

Results: Our data indicated that there were 13 differentially abundant miRNAs among all groups. Urinary miR-150 demonstrated the highest diagnostic value among all candidates. Urinary miR-185 and miR-133a increased by ARCO staging. The levels of urinary miR-4824 abruptly decreased after femoral head collapse (ARCO stage III and IV). Urinary miR-144 was the only marker that correlated with lesion size.

Conclusions: The levels of urinary miRNAs are valuable biomarkers for idiopathic ONFH. Given the noninvasive nature of this test, it is potentially useful for diagnosis and monitoring of idiopathic ONFH progression.

The translational potential of this article: This article gives novel methods for ONFH diagnosis and progression monitoring in a convenient and non-invasive way.

Introduction

Osteonecrosis of femoral head (ONFH) is a devastating disease and an increasing worldwide health problem [1,2]. ONFH is characterised by the necrosis of trabecular bones caused by loss of blood supply, which ultimately leads to the collapse of subchondral bone and limitation on limb motion [1,3]. The development of ONFH is generally attributed to genetic predisposition and risk factor exposure [4]. The risk factors of osteonecrosis include corticosteroid usage, alcohol intake, trauma, chemotherapy and antiretroviral therapy [3,5-10]. Some people without risk factor exposure can also develop ONFH, namely, idiopathic ONFH. Idiopathic ONFH accounted for 15–40% of overall ONFH patients according to previous reports [11-13].

miRNAs are a group of noncoding small RNAs containing approximately 22 nucleotides, which silence complementary target mRNAs. Many previous reports have shown that extracellular miRNAs are stable in biological fluids, including blood, urine and cerebrospinal fluid, and associated with multiple diseases [14-17]. An urinary miRNA test might be more superior to those tests on other fluids because urine could be collected noninvasively. The profile of urinary miRNAs in ONFH patients is currently unknown. It is also interesting to compare the urinary profile with the serum profile revealed by a previous study [18]. miRNAs play important roles in many aspects of ONFH pathogenesis. For example, Jia et al reported that miR-17-5p expression level was lower in mesenchymal stem cells (MSCs) isolated from ONFH patients and inhibition of miR-17-5p contributed to ONFH pathogenesis by limiting...
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MicroRNA Purification kit (Norgen Biotek, Canada) according to the manufacturers’ instructions. The concentration of RNA was detected by Nanodrop2000 and RNA samples were then stored at –80 °C for further processing. Total RNA (300 ng) was reversely transcribed into cDNA using the miScript II RT kit (Qiagen). The custom applied biosystems (ABI) TaqMan MicroRNA Array kit (ThermoFisher, 1617 miRNAs) was used to detect miRNA levels by real-time PCR. The concentrations of the miRNAs were normalised to spike-in control in onchop-the ABI 7900HT (Applied Biosystems) was used in this study and data were analysed with SDS 2.4 (Applied Biosystems).

**Data and statistics**

All data were analysed using software SPSS22.0. Data were represented as mean ± SD. All statistical significance was defined as p < 0.05. Analysis of Receiver Operating Characteristic (ROC) curves was performed to calculate the area under the curve (AUC) value along with standard error and 95% confidence intervals (CIs). ROC curves were considered significant with an AUC value of >0.8 and a p value of <0.05.

**Results**

**Patient clinical information**

In the initial screening phase, we first randomly chose ten patients for each ARCO stage (I, II, III and IV) and ten healthy participants as control from the entire cohort. Age, BMI and lesion size did not vary by stages in this study. All urinary samples were collected immediately after diagnosis of idiopathic ONFH.

**Initial screening and ranking of candidate miRNAs**

We screened 1617 urinary miRNAs in all urine samples. However, the levels of 556 miRNAs were undetectable in urine by real-time PCR. We therefore only included 1061 miRNAs (Supplementary Table 1) for final analysis. Our data indicated that there were 13 differentially abundant miRNAs including miR-150, miR-185, miR-133a, miR-144, miR-4284, miR-124, miR-107, miR-1184, miR-1304, let-7c, miR-521, miR-502-3p and miR-32 among all groups with p < 0.05 (Table 1 and Figure 1).

**Urinary miRNAs for diagnosis of idiopathic ONFH**

The fold change served as the major criterion for candidate miRNAs as diagnostic markers. Greater fold change values are more likely to ensure a great power for diagnosing the disease. In addition, the marker should either increase or decrease in all stages compared with control. Specifically, the criterion for further verification in the entire cohort was that the mean fold change of every stage was more than 1.5 compared with the control group. According to the results of screening, we chose miR-150, miR-185, miR-133a and miR-144 for further investigation. The diagnostic power was assessed using AUC calculated through ROC analysis (Table 2 and Figure 2). Urinary miR-150 showed the highest diagnostic power (AUC = 0.94, 95% CI = 0.91–0.98). The optimal cut-off for miR-150 was 1.45 with sensitivity at 87.1% and specificity at 92.7%.

**Association between urinary miRNAs and stages of idiopathic ONFH**

We then explored the association between urinary miRNAs and stages of idiopathic ONFH to seek biomarkers, which could monitor the progression of ONFH. Notably, we additionally chose miR-4284 for verification because it abruptly decreased after femoral head collapse (ARCO stage III and IV), a milestone of ONFH progression. The basic information of each group is summarised in Table 3. Urinary miR-185 and miR-133a increased by ARCO staging (miR-185: stage I = 1.64 ± 0.47, stage II = 1.91 ± 0.42, stage III = 1.96 ± 0.57 and stage IV = 2.22 ± 0.51; miR-133a: stage I = 1.56 ± 0.33, stage II = 1.72 ± 0.66, stage
ROC analysis for diagnostic power of four candidate miRNAs.

Table 1

| Patient characteristics | Control | Stage I | Stage II | Stage III | Stage IV | p value |
|-------------------------|---------|---------|----------|-----------|----------|---------|
| Male                    | 5       | 6       | 4        | 4         | 6        | 0.81    |
| Female                  | 5       | 4       | 6        | 6         | 4        | 0.82    |
| Age (years)             | 31.0 ± 8.8 | 32.9 ± 10.1 | 35.8 ± 7.7 | 33.6 ± 9.3 | 34.5 ± 9.7 | 0.86    |
| BMI                     | 23.7 ± 4.3 | 20.2 ± 6.9 | 22.9 ± 7.4 | 22.2 ± 8.8 | 22.2 ± 8.1 | 0.58    |
| Lesion size (%)         | N/A     | 22.5 ± 8.3 | 19.8 ± 7.6 | 23.85 ± 7.1 | 24.2 ± 5.9 | 0.54    |

Fold change of urinary miRNAs

- miR-150: 1.00 ± 0.42
- miR-185: 1.00 ± 0.37
- miR-133a: 1.00 ± 0.45
- miR-144: 1.00 ± 0.39
- miR-4284: 1.00 ± 0.57
- miR-124: 1.00 ± 0.56
- miR-107: 1.00 ± 0.55
- miR-1184: 1.00 ± 0.51
- miR-1304: 1.00 ± 0.41
- let-7c: 1.00 ± 0.45
- miR-521: 1.00 ± 0.48
- miR-502-3p: 1.00 ± 0.55
- miR-32: 1.00 ± 0.45

Table 2

| Gene name | AUC | p value | 95% confidence interval |
|-----------|-----|---------|------------------------|
| miR150    | 0.94 | <0.01  | 0.91-0.98              |
| miR185    | 0.93 | <0.01  | 0.87-0.95              |
| miR133a   | 0.89 | <0.01  | 0.82-0.93              |
| miR144    | 0.25 | <0.01  | 0.15-0.36              |

Association between urinary miRNAs and lesion size of idiopathic ONFH

We then analysed the association between urinary miRNAs and lesion size of idiopathic ONFH (Table 4). A linear regression was conducted and we found a strong correlation between miR-144 level and lesion size (R2 = 0.52, p < 0.01, Figure 3). The level of urinary miR-144 significantly decreased by the lesion size.

Table 2

ROC analysis for diagnostic power of four candidate miRNAs.

Figure 1. Heatmap of 13 differentially expressed miRNAs.

III = 1.93 ± 0.49 and stage IV = 2.02 ± 0.41). Consistent with initial screening, the levels of urinary miR-4824 abruptly decreased after femoral head collapse (ARCO stage III and IV).

Discussion

As we mentioned above, previous studies indicated that miRNAs played important roles in the development of ONFH in many different aspects. Several previous studies [35–37] performed miRNA microarray to profile miRNA expression in the serum of ONFH and the serum of healthy controls. However, all these studies did not verify their findings in a large cohort. In this study, we not only screened the differentially expressed miRNAs but also verified the results in a multicentre cohort (consisting of 155 patients). Interestingly, differentially abundant miRNAs in serum were not consistent with those in urine. The mechanism causing this difference remains unclear.

Hip-preserving treatment requires a pre-collapse femoral head (ARCO stage I and II) for therapeutic success [38]. However, the ONFH is a latent disease before collapse and stage-I and stage-II ONFH are usually asymptomatic. MRI is still the most conclusive method for diagnosis of ONFH because stage-I ONFH could not be detected by CT scanning and radiograph. However, the cost of MRI scanning is high causing a heavy burden on healthcare system [24–26], highlighting the need for a convenient laboratory test to detect ONFH development. To our...
knowledge, urinary miR-150 showed the highest diagnostic power among all known biomarkers for idiopathic ONFH [10,39–44].

Multiple studies on nonoperative treatments for small-lesion and pre-collapse ONFH demonstrate promising results [45–48]. However, monitoring the progression of ONFH is challenging for these patients and mostly relied on repeated MRI and CT scanning. In this study, we showed that urinary miR-185 and miR-133a increased by ARCO staging. In addition, we showed that the levels of urinary miR-4824 abruptly decreased after femoral head collapse, the milestone of ONFH progression. It is highly possible that these three urinary miRNAs could be a biomarker for monitoring ONFH progression. Follow-up and consecutive assessment on these miRNAs in a cohort of patients who received nonoperative treatment should be performed for further confirmation.

Another interesting finding is that there was a strong correlation between miR-144 level and lesion size. Lesion size is a key factor for clinical decision-making because 7% of small and 80% of large lesions resulted in a collapsing by eight years [10]. Current lesion size assessment requires MRI scanning and prolonged analysis. A laboratory test is clearly more convenient and urinary miR-144 is a promising candidate for lesion size measurement after further evaluation on its prognostic value.

### Table 3
Level of five candidate urinary miRNAs by idiopathic ONFH staging.

| Patient characteristics | Control (n = 41) | Stage I (n = 41) | Stage II (n = 36) | Stage III (n = 43) | Stage IV (n = 35) | p value |
|-------------------------|-----------------|-----------------|------------------|-------------------|------------------|---------|
| Male        | 23              | 21              | 19               | 19                | 17               | 0.86    |
| Female      | 18              | 20              | 17               | 24                | 18               | 0.59    |
| Age (years) | 33.8 ± 8.4      | 34.8 ± 9.5      | 36.4 ± 8.3       | 33.4 ± 8.8        | 33.6 ± 10.2      | 0.27    |
| BMI         | 22.1 ± 4.9      | 20.2 ± 6.4      | 23.8 ± 7.0       | 22.3 ± 8.6        | 22.4 ± 7.8       | 0.33    |
| Lesion size (%) | N/A            | 23.8 ± 7.8      | 22.6 ± 5.7       | 25.2 ± 6.0        | 24.5 ± 5.8       | 0.33    |
| Fold change of urinary miRNAs |                      |                  |                  |                   |                  |         |
| miR-150     | 1.00 ± 0.38     | 2.18 ± 0.81     | 2.52 ± 0.76      | 2.03 ± 0.74       | 2.08 ± 0.68      | < 0.01  |
| miR-185     | 1.00 ± 0.40     | 1.64 ± 0.47     | 1.91 ± 0.42      | 1.96 ± 0.57       | 2.22 ± 0.51      | < 0.01  |
| miR-133a    | 1.00 ± 0.47     | 1.56 ± 0.33     | 1.72 ± 0.66      | 1.93 ± 0.49       | 2.02 ± 0.41      | < 0.01  |
| miR-144     | 1.00 ± 0.39     | 0.60 ± 0.12     | 0.61 ± 0.22      | 0.65 ± 0.24       | 0.73 ± 0.27      | < 0.01  |
| miR-4284    | 1.00 ± 0.55     | 1.04 ± 0.19     | 1.13 ± 0.39      | 0.54 ± 0.33       | 0.48 ± 0.31      | < 0.01  |

### Table 4
Linear regression analysis on the association between five candidate urinary miRNAs and lesion size.

| Gene name | $R^2$ | p value |
|-----------|-------|---------|
| miR-150   | 0.01  | 0.37    |
| miR-185   | 0.06  | < 0.01  |
| miR-133a  | 0.01  | 0.34    |
| miR-144   | 0.52  | < 0.01  |
| miR-4284  | 0.02  | 1.20    |

Figure 2. The diagnostic powers of miR-150, miR-185, miR-133a and miR-144 were assessed using AUC calculated through ROC analysis.

Figure 3. The linear regression demonstrated a strong correlation between miR-144 level and lesion size.
Interruption of bone circulation was the basic mechanism for ONFH development [1,3]. However, the progression of ONFH involves several different pathophysiological mechanisms. A “vicious cycle” theory was proposed involving bone homeostasis alteration, cell injury and subsequent further blood flow impairment [10]. Our study also provides a useful insight into the pathogenesis of ONFH.

Previous study revealed that miR-144 was found to inhibit bone tissue mineralisation by promoting osteoclast formation and proliferation [49,50]. Increased urinary miR-144 might reflect increased osteoclast activity, which was observed in the necrotic region.

This study has several limitations. First, it used a screening confirmation design. Thus, many potentially significant miRNAs might be missed by initial screening due to the sample size limitation. Second, the healthy control group included the volunteers of this study. This design might cause potential selection bias.

Conclusion

The levels of urinary miRNAs are valuable biomarkers for idiopathic ONFH. Given the noninvasive nature of this test, it is potentially useful for monitoring idiopathic ONFH progression.

Conflict of Interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2020.01.008.

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