MicroRNAs as potential biopredictors for osteoporosis

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Osteoporosis is a highly prevalent disease worldwide with high morbidity and mortality due to fractures. The cause of osteoporosis is the predominance of osteoresorption over bone remodelling. The study of novel epigenetic factors associated with this disease may increase knowledge of the pathogenesis and epidemiology of this disease. Among the known epigenetic mechanisms, micro RNA (miRNA) are one of the most studied regulators of gene expression. miRNAs have a key role in the regulation of bone homeostasis and metabolism. They are present in body fluids, including serum, suggesting that circulating miRNAs could serve as simple non-invasive biomarkers for the diagnosis of osteoporosis. The aim of this review article was to highlight the biogenesis, detection, significance of miRNAs as novel biomarkers associated with osteoporosis.

Key words: biogenesis, epigenetics, miRNA, osteoporosis.

MikroRNA ako potencionálne biopredikty pre osteoporózu

Osteoporóza je celosvetovo vysoko frekventované ochorenie s vysokou morbiditou a mortalitou v dôsledku zlomenín. Príčinou osteoporózy je prevaha osteoresorpcie nad novotvorbou kostí. Štúdium nových epigenetických faktorov spojených s týmto ochorením môže rozšíriť poznatky o patogenéze a epidemiológii tohto ochorenia. Spomedzi známych epigenetických mechanizmov sú miRNA jedným z najviac študovaných regulátorov génovej expresie. miRNA majú kľúčovú úlohu pri regulácii kostnej homeostázy a metabolizme.
Sú prítomné v telesných tekutinách vrátane séra, čo naznačuje, že cirkulujúce miRNA by mohli slúžiť ako jednoduché neinvazívne biomarkery pre diagnostiku osteoporózy. Cieľom tohto prehľadového článku bolo poukázať na biogenézu, detekciu, význam miRNA ako nových biomarkerov asociovaných s osteoporózou.

Kľúčové slová: biogenéza, epigenetika, miRNA, osteoporóza.

Introduction

Osteoporosis is a multifactorial disease characterized by reduction of bone mass and alteration of bone architecture, leading to increased bone fragility and increased risk of fractures (1). BMD loss associated with declining estrogen levels increases the risk of fractures in postmenopausal women, which account for the majority of osteoporosis cases (2). Fragility fractures are one of the most common causes of disability and a major contributor to rising medical care costs in all areas of the world (3). As the prevalence of fractures increases in parallel with the lengthening life expectancy of the population, osteoporosis is becoming an increasingly significant public health burden (4, 5, 6). However, the precise epigenetic mechanisms underlying osteoporosis remain unclear. A growing body of research suggests that epigenetic changes could act as important mediators, linking genetic tendencies and environmental influences, thereby increasing the risk of osteoporosis. Within epigenetic factors, microRNAs (miRNA) have been recognized as key regulatory elements (7). miRNAs are small non-coding and single-stranded RNA molecules that play an important role in mRNA expression through direct binding to the 3'-UTR (3'-untranslated region) of target mRNAs (messenger RNAs) (8). miRNAs are involved in a variety of biological processes including cell differentiation, migration, invasion and apoptosis. miRNAs regulate gene expression at the posttranscrip-

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tional level through mRNA degradation or translation inhibition (9). In the field of skeletal biology, studies have shown that the differentiation and function of osteoblasts and osteoclasts are regulated by miRNAs, suggesting their regulatory role in bone formation and resorption, bone remodelling, and bone repair (10). Their dysregulation is associated with metabolic diseases including osteoporosis (9).

The aim of this review article was to highlight the biogenesis, detection, significance of miRNAs as novel biomarkers studied in bone diseases, especially in the context of osteoporosis.

Epigenetics

Epigenetic mechanisms include DNA methylation (deoxyribonucleic acid) and histone modifications that regulate gene transcription and non-coding RNA (ncRNA) that acts at the post-transcriptional level. Among the known epigenetic mechanisms, miRNAs are one of the most studied regulators of gene expression in both physiological and pathological conditions. miRNAs have a key role in the regulation of bone homeostasis and metabolism. MicroRNAs can affect differentiation, function, apoptosis and proliferation of osteoblasts and osteoclasts (Table 1) (13). One such miRNA is miR-9-5p, whose expression is increased in osteoporotic patients. By directly binding to Wnt3, it can decrease osteogenesis and increase both adipogenesis and osteoclastogenesis. Furthermore, miR-21 has been found to promote osteoclastogenesis. Osteoclastogenic factor (c-Fos) upregulates miR-21, which in turn stimulates RANKL-induced osteoclastogenesis. Osteoporotic women with vertebral fractures show significantly reduced miR-21-5p expression (14). Associations between hsa-miR-19 b-3p and the bone formation markers OC (osteocalcin), ALP (alkaline phosphatase), and the bone resorption marker CTx (beta-Cross-Laps) were pointed out by Zarecki et al. (15). Mohammadisina et al (16) concluded in their study in postmenopausal women that higher levels of miRNA-21 and miRNA-422a expression increase the likelihood of osteoporosis in postmenopausal women. miRNA-21 affects both osteoclast and osteoblast genes, with c-Fos (a Fos family transcription factor) upregulating miRNA-21 expression. Lv, Sun and Zhang (17) found that miR-133 negatively correlated with bone mineral density, precisely why (on this basis) it is considered a potential biomarker associated with postmenopausal osteoporosis. The expression of miR-133 was significantly increased due to estrogen deficiency. Pala and Denkceken (18) concluded that miR-133a was upregulated during osteoclastogenesis. miR-133a is involved in the regulation of postmenopausal osteoporosis by osteoclast differentiation. Kong et al. (19) in a study they conducted in postmenopausal women concluded that miR-320a downregulates osteoblast function and induces oxidative stress. miR-320a was overexpressed in patients with postmenopausal osteoporosis and showed inhibitory effects on the activity and differentiation of M3T3-E1 cells; it also affected apoptosis of MC3T3-E1 cells. miR-155 downregulates an essential transcription factor in osteoclast differentiation (MITF), this finding reflects the positive and negative regulation of miRNAs at the stage of two different cell types. miR-155 functions as a molecular switch between the macrophage and osteoclast lineages. miR-21 is upregulated during RANKL-induced differentiation of mononuclear to multinucleated cells, inhibiting the PDCD4 protein that derepresses c-Fos (20).

Tab. 1. *MicroRNAs affecting osteoblast and osteoclast differentiation*

MiRNAs affecting osteoblast differentiation		
miRNA	**Regulation (↑ ↓)**	**Signalling pathway**
miR-210	↑	VEGF
miR-103a	↓	Runx2
miR-17-5p	↓	BMP2
miR-106a	↓	BMP2
miR-29a	↑	Runx2
miR-20a	↑	BMP/Runx2
miR-125b	↓	Osterix
miR-138	↓	Focal adhesion kinase signaling pathway
miR-141-3p	↓	Wnt signaling pathway
miR-203a-3p	↓	Smad9, Wnt/β-catenin signaling pathway
miR-216a	↑	BMP/TGF-β signaling pathway
miR-375	↓	Runx2
miR-194	↑	Runx2
miR-100	↓	Smad1
MiRNAs affecting osteoclast differentiation		
miR-503	↓	RANKL
miR-20a	↓	RANKL
miR-214	↑	PI3K/Akt
miR-148a	↑	RANKL
miR-17	↓	RANKL
miR-141	↓	Calcr
miR-19a	↑	Runx2, TWIST
miR-21	↑	RANKL, PI3K/Akt pathway
miR-183	↑	RANKL

↑ – upregulation; ↓ – downregulation; VEGF – vascular endothelial growth factor; Runx2 – Runt-related transcription factor 2; BMP2 – bone morphogenetic protein 2; RANKL – NF-κB receptor activator; PI3K/Akt – phosphatidylinositol 3-kinase/Akt; BMP – bone morphogenetic protein; TGF-β – transforming growth factor beta; Smad9 – mothers against decapentaplegic homolog 9; Calcr – calcitonin receptor; TWIST – basic helix-loop-helix transcription factor; miR – microRNA
Modified by (11,12)

MicroRNA (miRNA)

MicroRNAs are short single-stranded RNAs of 22/23–25 nucleotides that block the expression of complementary or partially complementary mRNAs (21). Although miRNAs make up only 1 to 5 % of the human genome, 60 % of protein-coding genes can be modulated by miRNAs, making these non-coding RNA molecules master epigenetic regulators in the cell (22). They are significantly involved in the development and progression of various diseases. Mutations, dysfunctions or dysregulations of miRNA biogenesis and their targets lead to the blockage of physiological and biochemical pathways involved in the development of civilization diseases in humans (23).

Biogenesis of miRNAs

The biogenesis of microRNAs occurs through a multi-step process requiring both nuclear and cytoplasmic phases (24). This process is regulated at multiple levels (Figure 1), starting from the initial transcription of the miRNA and processing by RNase Drosha in the nucleus, further processing by RNase Dicer followed by modification in the cytoplasm, plating on the RNA-induced silencing complex to finally degradation of the RNA (25). The process of biogenesis begins with the processing

of transcripts by RNA polymerases II or III post- or co-transcriptionally. Approximately half of all currently identified microRNAs are intragenic and processed mostly from introns and a relatively small number of exons, the protein-coding genes, while the remainder are intergenic, i. e. transcribed independently of the host gene and regulated by their own promoters. Sometimes miRNAs are transcribed as a single long transcript called a cluster. The biogenesis of miRNAs is divided into canonical and non-canonical pathways (26).

Canonical pathway

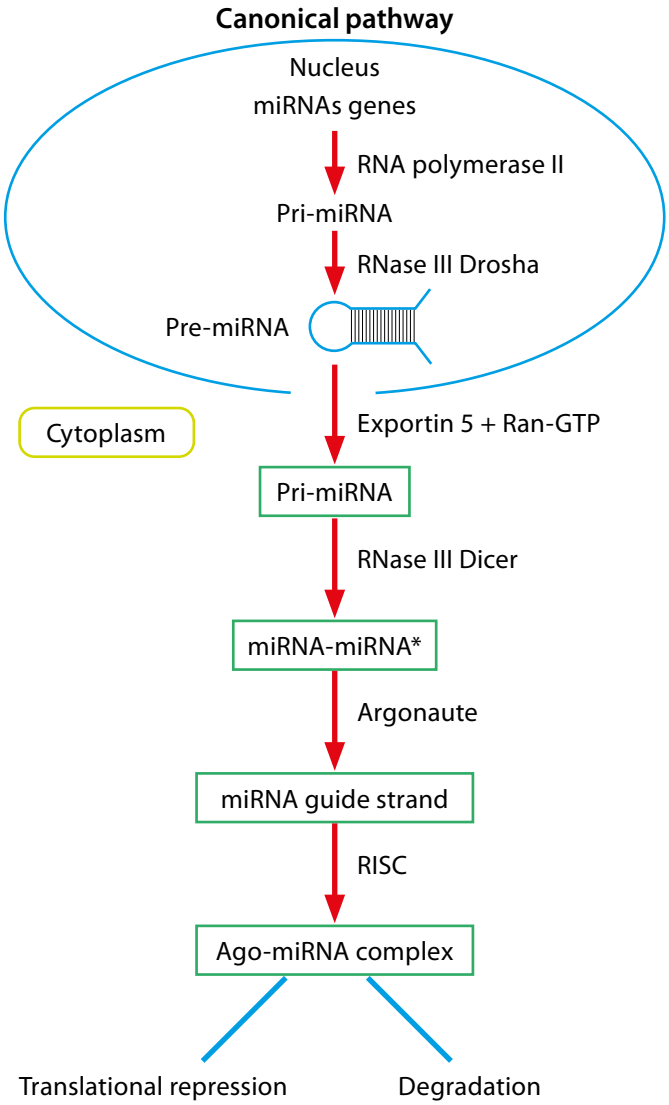
Canonical miRNA biogenesis is initiated by transcription of the miRNA host gene by RNA polymerase II (RNAPII) to produce the primary miRNA transcript (pri-miRNA). Pri-miRNAs contain a stem-loop locus structure that encodes a miRNA duplex. Subsequently, cleavage of the hairpin by the RNase III enzyme Drosha and its RNA-binding protein cofactor DiGeorge Syndrome Critical Region 8 (DGCR8), collectively referred to as the microRNA complex, occurs, forming a 60–80 nucleotide intermediate hairpin known as pre-miRNA. Nuclear export of pre-miRNA is facilitated by Exportin 5 and Ran-GTP (a nuclear GTP-binding protein), and other export mechanisms are also thought to be present. The newly transcribed pre-miRNA with the typical 5′ phosphate and ~2-nucleotide 3′ overlap is subsequently exported to the cytoplasm by Exportin 5 and the Ran-dependent nuclear transport receptor protein. In the cytoplasm, pre-miRNAs are processed into mature ~18- to 23-nucleotide long miRNA duplexes by the RNase III enzyme, Dicer-1, with the help of dsRNA-binding proteins (double-stranded RNA), RNA protein kinase activator, and transactivation-binding protein (28, 29).

The miRNA duplex is inserted into Argonaute (AGO) proteins. The 2 strands of the miRNA separate depending on various factors, such as the stability of the base pairing at the 5′ end or the thermodynamic asymmetry of the duplex. After insertion, only one strand is retained, referred to as the guide strand, whose 5′-nucleotide interacts with the MID domain of the AGO proteins to form the final AGO-miRNA complex, called the RNA-induced silencing complex (RISC). The choice of guide strand depends on the identity of the 5′-nucleotide and the thermodynamic stability of the two ends of the miRNA duplex; a strand with a 5′-uridine or 5′-adenosine and thermodynamically unstable 5′ ends is considered preferable. The resulting RISC binds to the target mRNA, mainly through sequence complementarity between the germline sequences and the target sites in the mRNA. TNRC6 (GW182) proteins, AGO interacting partners, play an important role in target repression by interacting with poly(A)-binding protein and recruiting the deadenylation complexes PAN2-PAN3 and CCR4-NOT to target mRNAs (30).

Non-canonical pathway

While the majority of identified microRNAs are produced by the canonical pathway, there are several examples of non-canonical pathways. The most common variation is the miRtron class of miRNAs (31). MiRtrons generate cloned ~22 nt RNAs that adopt miRNA/miRNA* duplexes with 3′ overhangs. Their distinguishing feature is that the genomic ends of miRNA/miRNA* read pairs are precisely defined by downstream donor and acceptor sites. In this configuration, the splicing machinery generates hairpins, thereby bypassing Drosha cleavage (32). All miRtrons are initially processed by the nuclear splicing mechanism as typical introns and form stable hairpins with a shorter stem compared to canonical pri-miRNAs, these shorter hairpin structures cannot be processed by Drosha/DGCR8. Instead, they are subject to branching by the branching enzyme 1 (DBR1). Intron-derived miRNAs persist in cells that are deficient for Drosha or DGCR8 activity. Similar to canonical miRNAs, pre-miRNAs derived from miRtrons are bound by XPO-5, translocated to the cytoplasm, and cleaved by Dicer (33). Once incorporated into RISC, miRNAs generally function as a guide that base-pairs with the 3′ UTR of their target mRNAs, whereupon the double-stranded miRNA-mRNA complex induces downstream translational repression and mRNA degradation, thereby silencing the target mRNAs. Altered miRNA expression can passively indicate the mechanism of disease, and it is also possible to actively control the disease by altering miRNA regulation (34, 35). The pre-miR-451 stem-loop structure is too short to be cleaved by Dicer on the basis that it can bypass Dicer processing and instead relies on Ago2 (Argonaute RISC catalytic component 2) for

Fig. 1. *Scheme of miRNA biogenesis. Modified from (27)*



Ago – Argonaute; Pre-miRNA – precursor miRNA; Pri-miRNA – primary miRNA; miRNA – microRNA; RISC – RNA-induced silencing complex

maturation. Examination of the miR-451 precursor revealed an unusual property. miR-451 is highly expressed in erythrocytes, regulating the differentiation of erythroblasts into mature red blood cells (36, 37). Pri-miR-451 is initially cleaved by Drosha/DGCR8 to form a short pre--miR-451 with ~18 bp of duplex stem, which is, however, too short to serve as a substrate for Dicer. Instead, pre-miR-451 is inserted directly into the Ago proteins. Filaments that enter the noncleaved Ago proteins cannot further mature, but those that affect Ago2 are cut into their 3'filament arm, which is guided by the 5′ end of the filament, yielding a 30nt Ago-cleaved species (26, 32).

Detection of miRNA

Extracellular miRNAs are easily detectable; they can serve as mediators of intercellular communication. Scientific studies report that these miRNAs originate from three main pathways. First, they could be transported by extracellular vesicles, either by exocytosis or by direct budding of the cell plasma membrane. Second, they could bind to specific proteins, such as lipoproteins and ribonucleoproteins, which are subsequently secreted as protein-miRNA complexes. In addition, they may originate from damaged or dead cells (25, 38, 39). Several methods are used for the detection and quantification of miRNAs, such as quantitative real-time PCR (qRT-PCR), microarray analysis, and next-generation sequencing (NGS) (27). Microarrays are a universal analytical tool for miRNA expression profiling and are used to compare miRNA expression profiles. Using microarrays, we can evaluate the expression patterns of hundreds of miRNA genes in a single assay. They are typically solid-surface slides with hundreds of probes arranged in a grid. miRNA arrays involve several main steps, namely: purification of mature miRNA, reverse transcription and simultaneous labelling of cDNA, and hybridization of cDNA with high--affinity probes on a glass slide. RT-qPCR is the most used method for detecting mature miRNAs. This method can distinguish between miRNA species that differ by as little as a single nucleotide. The target miRNA is reverse transcribed by binding a stem-loop primer to the 3′ end of the miRNA for reverse transcription. This miRNA is then used as a template for miRNA amplification with a specific forward primer and a universal reverse primer or a TaqMan probe. Next-generation sequencing (NGS) is not only capable of profiling the expression of known miRNAs but is also suitable for identifying various unknown miRNA variants. The basic procedure for NGS miRNA sequencing is similar to DNA sequencing with additional steps that consider the generation of an RNA library. The initial step usually requires the enrichment of small RNAs that are simultaneously ligated to 3′ and 5′ adapters. The total small cDNA is generated by reverse transcription. Reverse transcription is followed by amplification and subsequent sequencing. NGS generates millions of reads, and after processing the raw data, it is possible to quantify the expression of thousands of miRNAs in a single experiment (40).

Significance and modes of action of miRNAs

In humans, more than 2 500 microRNAs have now been described and are involved in virtually all biological processes. Given their role as regulators of gene expression, they are thought to be associated with specific and physiological and pathological conditions and have been proposed as biomarkers in various diseases. miRNAs found in biological fluids are referred to as circulating, the expression profile is specific to each cell type. The main advantages of using circulating miRNAs as biomarkers include the high stability of the molecules and the wide availability of samples. Due to the individual nature of expression changes, these molecules have high potential in personalized medicine. Determination of miRNA expression profile can support disease recognition and diagnosis and can be used to monitor therapeutic responses and determine the prognosis of the disease in the patient, as well as in the selection of adequate treatment (37). The miRBase database is one of the major public repositories and online sources of microRNA sequences and annotations. Established in 2002, this database is responsible for the nomenclature of microRNA genes. miRBase provides a wide range of information on published microRNAs, including their sequences, precursors, biogenesis, genome coordinates, literature references, expression data obtained by deep sequencing (41). miRNAs regulate almost all cellular functions including cell proliferation, growth, differentiation and apoptosis. The expression of miRNAs in a specific cell type can be a useful marker to identify a particular cell type (42). The maintenance of bone as a dynamic tissue depends on a balance between osteoblast-mediated bone formation and osteoclast-mediated bone resorption (43). Osteoblasts are involved in bone matrix formation and secrete many osteogenic factors such as osteopontin, osteocalcin and alkaline phosphatase (44). Several specific miRNAs are involved in the differentiation process of BMSCs (bone marrow mesenchymal stem cells) into osteoblasts. At the same time, miR-29 is involved in the regulation of bone remodelling and collagen synthesis, which influences the risk of osteoporosis. miR-29b modulates extracellular matrix proteins to regulate osteoblastogenesis (45). miR-20a promotes differentiation of BMSCs into osteoblasts as it directly binds to the 3'UTR of BMP-2 (bone morphogenetic protein 2) and activates BMP/Runx2 (Runt-related transcription factor 2) signalling through receptor silencing (11). The activation of FoxO1, which protects cells in bone from active oxygen species, stimulates osteoblast proliferation and differentiation. miR-182 negatively regulates osteoblastogenesis through suppression of FoxO1 in cells. Overexpression of miR-182 can inhibit osteoblast differentiation. miR-93 and miR-214 negatively regulate osteoblast mineralization. During osteoblast mineralization, miR-93 is down-regulated, its over-expression can suppress Sp7 protein expression during osteoblast mineralization (46).

Osteoclasts are multinucleated bone-resorbing cells derived from the myeloid lineage and are crucial for normal skeletal development and homeostasis. Osteoclast differentiation requires macrophage colony-stimulating factor (MCSF), which promotes proliferation and survival, as well as RANKL, which induces differentiation. During osteoclastogenesis, many miRNAs are differentially expressed, regulating osteoclast differentiation and function. As a master regulator of osteoclastogenesis, NFATc1 (nuclear factor of activated T cells) is a critical component of osteoclast differentiation (47). miR-31 is induced by RANKL, which positively regulates cytoskeleton organization during osteoclastogenesis and influences bone resorption activity. It targets the expression of RhoA (a transforming protein) to optimize actin ring formation in

osteoclasts, which is critical for both cytoskeleton organization and bone resorption (48). MiRNAs are involved in skeletal development and play an important role in regulating bone homeostasis. MicroRNAs are closely related to various diseases of the skeletal system and have an impact on the diagnosis, prognosis and treatment of these diseases (49).

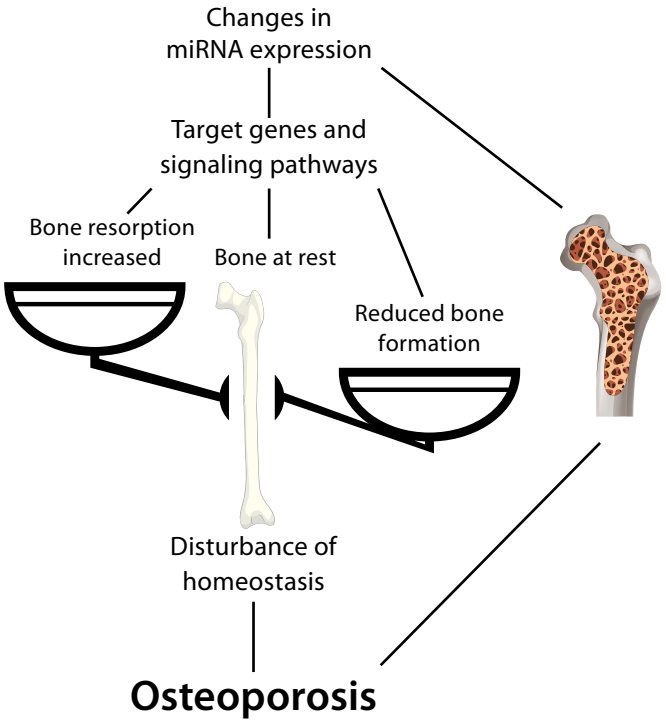
The mode of action of miRNAs involves the recognition of the 3'-UTR of target mRNAs by sequence complementarity and the recruitment of nucleases to repress mRNA expression. Two to eight nucleotides at the 5'-end of the miRNA is the germline region that is conserved in the miRNA family, controlling target recognition. Complementarity with the miRNA controls the fate of target mRNAs. High complementarity causes mRNA degradation; conversely, partial complementarity prevents translation of target mRNAs. Approximately 60 % of human mRNAs are conserved miRNA targets (50).

Bone tissue is constantly remodelling during an individual's lifetime. Bone tissue is made up of four specific cell types; osteocytes, osteoblasts, osteoclasts in bone, and chondrocytes found in cartilage. Bone homeostasis is maintained through a balance between osteoclastic bone resorption and osteoblastic bone formation. miRNAs are important regulators of bone resorption activity maintained by osteoclasts as well as osteoblast proliferation and differentiation (Table 1). Dysregulation of microRNAs is a pathological factor in bone degradation and resorption, as well as in bone-related diseases (51).

Bone metabolism is a multifaceted process that involves different types of bone cells, such as bone marrow mesenchymal stem cells, osteoblasts, osteocytes, and various signalling pathways, including the Wnt/ß-catenin signalling pathway, transforming growth factor ß (TGF-ß), Smad2/COL4A1 signalling pathway, and bone morphogenetic protein. Deviation in the regulation of signalling pathways or cells can result in various pathological conditions such as osteoporosis. Osteoporosis is characterized by low bone mineral density due to dysregulation in osteoblast-mediated bone formation and osteoclast-mediated bone resorption (52). It is a bone disorder that is characterized by loss of bone strength due to an imbalance between bone resorption and bone formation mechanisms, leading to increased fragility and fracture formation (53). Osteoporosis affects approximately 200 million people worldwide, with postmenopausal women being the most affected. Patients with postmenopausal osteoporosis are mostly free of clinical symptoms until they suffer their first fracture. Advanced disease manifests with back pain or decreased body height due to spinal deformities caused by fractures and compression of vertebral bodies (54). The factures occur mainly in the hip, vertebrae and distal forearm (1). Osteoporosis is divided into primary and secondary. Primary osteoporosis is a metabolic bone disease that is most associated with postmenopausal status or age.

The incidence of primary osteoporosis is significantly higher than that of secondary osteoporosis. Secondary osteoporosis arises because of another disease or external influences (55). Postmenopausal osteoporosis occurs approximately 15 to 20 years after menopause; the trabecular bone is most affected. Among the pathophysiological mechanisms, oestrogen deficiency has the greatest contribution to the development of postmenopausal osteoporosis (54). In connection with the biochemical diagnosis of the disease, bone markers of osteo-

Fig. 2. *Effect of miRNAs on the development of osteoporosis. Modified from (54)*



formation - ALP, osteocalcin and P1NP (aminoterminal propeptide of procollagen type 1), osteoresorption (CTX - β - Cross Laps, TRAP5b - tartrate-resistant phosphatase 5 b), hormone 25(OH)D, mineral elements calcium, phosphorus and others are determined. Molecular biomarkers of osteoporosis may contribute to the development of alternative therapeutic strategies.

In recent years, some RNAs (ribonucleic acid), such as miRNAs, have been found to regulate gene expression and influence many biological processes (Figure 2), including bone metabolism (56).

Conclusion

Osteoporosis is the most common metabolic bone disorder. It is often diagnosed after the occurrence of the first fracture with important health consequences. Therefore, the search for adequate markers that could assist in finding patients at risk is still needed. Using molecular genetic analyses, researchers have been able to discover new biomarkers for osteoporosis, which include miRNAs. Using miRNAs, differences in the expression of genes involved in the disease are being monitored. However, research on miRNAs as biomarkers is still at an early stage as many findings generally lack reproducibility. miRNAs meet most of the required criteria to be adequate biomarkers - availability, high specificity and sensitivity. Despite current limitations, miRNAs as biomarkers remain an impressive area of research. With the rapid development of molecular genetic techniques, it is anticipated that miRNAs will become a routine approach in the development of personalised profiles of patients with CVD (which includes osteoporosis), allowing for more specific therapeutic interventions.

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