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Introduction

miRNAs have been extensively studied in bone research, particularly their relationship to osteoporosis (De-Ugarte et al, BMC Med Genomics, 2015; Seeliger et al, J Bone Min Res, 2014). However, the miRNA expression signatures described in patients with osteoporosis do not provide evidence of causality because the altered pattern could be a consequence of the disease or even unrelated to the pathogenesis. Another approach in miRNAs studies is the association analysis between one SNP within a candidate miRNA (miR-SNP) or in a miRNA target site, and one disease related-outcome. In this case, the associated variant is likely involved in the pathophysiology or confers susceptibility to develop the disease (Estrada et al, Nat Genet, 2012).

Methods

SNPs located in pre-miRNA sequences that (1) bind to the mRNA 3'UTR of genes related with bone metabolism or (2) found to be highly expressed in human osteoblasts were selected. Validated SNPs with a MAF>0.01 (n=5) were genotyped in the OSTEOMED2 cohort (Table 1) to assess their association with LS BMD and FN BMD (Table 3). Multivariate linear regression models were fitted to assess the association between genotyped SNPs and BMD. Potential confounders considered for adjustment were densitometer devices, body mass index (BMI) and age. miRNAs which harbored BMD-associated SNPs were quantified by qPCR in order to compare the expression levels between OP and non-OP bone samples (Table 2). Mann-Whitney U test was performed for OP and non-OP group comparisons. Human primary osteoblasts were cultured for DNA and RNA extraction and sorted by genotype for both rs6430498 and rs12512664. The correlation between expression levels of mature miRNA miR-3679-3p and miR-4274 with its corresponding genotypes was analyzed using linear regression. All analyses were two-tailed, and p-values<0.05 were considered significant.

Table 1. Baseline characteristics of the OSTEOMED2 cohort.

| Patient characteristic | Mean ± SD | |
|--------------------------|------------------|------------------|
| | LS BMD n=2183 | FN BMD n=2015 |
| Age (years) | 57.61 ± 9.26 | 58.80 ± 8.99 |
| Age of menopause (years) | 48.7 ± 3.94 | 48.7 ± 3.92 |
| BMI (kg/m ²) | 26.56 ± 4.18 | 26.48 ± 4.13 |
| BMD (g/cm ²) | 0.870 ± 0.16 | 0.707 ± 0.14 |

Abbreviations: BMI=body mass index; BMD=bone mineral density; LS=lumbar spine; FN=femoral neck

Table 2. Patient characteristics for osteoporotic fracture and non-osteoporotic groups.

| | n | Age (Mean ± SD) | BMI (kg/m ²) (Mean ± SD) | BMD (g/cm ²) (Mean ± SD) |
|--------------------------|----|--------------------|---|---|
| Biological groups | | | | |
| Osteoporotic | 10 | 75.6 ± 6.38 | 27.11 ± 2.94 | Fragility fracture |
| Non-osteoporotic | 10 | 71.7 ± 7.36 | 27.42 ± 3.15 | 0.882 ± 0.137 |

Abbreviations: SD: Standard Deviation; BMI: Body Mass Index; BMD: Bone Mineral Density

Objectives

The aims of this study were (1) to identify SNPs within candidate miRNAs in order to perform an association study with bone mineral density (BMD), the main outcome used to define osteoporosis and (2) to validate in bone cells this miR-SNP association with the osteoporotic phenotype.

Results

SNP rs6430498 in the miR-3679 and rs12512664 in the miR-4274 were significantly associated with FN BMD (Table 4). The A alleles for rs6430498 (minority allele) and rs12512664 (majority allele) were found to be associated with lower BMD values.

Table 4. SNPs associated with FN BMD in linear-regression analysis

| SNP ID | miRNA | Genotyping efficiency (%) | HWE | Beta coefficient ^a [95% CI] | p value |
|------------|----------|---------------------------|------|---|--------------------|
| rs6430498 | miR-3679 | 97.96 | 1 | -0.017 [-0.032 to -0.003] | 0.021 ^R |
| rs12512664 | miR-4274 | 98.72 | 0.47 | 0.015 [0.004 to 0.027] | 0.01 ^R |

^aAdjusted for DXA device, age and BMI

Abbreviations: FN BMD=femoral neck bone mineral density; HWE=Hardy-Weinberg equilibrium; R=recessive model

Both miRNAs miR-3679-3p and miR-4274 were significantly overexpressed in the OP samples (Table 5).

Table 5. miRNA expression levels, comparison between osteoporotic and non-osteoporotic bone samples

| miRNA | Biological Group | RQ (Median) | IQR | P value |
|----------|------------------|-------------|---------|---------|
| miR-3679 | Osteoporotic | 89.601 | 220.636 | 0.001 |
| | Control | 1.423 | 0.964 | |
| miR-4274 | Osteoporotic | 144.268 | 318.409 | 0.001 |
| | Control | 1.197 | 2.154 | |

Abbreviations: RQ=Relative quantification; IQR=Interquartile Range

A significant correlation was observed between miRNA levels and the genetic variant (Figure 1). The A allele for both SNPs was associated with higher expression of each corresponding miRNA (miR-3674; log-additive model; p-value=0.015, and miR-4274; dominant model; p-value=0.013). Additionally, in order to corroborate that the differences among expression levels are due to genotypes "per se" and not for other cellular circumstances, another bone-related miRNAs were checked in these cells and no differences in expression were found irrespective of genotypes.

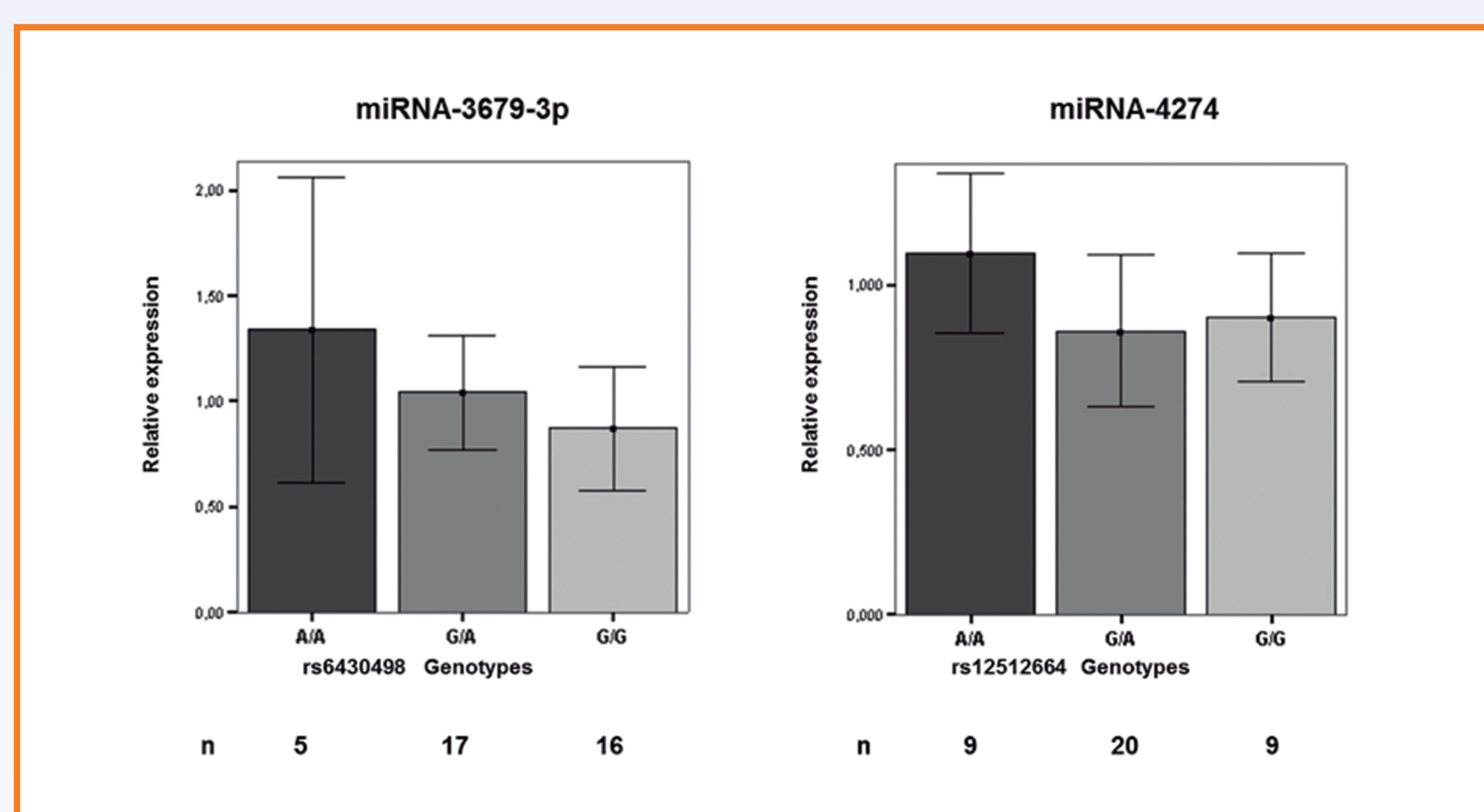


Figure 1. Correlation between miRNA expression levels and genotypes for miR-3679 and miR-4274. miRNA expression levels are represented as a mean±SD of the relative expression in Real-Time PCR. U6 was used for normalization. Samples of 38 human primary osteoblasts were used for experiments. (n) is the number of samples for each genotype group.

Conclusions

- Genetic variants in miR-3679 and miR-4274 are associated with femoral neck bone mineral density. In both cases, alleles associated with lower BMD correlated with higher expression levels of respective mature miRNAs in human osteoblastic cells which were found overexpressed in fractured bone samples.
- Our results open new exploratory avenues for future studies in the bone field and the treatment of osteoporosis.

References

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Disclosures

The authors state that they have no conflict of interest.

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Table 3. Validation of miR-SNPs for the BMD association analysis.

| TARGET GENE | miRNA | SNP | MAFB |
|--------------------------|-------------|-------------|------------|
| ESR1 | miR-106b | rs72631827 | not polym. |
| | miR-130b | rs72631822 | not polym. |
| | miR-148b | rs74878365 | not polym. |
| | miR-18a | rs41275866 | not polym. |
| | miR-222 | rs72631825 | not polym. |
| | miR-373 | rs80338016 | not polym. |
| | miR-520c | rs7255628 | not polym. |
| | miR-93 | rs72631824 | not polym. |
| | miR-96 | rs41274239 | 0.0033 |
| | | | rs73159662 |
| TGFB2 | miR-141 | rs34385807 | not polym. |
| | miR-149 | rs71428439 | not polym. |
| | miR-182 | rs77586312 | not polym. |
| | | rs75953509 | not polym. |
| | | rs80041074 | 0.0033 |
| | miR-199b | rs72631835 | not polym. |
| | miR-193a | rs60406007 | not polym. |
| | miR-200b | rs72563729 | not polym. |
| | miR-33a | rs77809319 | not polym. |
| | miR-431 | rs76090066 | 0.00083 |
| | rs128840'05 | not polym. | |
| | rs6971711 | not polym. | |
| | rs76662330 | not polym. | |
| | rs41276930 | 0.005 | |
| | rs75737367 | not polym. | |
| PTH1R | miR-339 | rs13232101 | not polym. |
| | | rs72631820 | not polym. |
| | | rs72631831 | not polym. |
| RUNX2 | miR-122 | rs41292412 | 0.0033 |
| | miR-154 | rs41286570 | 0.0004 |
| IL6R | miR-124-2 | rs72631829 | not polym. |
| | miR-124-3 | rs34059726 | not polym. |
| | miR-125a | rs12975333 | not polym. |
| | miR-140 | rs7205289 | not polym. |
| | miR-320d-1 | rs74826059 | not polym. |
| | miR-499 | rs3746444 | 0.21 |
| | rs7267163 | 0.0025 | |
| LRP5 | miR-27a | rs11671784 | 0.0162 |
| IL6 | miR-146a | rs2910164 | 0.26 |
| | miR-146b | rs76149940 | not polym. |
| | miR-202 | rs12355840 | not polym. |
| | miR-365-2 | rs35143473 | not polym. |
| VDR | miR-10a | rs72631828 | not polym. |
| | miR-223 | rs34952329 | not polym. |
| CYP24A1 | miR-30b | rs111424617 | not polym. |
| | miR-30e | rs112439044 | not polym. |
| | miR-183 | rs72631833 | not polym. |
| | | rs41281222 | not polym. |
| | miR-101-2 | rs78851134 | 0.0004 |
| Highly expressed in HObs | miR-1282 | rs11269 | not polym. |
| | miR-3679 | rs6430498 | 0.35 |
| | miR-4274 | rs10175383 | not polym. |
| | | rs12512664 | 0.47 |

MAFB: Minor allele frequency in BARCOS cohort

In bold; Validated SNPs for genotyping in total OSTEOMED2 cohort