Increased circulating microRNA-21 level as a potential indicator for predicting a higher risk of incident fragility fractures

Abstract

Context: As a common disease in the elderly, osteoporosis clearly increases the risk of fractures, leading to higher mortality, but the current markers to estimate the risk of fractures are limited. MicroRNA-21 (miR-21) may play an important role in osteoporosis, but the link of this biomarker with fractures was undetermined.

Objectives: We aimed to investigate the association between miR-21 levels and the presence of fragility fractures.

Methods: A total of 200 patients were recruited and miR-21 was collected from baseline serum. The correlation between miR-21 and the fracture risk assessment tool (FRAX) score was analyzed. The incidence of fragility fractures was presented by Kaplan-Meier analysis, and Cox regression analysis was utilized to evaluate risk factors. The diagnostic value of miR-21 was conducted by the area under curve (AUC).

Results: The FRAX score was significantly associated with miR-21 level (p<0.001). According to the 50th percentile of miR-21 content in the overall distribution, the cumulative incidence of fragility fractures was significantly higher in patients with higher miR-21 levels than those with lower levels (75.4, 95% CI: 69.0–81.8 vs. 59.2, 95% CI: 42.1–76.3, p<0.001). The results of the Cox regression analysis showed that the miR-21 level was an independent risk factor linked to the incidence of fracture (p=0.005). The optimal cut-off value of the miR-21 was 6.08, and the AUC for predicting fracture was 0.718 (95% CI, 0.645–0.790).

Conclusions: This study showed that miR-21 has optimal diagnostic performance in the discrimination of fragility fracture, and the circulating miR-21 level in predicting the risk of fragility fracture may have a certain value.

Keywords: circulating biomarker; fracture risk; fracture risk assessment tool (FRAX); microRNA; osteoporosis

Osteoporosis has been regarded as one of the most common diseases worldwide and can feature skeletal damage [1, 2]. There can be a high rate of osteoporosis (up to 70%) in people over 80 years old [3]. Patients with osteoporosis usually have decreased bone density and damage to the microstructure of their bone tissue, leading to increased bone brittleness. As the global population ages, osteoporosis in the elderly often leads to a growing incidence of disability and even mortality [4]. As a result, the treatment-related costs of osteoporosis have been imposing an extreme economic burden on us globally [5].

It is well known that osteoporosis clearly increases the risk of fragility fracture. So far, dual-energy X-ray absorptiometry (DXA) has been regarded as the gold standard for the diagnosis of fragility fracture [6]. Despite a significant amount of research devoted to this condition in recent years, the application was still limited by the availability of this tool [3]. What’s more, risk stratification of fragility fracture was based on the fracture risk assessment tool (FRAX) [7]; however, a recent study showed that the FRAX tool may underestimate the fracture risk in some populations [8, 9]. Current bone turnover markers still had limited power in differentiating individuals at high risk of fragility fractures, which showed a moderate association with bone strength [10]. Thus, new reliable biomarkers were anticipated.

Over the past decade, research elucidating the mechanism of bone metabolism has focused on microRNAs (miRNAs), which can be alternative biomarkers of osteoporosis, and also to elucidate the molecular mechanism involved in skeletal metabolism [11]. Thus, evaluation of circulating miRNAs may forecast the individual predisposition to incident
fragility fractures, either alone or concomitant utilized with existing biomarkers of osteoporosis. For the past few years, miR-21 has been a major concern from people as an emerging alternative index in skeletal disorders, which may be closely associated with the disease onset and progress of osteoblasts, osteoclasts, and osteoporosis via various mechanisms [8, 12, 13]. A previous study showed that miR-21 was correlated with bone turnover biomarkers and up-regulated in the population with osteoporotic hip fracture [14, 15]. Additionally, the study of Ciuffi et al. [16] found that the serum level of miR-21 was significantly upregulated in patients with osteoporosis, but with insignificant association with either vertebral fracture, femur fracture, or total fracture.

Nevertheless, the accuracy of miR-21 in the prediction of fragility fractures remains undetermined, and further investigation is warranted to evaluate the role of this biomarker. Herein, we presented this study to investigate the association between miR-21 expression and the presence of fragility fractures.

**Methods**

**Study cohort**

A total of 200 patients were recruited from the Department of Orthopedics, The Second Affiliated Hospital of Harbin Medical University from January 2015 to December 2017. In all enrolled patients, medical histories were retrospectively documented, and physical examination was performed at admission. Moreover, peripheral blood (PB) of all subjects was also collected, and serum was retained and stored at −80 °C until the following analysis.

Patients who had experienced at least one episode of incident low-traumatic fragility fracture without any ascertainable reason and identifiable trauma were included in this study. The history of fragility fractures was reported either by questionnaire or skeletal digital X-rays. We excluded patients with any other bone-affecting conditions at the beginning, such as chronic liver, kidney, or gastrointestinal disease and uncontrolled thyroid disease.

The study was approved by the Ethics Committee of The Second Affiliated Hospital of Harbin Medical University, and all patients had signed the informed consent before enrollment (IRB number: 2022XJSS140).

**MicroRNA analysis**

To avoid the influence of fracture healing on the profiles of miRNA, the duration from this study to the last fractures should be at least six months. Total RNA was collected and purified by the miRNasy Serum/Plasma Kit (QIAGEN). Stored serum samples were centrifuged at 12,000×g for 5 min. For real-time quantitative PCR (RT-qPCR) analysis, cDNA samples were diluted and utilized in individual PCR reactions by SYBR Green master mix and miRNA primer assays (Sangon). The amplification reactions were incubated at 95 °C for 30 min followed by 40 cycles at 94 °C for 15 s, 55 °C for 30 s, and 70 °C for 30 s on the RT-qPCR system (Roche). Relative gene expression was presented by comparing the cycle times for the target gene. RNU6B was conducted as the miRNA internal control. Cycle threshold (Ct) values were normalized to the internal control, and expression of miRNA was calculated as ΔCt=(Ct gene of interest-Ct internal control). All analysis was based on the method of baseline value (2−ΔΔCt).

**Calculation of FRAX and measurement of bone mineral density**

Bone mineral density (BMD) was measured utilizing DXA at the lumbar spine (LS), femoral neck (FN), and total hip (TH) at baseline and during yearly follow-up for the study population. The probability of fracture risk was evaluated by the FRAX tool [17]. Via self-reported questionnaires, we collected baseline characteristics regarding age, gender, body mass index (BMI), history of diabetes mellitus (DM), daily alcohol consumption, and smoking status. Moreover, other information, such as the history of glucocorticoid use, prior fractures, and parental hip fracture, were also analyzed.

**Statistical analysis**

Data with skewed distribution were given as median and range and analyzed by the Mann-Whitney U test. Categorical variables were described with percentages and compared utilizing the chi-square test. The Spearman’s rank correlation was utilized to evaluate the association between FRAX scores and the level of circulating miR-21. The cumulative incidence of fragility fractures was presented by the Kaplan-Meier method and compared utilizing the log-rank test. Next we tested for the associations between the baseline miR-21 level and incident fragility fracture utilizing Cox proportional hazard models. The area under the receiver operating characteristic (ROC) curve was also computed to investigate the diagnostic value of miR-21 in differentiating people who experienced fragility fractures during the follow-up. p<0.05 was statistically significant. SPSS version 20.0 was utilized for statistical analysis and GraphPad Prism 9.0 was carried out to create graphics.

**Results**

**Baseline characteristics**

Of the 200 participants in this study, the median age was 58 years old (interquartile range [IQR]: 37–86) and 76 (38.0 %) were men. Half (50 %, 100/200) of the patients experienced at least one incident fragility fracture during a median follow-up time of 52 (IQR: 18–72) months. Moreover, the first incidence of fragility fractures occurred on a median of 1,440 days post the baseline visit. The baseline characteristics were shown in Table 1, and all included patients were dichotomized into low and high groups according to the 50th percentile of miR-21 concentrations in the overall distribution (set at 10.2 of miR-21 level). Generally, the
two groups were well-matched in age, gender, and current alcohol intake at baseline. However, the history of DM, current smoking, BMI $\geq 28$ and falls $\geq 2$ times in the past year were more common in patients with higher levels of miR-21 (all $p<0.05$); moreover, patients in this group also had a higher incidence of glucocorticoid use as well as parental history of hip fracture (all $p<0.05$).

**MiR-21 content is a potential risk factor for the occurrence of fragility fractures**

Figure 1 showed the correlation analysis between FRAX score and miR-21 content, and miR-21 was significantly associated with the miR-21 level ($p<0.001$). After being divided into low and high groups according to the 50th percentile of miR-21 content in the overall distribution, Figure 2 showed that the cumulative incidence of fragility fractures was significantly higher among patients with higher levels of miR-21 (75.4, 95% CI: 69.0–81.8 vs. 59.2, 95% CI: 42.1–76.3, $p<0.001$).

The result of the multivariable Cox regression analysis demonstrated that the miR-21 level was significantly linked to the occurrence of incident fragility fractures (HR: 1.06, 95% CI: 1.03–1.09, $p<0.001$). After adjustments for potential risk factors, such as daily oral glucocorticoid use, history of DM, and BMI level, circulating miR-21 remained a significant determinant for incident fragility fractures (HR: 1.05, 95% CI: 1.01–1.08, $p=0.005$) (Table 2).

**Diagnostic potential of miR-21 in patients presenting with fragility fractures**

ROC analysis was computed to evaluate the potential role of miR-21 as a biomarker for patients with incident fragility fractures (Figure 3). The optimal cut-off value of the miR-21 was 6.08 with a sensitivity of 96.0% and a specificity of 66.0%. The area under the curve (AUC) was 0.718 (95% CI: 0.645–0.790, $p<0.001$).
Table 1: Clinical characteristics of included patients at baseline.

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>MIR-21&lt;10.2 (n=100)</th>
<th>MIR-21≥10.2 (n=100)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range), y</td>
<td>58 (37–86)</td>
<td>60 (40–87)</td>
<td>0.371</td>
</tr>
<tr>
<td>Men, No., %</td>
<td>42 (42.0)</td>
<td>34 (34.0)</td>
<td>0.244</td>
</tr>
<tr>
<td>Alcohol intake, No., %</td>
<td>53 (53.0)</td>
<td>50 (50.0)</td>
<td>0.671</td>
</tr>
<tr>
<td>Diabetes mellitus, No., %</td>
<td>28 (28.0)</td>
<td>50 (50.0)</td>
<td>0.001</td>
</tr>
<tr>
<td>Current smoking, No., %</td>
<td>13 (13.0)</td>
<td>30 (30.0)</td>
<td>0.003</td>
</tr>
<tr>
<td>Falls≥2 times in the past year, No., %</td>
<td>10 (10.0)</td>
<td>21 (21.0)</td>
<td>0.032</td>
</tr>
<tr>
<td>Daily oral glucocorticoid use, No., %</td>
<td>5 (5.0)</td>
<td>25 (25.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Parental history of hip fracture, No., %</td>
<td>16 (16.0)</td>
<td>28 (28.0)</td>
<td>0.041</td>
</tr>
<tr>
<td>BMI≥28, No., %</td>
<td>32 (32.0)</td>
<td>56 (56.0)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

BMI, body mass index; MIR-21, microRNA-21.

Discussion

To our knowledge, this is the first study to clarify the diagnostic and predictive role of circulating miR-21 in incident fragility fractures. The results showed that miR-21 may predict the occurrence of fragility fractures with high sensitivity, and it may be regarded as an independent risk factor for fragility fractures. Additionally, this study also indicated that the miR-21 levels were altered regardless of sex and age, proving a notable signature of miRNA in patients who experienced incident fragility fractures. Furthermore, the AUC values of miR-21 also reflected an obvious distinction of patients from the controls, and the result of multivariate Cox regression showed that miR-21 was an independent risk factor of incident fragility fractures.

Osteoporosis represented a serious problem for public health and significantly increased the risk of incident fragility fractures. For individuals, fractures have been regarded as the most serious complication of patients with osteoporosis and lead to a dramatic deterioration in quality of life and subsequent poor clinical outcomes for these patients. Thus, it is of great importance to identify patients with increased fracture risk. Although the DXA scans give the estimation of skeletal status by measuring the BMD, the application of this tool is still limited by its availability in some medical centers. Besides, another way to assess the risk of incident fractures is via the FRAX calculation tool, a web-based risk evaluation method that forecasts the fracture risk [18]. However, FRAX has been reported with a potentially limited predictive value [19], and separate FRAX models may be needed for different races because of various rates of major osteoporotic fracture (MOF) in separate ethnic groups [7].

Table 2: Univariable and multivariable model for predicting risk factors of fragility fracture.

<table>
<thead>
<tr>
<th>Univariable</th>
<th>Multivariable</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (95 % CI)</td>
<td>p-Value</td>
</tr>
<tr>
<td>MIR-21 level</td>
<td>1.06</td>
</tr>
<tr>
<td>(1.03–1.09)</td>
<td>(1.01–1.08)</td>
</tr>
<tr>
<td>Daily oral glucocorticoid use</td>
<td>1.99</td>
</tr>
<tr>
<td>(1.25–3.17)</td>
<td>(0.95–2.54)</td>
</tr>
<tr>
<td>Falls≥2 times in the past year</td>
<td>1.45</td>
</tr>
<tr>
<td>(0.88–2.39)</td>
<td>–</td>
</tr>
<tr>
<td>Current smoking</td>
<td>1.34</td>
</tr>
<tr>
<td>(0.84–2.13)</td>
<td>–</td>
</tr>
<tr>
<td>Alcohol intake</td>
<td>0.63</td>
</tr>
<tr>
<td>(0.26–1.54)</td>
<td>–</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.53</td>
</tr>
<tr>
<td>(1.03–2.27)</td>
<td>(0.46–2.60)</td>
</tr>
<tr>
<td>Parental history of hip fracture</td>
<td>1.19</td>
</tr>
<tr>
<td>(0.75–1.89)</td>
<td>–</td>
</tr>
<tr>
<td>BMI≥28</td>
<td>1.55</td>
</tr>
<tr>
<td>(1.05–2.30)</td>
<td>(0.50–2.79)</td>
</tr>
</tbody>
</table>

BMI, body mass index; MIR-21, microRNA-21.

The pathogenesis of osteoporosis is triggered by the imbalance between osteoclast-relevant bone resorption and osteoblast-relevant bone formation [12]. MiR-21 plays an important role in both osteoblasts and osteoclasts, so this biomarker may play a strong part in the pathogenesis of osteoporosis. A study by Zarecki et al. [20] indicated that patients with high levels of miR-21 were linked to lower BMD, but they failed to find any association between miR-21 and bone turnover markers as well as the occurrence of fractures [20]. The findings from another research by Yavropoulou et al. [21] were inconsistent with the study mentioned above, and the miR-21 levels were lower in people who experienced incident fractures when compared to those who were without fractures.

Limitations

This study also had several limitations. First, this study included relatively small sample sizes, so its reliability and validity are limited. Second, this study was unable to clarify a causal relationship between circulating miR-21 levels and the presence of fragility fractures. Third, the use of miR-21 in clinical practice is still challenged by pre-analytical methods, such as sampling procedures, associated diseases, and concomitant use of drugs, as well as various analytical methods and ways of interpreting results among different laboratories. Therefore, some steps should be adopted to minimize variability of this biomarker and hence to provide better results.
Conclusions

In conclusion, miR-21 is differentially expressed in patients with incident fragility fractures and is closely correlated to the current evaluation index system of risks. This study showed that miR-21 has optimal diagnostic performance in the discrimination of fragility fracture, and the circulating miR-21 level in predicting the risk of fragility fracture may have a certain value. However, further study is warranted to unveil the mechanisms by which this miRNA may be linked to fragility fractures in a larger cohort of patients.

Research ethics: The study was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University (IRB number: 2022XJSS140).

Informed consent: All patients in this study provided written informed consent prior to participation.

Author contributions: All authors provided substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; all authors drafted the article or revised it critically for important intellectual content; all authors gave final approval of the version of the article to be published; and all authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Competing interests: None declared.

Research funding: None declared.

Data availability: The raw data of this study are available from the corresponding author upon request.

References