Circulating MicroRNAs as Novel Potential Diagnostic Biomarkers for Osteosarcoma: A Systematic Review

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Abstract: Osteosarcoma (OS) is a fast-progressing bone tumor with high incidence in children and adolescents. The main diagnostic methods for OS are imaging exams and biopsies. In spite of the several resources available for detecting the disease, establishing an early diagnosis is still difficult, resulting in worse prognosis and lower survival rates for patients with OS. The identification of novel biomarkers would be helpful, and recently, circulating microRNAs (miRNAs) have been pointed to as possible non-invasive biomarkers. In order to assess the effectiveness of miRNA research, we performed a systematic review to assess the potential role of circulating miRNAs as biomarkers for OS diagnosis. We performed a search in various databases—PubMed, LILACS (Literatura Latino-americana e do Caribe em Ciências da Saúde), VHL (Virtual Health Library), Elsevier, Web of Science, Gale Academic One File—using the terms: “Circulating microRNAs” OR “plasma microRNAs” OR “serum microRNAs” OR “blood microRNAs” OR “cell-free microRNAs” OR “exosome microRNAs” OR “extracellular vesicles microRNAs” OR “liquid biopsy” AND “osteosarcoma” AND “diagnostic”.

We found 35 eligible studies that were independently identified and had had their quality assessed according to Quality Assessment of Diagnostic Accuracy Studies (QUADAS-2) guidelines. Despite the useful number of publications on this subject and the fact that several microRNAs showed excellent diagnostic performance for OS, the lack of consistency in results suggests that additional prospective studies are needed to confirm the role of circulating miRNAs as non-invasive biomarkers in OS.

Keywords: circulating microRNAs; microRNAs; osteosarcoma; diagnostic

1. Introduction

Osteosarcoma (OS), the most common type of cancer in children and adolescents, is a rapidly progressing bone tumor that is characterized by the production of osteoids by malignant cells [1]. Epidemiological data indicate that the incidence of OS is 0.2–0.3 per 100,000 cases per year, increasing to 0.8–1.1 per 100,000 cases per year in the population aged 15 to 19 [2].

Currently, diagnostic methods for OS consist of imaging examinations, such as X-rays, computed tomography and magnetic resonance imaging, and biopsy, which is considered the gold standard for diagnostic confirmation [2,3]. Although there are several resources for detecting the disease, establishing an early diagnosis is still difficult, which results in worse prognosis and lower survival rates for patients. Therefore, it would be helpful to discover new, less invasive diagnostic methods, with greater sensitivity and specificity, capable of detecting OS in early stages and with greater reliability [4]. In this context, studies involving microRNAs point to them as a good strategy to guarantee the early detection of OS, enabling better prognosis and increasing the survival of patients.
MicroRNAs are described as possible biomarkers because of their contribution to the pathogenesis of different types of cancer. They show an aberrant expression that is supposed to affect several biological processes, such as cell proliferation, differentiation and apoptosis, by controlling the expression of target genes and acting as tumor suppressors or oncogenes [5]. Circulating microRNAs, which are particularly stable in body fluids and, because of this, are proposed as excellent non-invasive cancer biomarkers, have been investigated for their diagnostic and prognostic potential [6]. These molecules are released from tissues into the circulatory system as a pathology develops. Furthermore, differential expressions of circulating microRNAs have been reported in several pathological conditions, including cancer [7,8].

Many studies on the use of circulating microRNAs as biomarkers in the diagnosis of OS have been carried out and published. They concluded that several microRNAs were found to be differentially expressed—either up- or downregulated [4,9]. Despite the useful number of publications on this subject, there are some inconsistencies between them, due to differences in the detection methods, the analysis of microRNAs expression, etc.

Considering this, we have gathered several studies to help in understanding the current research scenario on this topic through a systematic review. Systematic reviews are important because they concern important and published scientific studies, providing ample review of the state-of-the-art research on the topic, and allowing suggestions for improvements to obtain more striking results. In this context, it is imperative to develop a rigorous strategy for microRNA quantitation experiments. We also intend to suggest guidelines to avoid the potential bias introduced by differences in the amounts of starting material, sample collection and quality, RNA isolation, and PCR efficiency, since reverse transcription quantitative polymerase chain reaction (RT-qPCR), which is the most commonly used method for quantifying circulating microRNAs, could compromise the use of microRNAs as cancer biomarkers in serum or plasma samples.

2. Material and Methods

2.1. Literature Search Strategy

In accordance with predefined protocols and aiming at the identification of studies related to circulating microRNAs as diagnostic biomarkers for the detection of osteosarcoma, we performed a systematic literature search on the PubMed, LILACS (Literatura Latino-americana e do Caribe em Ciências da Saúde), VHL (Virtual Health Library), Elsevier, Web of Science, and Gale Academic One File databases for eligible articles published between 1 January 2015 and 29 April 2020. The search terms were: “Circulating microRNAs” OR “plasma microRNAs” OR “serum microRNAs” OR “blood microRNAs” OR “cell-free microRNAs” OR “exosome microRNAs” OR “extracellular vesicles microRNAs” OR “liquid biopsy” AND “osteosarcoma” AND “diagnostic”.

2.2. Eligibility Criteria

We considered studies eligible if they met the following criteria: (1) studies evaluating circulating microRNAs expression in human samples, comparing OS patients with healthy Control subjects; (2) studies that employed blood specimens, including serum, plasma and exosome vesicles; (3) studies that made a definitive diagnosis of OS through histopathological examination. The exclusion criteria were the following: (1) non-original articles or articles published in the form of letters to the editor, opinion pieces, reviews, editorials, case reports, expert opinions, protocols, conference or meeting abstracts, comments, or meta-analyses; (2) studies not related to circulating microRNAs’ expression or that evaluated microRNA expression only in cell lines or tissues; (3) studies with experiments on animal models only; (4) studies with insufficient or unqualified data.

2.3. Data Extraction and Statistical Analysis

Only articles in English, Portuguese or Spanish were included. Duplicate publications were also removed. Relevant and qualified studies were independently selected by two
investigators who were also responsible for data extraction. The following data were retrieved from all included studies: basic information (first author, year of publication and country of research), patients’ characteristics (ethnicity of research population and number of participants, mean or median age, gender, histologic type and stage of the tumor), type of sample (total blood, plasma, serum or exosome vesicles), total number of cases and controls, target microRNAs or microRNA panels investigated, detection or measurement method, endogenous control used for normalization analysis, normalization method and diagnostic related parameters, such as AUC, sensitivity, specificity or expression variation. Any inconsistency was analyzed by further discussion among the authors.

2.4. Quality Assessment of the Included Studies

The search method and the quality of the articles were evaluated by two reviewers, and any disagreements were resolved by a third person. Quality assessment of the eligible studies was performed by two independent investigators and was conducted using QUADAS-2 (Quality Assessment Tool for Diagnostic Accuracy Studies) [10] to estimate the risk level of bias. Basically, this evaluates four components: (a) patient selection; (b) index test; (c) reference standard and (d) flow and timing. The risk level of bias is classified as “low”, “high” or “unclear” based on the answers to questions included in each component. The tool also allows for evaluating the clinical applicability, which can also be judged as “low”, “high” or “unclear”.

2.5. Study Registration

The retrieved studies were assessed following the criteria established by the preferred reporting items for systematic review and meta-analysis (PRISMA) guidelines for systematic review [11], with a PROSPERO registration number of CRD42020192655.

3. Results

3.1. Literature Search Results

As shown in Figure 1, via the initial literature search, we selected 282 articles. After removing 64 duplicates, the remaining 218 articles were submitted to title and abstract assessment. A further 141 articles were excluded, because they were letters, reviews, editorials, case reports, expert opinions, protocols, conference or meeting abstracts, comments or meta-analyses, or articles not related to our topic. The remaining 77 articles were submitted to full-text review, of which 42 articles were excluded because they were not realized in human samples or because they were not about circulating microRNAs. Finally, 35 studies were included in this systematic review. The characteristics of these studies are displayed in Tables 1 and 2.

Table 1. MicroRNA candidate selection methods found in the included articles.

| Author            | Year | Type of Global MicroRNA Expression Profiling | Were Samples Pooled? | If Yes, What Was the Number of Samples Per Pool? | If Samples Were Not Pooled, How Many Samples Per Group Were Analyzed in Large-Scale Analysis? | Were Candidate MicroRNAs Selected by Analysis of Public MicroRNAs Datasets? | Were Candidate MicroRNAs Selected by Literature Review? |
|-------------------|------|--------------------------------------------|----------------------|-----------------------------------------------|----------------------------------------------------------------------------------|------------------------------------------------------------------------|-----------------------------------------------|
| Allen-Rhoades et al. [12] | 2015 | MicroRNA PCR panel                         | Conducted using non-human samples | N/A                                           | N/A                                                                               | No                                                                       | No                                            |
| Cai et al. [13]    | 2015 | N/A                                        | N/A                  | N/A                                           | N/A                                                                               | No                                                                       | Yes                                           |
| Hui et al. [14]    | 2015 | MicroRNA PCR panel                         | No                   | N/A                                           | 3 per group                                                                       | No                                                                       | No                                            |
| Lian et al. [15]   | 2015 | MicroRNA PCR panel                         | Yes                  | 2 pools with 10 samples each                  | N/A                                                                               | No                                                                       | Yes                                           |
| Tang et al. [16]   | 2015 | N/A                                        | N/A                  | N/A                                           | N/A                                                                               | No                                                                       | Yes                                           |
| Wang et al. [17]   | 2015 | N/A                                        | N/A                  | N/A                                           | N/A                                                                               | No                                                                       | Yes                                           |
| Wang et al. [18]   | 2015 | N/A                                        | N/A                  | N/A                                           | N/A                                                                               | No                                                                       | Yes                                           |
### Table 1. Cont.

| Author            | Year | Type of Global MicroRNA Expression Profiling | Were Samples Pooled? | If Yes, What Was the Number of Samples Per Pool? | If Samples Were Not Pooled, How Many Samples Per Group Were Analyzed in Large-Scale Analysis? | Were Candidate MicroRNAs Selected by Analysis of Public MicroRNAs Datasets? | Were Candidate MicroRNAs Selected by Literature Review? |
|-------------------|------|---------------------------------------------|----------------------|-------------------------------------------------|---------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|-----------------------------------------------------------|
| Yang et al. [19]  | 2015 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Zhou et al. [20]  | 2015 | MicroRNA PCR panel                          | Yes                  | 3 pools with 10 samples each                     | N/A                                                                                        | No                                                                            | No                                                        |
| Cao et al. [21]   | 2016 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Li et al. [3]     | 2016 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Niu et al. [22]   | 2016 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Pang et al. [23]  | 2016 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Sun et al. [24]   | 2016 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Zhou et al. [25]  | 2016 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Fujiwara et al. [26] | 2017 | Microarray                                  | No                   | N/A                                             | 10 per group                                                                               | No                                                                            | No                                                        |
| Liu et al. [27]   | 2017 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Wang et al. [28]  | 2017 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Xie et al. [29]   | 2017 | Sequencing                                  | No                   | N/A                                             | 3 OS and 10 control subjects                                                               | No                                                                            | No                                                        |
| Cong et al. [30]  | 2018 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Liu, Song et al. [31] | 2018 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Liu, Zhao et al. [32] | 2018 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | No                                                        |
| Monterde-Cruz et al. [2] | 2018 | MicroRNA PCR panel                          | Yes                  | 4 pools with 5 samples each                      | N/A                                                                                        | No                                                                            | No                                                        |
| Tian et al. [33]  | 2018 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | Yes                                                                           | No                                                        |
| Xu et al. [34]    | 2018 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Yao et al. [35]   | 2018 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Zhao et al. [36]  | 2018 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Zhou et al. [37]  | 2018 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Zhu et al. [38]   | 2018 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Cuscino et al. [9] | 2019 | Sequencing                                  | Conducted using cell lineages samples | N/A                                             | N/A                                                                                        | No                                                                            | No                                                        |
| Huang et al. [4]  | 2019 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | Yes                                                                           | No                                                        |
| Huang, Sun et al. [39] | 2019 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | No                                                        |
| Zhu et al. [1]    | 2019 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Shi et al. [40]   | 2020 | N/A                                         | N/A                  | N/A                                             | N/A                                                                                        | No                                                                            | Yes                                                       |
| Zhang et al. [41] | 2020 | Sequencing                                  | No                   | N/A                                             | 1 per group                                                                                  | No                                                                            | Yes                                                       |

### 3.2. Main Results and Study Quality Assessment

First, we were interested in determining how microRNA candidate selection occurs. As shown in Table 1, from the 35 included articles, 9 employed some type of high-throughput analysis for microRNA candidate selection, of which 1 performed microarray analysis, 3 used sequencing and 5 employed some type of microRNA PCR panel. Regarding the types of samples evaluated in these high-throughput analyses, four articles used non-pooled samples, three used pooled samples, one used a cell lineage, and in one article, the screening analyses were conducted in non-human samples. In total, 2 articles selected microRNA candidates by analyzing public microRNA expression datasets, and the remaining 24 selected target miRNAs from the literature.
Figure 1. Overview of the literature search and selection process.

As shown in Table 2, 32 of the 35 included articles were from East Asia, 1 was from Italy, 1 from the United States and 1 from Mexico, mostly published in 2015 and 2018. The median number (range) of control and case subjects was 43.91 (3–133) and 72.70 (3–185), respectively, and the majority cases included metastatic and non-metastatic patients, except for Tian et al. [33]. Of the 35 studies, 10 did not state whether metastatic patients were included. Serum samples were the evaluated specimens in 28 articles, 5 studies analyzed plasma samples, 1 analyzed PBMC (peripheral blood mononuclear cells) and 1 evaluated microRNA expression in extracellular vesicles. Quantitative real-time PCR was used to evaluate microRNAs expressions in almost all the studies, and microRNA U6 was the most used endogenous reference microRNA for normalization purposes. However, significant differences were found in the included studies, reinforcing the need to evaluate the choice of normalization method to minimize quantitation errors and technical variability in experiments.

In total, 20 studies reported the upregulation of 38 different microRNAs in OS patients, and 16 studies reported that 22 different microRNAs were downregulated in this group of patients. Among the 35 included studies, 10 only described whether the candidate microRNA expression was up- or downregulated, and 25 described microRNAs with diagnostic potential for osteosarcoma, with a diagnostic performance of ≥0.70 AUC. Among the 16 studies that reported sensitivity and specificity, both exceeded 80% in the 3 studies of individual miRNAs and the 1 study of miRNA combinations of four microRNAs, and both exceeded 70% among the 13 individual miRNAs studies and the 1 miRNA combination study.
Several microRNAs showed excellent diagnostic performances for osteosarcoma. The AUC values of MiR-195-5p, MiR-320a and MiR-374a-5p in Lian’s et al. (2015) study [15] were 0.9029, 0.9188 and 0.9173, respectively. In Wang et al.’s (2015) study [17], the AUC value of MiR-152 was 0.956. The best values of sensitivity and specificity were also found for MiR-152 in Wang et al.’s (2015) study, and were of 96.2% and 92.5% [17].

The MiR-320a and MiR-95-3p expressions were evaluated in three different studies each. MiR-320a expression was evaluated in Monterde-Cruz et al.’s (2018) [2], Hui et al.’s (2015) [14] and Lian et al.’s (2015) [15] studies, but only in Lian et al.’s (2015) study was a differential expression observed; MiR-320a was found to be more expressed in OS samples than in control subjects, and the AUC value was 0.9188. MiR-95-3p expression was evaluated in Hui et al.’s (2015) [14], Zhao et al.’s (2018) [36] and Niu et al.’s (2016) [22] studies, but conflicting results were observed. Zhao et al. (2018) found that MiR-95-3p expression was upregulated in OS samples, and did not comment on AUC, sensitivity or specificity values, but Niu et al. (2016) described a lower expression of this microRNA, with an AUC value of 0.863.

Figure 2 shows the QUADAS-2 quality evaluation results. The results indicate that the included studies had low to moderate scores because they had unclear information about patients and the reference standard selection, as well as low applicability concerns.

![Figure 2. Risk of bias assessment using the QUADAS-2 tool.](image-url)
Table 2. Summary of included studies using circulating miRNAs as biomarkers of osteosarcoma.

| Control Group | Case Group |
|---------------|------------|
| Author        | Year | Ethnicity | N | Sex | Mean Age (y) | N | Sex | Mean Age (y) | Metast | Specim | Det | Normaliz. | Method for Expression Level Calculation | Differentially Expressed MicroRNAs | Up- or Down-regulation Description Only | AUC | SEN | SPE |
| Allen-Rhoades et al. [12] | 2015 | American | 30 | N/I | 18 | 40 | 2M 17F | 13.41 | 20 yes, 19 no | plasma | qPCR | miR-320a + miR-15a-5p + CqUniSp2)/3 | 2−ΔCt | miR-205-5p miR-214 miR-335-5p miR-574-3p | No | MiR-205-5p: 0.71 | MiR-214: 0.8 | MiR-335-5p: 0.79 | MiR-574-3p: 0.88 |
| Cai et al. [13] | 2015 | Asian | 60 | N/I | 166 | 96M 70F | <55:72 ≥55:94 | 42 yes, 124 no | serum | qPCR | U6 | 2−ΔΔCt | MiR-195 | No | 0.892 | 88.0% | 83.3% |
| Hui et al. [14] | 2015 | Asian | 20 | 12M 8F | 14.3 | 20 | 13M 7F | 13 | 2 yes, 11 no | serum | qPCR | cel-miR-39 | 2−ΔΔCt | miR-106a-5p miR-16-5p miR-20a-5p miR-25-3p miR-425-5p miR-451a miR-139-5p | No | miR-106a-5p: 0.7255 miR-16-5p: 0.7686 miR-20a-5p: 0.8421 miR-25-3p: 0.7961 miR-425-5p: 0.7765 miR-451a: 0.7961 miR-139-5p: 0.7998 |
| Lian et al. [15] | 2015 | Asian | 90 | 44M 46 F | 16.2 | 90 | 43M 47F | 15.8 | 18 yes, 72 no | plasma | qPCR | comparison of the miRNA concentra. to the serum volume | comparison of the miRNA concentra. to the serum volume | miR-195-5p miR-199a-3p miR-320a miR-374a-5p | No | miR-195-5p: 0.9029 miR-199a-3p: 0.9025 miR-320a: 0.9188 miR-374a-5p: 0.9173 4-miRNAs: 0.608 |
| Tang et al. [16] | 2015 | Asian | 60 | N/I | 116 | 96M 70F | <55:72 ≥55:94 | 42 yes, 124 no | serum | qPCR | U6 | 2−ΔΔCt | MiR-27a | No | 0.867 | 70.01% | 98.30% |
| Wang et al. [17] | 2015 | Asian | 20 | N/I | 80 | 40M 40F | <19:40 ≥19:40 | 12 yes, 68 no | serum | qPCR | U6 | 2−ΔΔCt | MiR-152 | No | 0.956 | 96.2% | 92.5% |
| Wang et al. [18] | 2015 | Asian | 20 | N/I | 100 | 66M 34F | <20:69 ≥20:31 | 42 yes, 58 no | serum | qPCR | U6 | 2−ΔΔCt | MiR-191 | No | 0.858 | 74.00% | 100.0% |
| Yang et al. [19] | 2015 | Asian | 50 | N/I | 108 | 78M 30F | <20:40 ≥20:68 | 40 yes, 68 no | serum | qPCR | RNU6 | 2−ΔΔCt | MiR-221 | No | 0.844 | 65.7% | 100.0% |
| Zhou et al. [20] | 2015 | Asian | 60 | 38M 22F | ≥20:23 ≤20:37 | 60 | 38M 22F | ≥20:23 ≤20:37 | 8 yes, 52 no | serum | qPCR | comparison of the miRNA concentra. to the serum volume | comparison of the miRNA concentra. to the serum volume | MiR-199α-5p | No | 0.8606 | 88.33% | 76.67% |
| Author            | Year | Ethnicity | N Sex | Mean Age (y) | N Sex | Mean Age (y) | Metast Specim | Det Met | Specim | Normaliz. | Method for Expression Level Calculation | Differentially Expressed MicroRNAs | Up- or Down-regulation Description Only | AUC     | SEN    | SPE    |
|-------------------|------|-----------|-------|--------------|-------|--------------|---------------|---------|--------|-----------|----------------------------------------|---------------------------------|-----------------------------------|---------|-------|-------|
| Cao et al. [21]   | 2016 | Asian     | 20    | N/I          | 60    | 32M 28F      | ≤18:37>18:23  | 9 yes, 51 no | serum  | qPCR   | RNU48     | 2−ΔΔCt                                | MiR-326                        | No                                | 0.897   | 83.7% | 94.5% |
| Li et al. [3]     | 2016 | Asian     | 46    | 27M 19F      | 19.6  | 46 27M 19F   | 19.6          | N/I     | serum  | U6       | 2−ΔΔCt                                | MiR-17                        | Yes                               | N/I     | N/I   | N/I   |
| Niu et al. [22]   | 2016 | Asian     | 133   | 71M 62F      | 133   | 133 71M 62F  | ≤15:59>15:74  | 68 yes, 65 no| serum  | qPCR   | U6       | 2−ΔΔCt                                | MiR-95-3p                     | No                                | 0.863   | N/I   | N/I   |
| Pang et al. [33]  | 2016 | Asian     | 130   | N/I          | 185   | 110M 73F    | ≤55:73≥55:112 | 57 yes, 128 no| serum  | qPCR   | U6       | 2−ΔΔCt                                | MiR-497                       | No                                | 0.848   | N/I   | N/I   |
| Sun et al. [24]   | 2016 | Asian     | 62    | N/I          | 62    | N/I          | ≤15:59>15:74  | N/I     | serum  | U6       | 2−ΔΔCt                                | MiR-24                        | Yes                               | N/C     | N/C   | N/C   |
| Zhou et al. [25]  | 2016 | Asian     | 40    | N/I          | 40    | 25M 15F     | >15:27<15:13  | N/I     | serum  | U6       | 2−ΔΔCt                                | MiR-421                       | Yes                               | N/C     | N/C   | N/C   |
| Fujiwara et al. [36] | 2017 | Asian     | 8     | 4M 4F        | 14    | 7M 7F       | 0-10:2 11-20:8≥21:4 | 1 yes, 13 no | serum  | qPCR   | N/I       | 2−ΔΔCt                                | miR-25-3p                      | No                                | MiR-25-3p: 0.868, miR-17-3p: 0.720 | MiR-25-3p: 71.4%, MiR-17-3p: 64.3%, MiR-25-3p: 92.3%, MiR-17-3p: 84.6% |
| Liu et al. [27]   | 2017 | Asian     | 10    | N/I          | 20    | N/I          | N/I           | N/I     | serum  | N/I       | N/I                                  | N/I                             | MiR-598                           | Yes                               | N/C     | N/C   | N/C   |
| Wang et al. [38]  | 2017 | Asian     | 20    | 8M 12F       | 24.5  | 102 54M 48F | Low: 17.3 High: 16.4 | 36 yes, 66 no | serum  | RNU6B | N/I       | N/I                                  | MiR-491                        | Yes                               | N/I     | N/I   | N/I   |
| Xie et al. [29]   | 2017 | Asian     | 3     | N/I          | 3     | N/I          | N/I           | PBMC    | qPCR   | U6       | 2−ΔΔCt                                | hsa-miR-221-5p                  | Yes                               | N/C     | N/C   | N/C   |
| Cong et al. [30]  | 2018 | Asian     | 50    | N/I          | 114   | 62M 52F     | ≥18:71<18:43  | 60 yes, 54 no| serum  | qPCR   | RNU6   | 2−ΔΔCt                                | MiR-124                        | No                                | 0.846   | 79.8% | 86%   |
| Li, Song et al. [31] | 2018 | Asian     | 76    | N/I          | 76    | N/I          | N/I           | N/I     | plasma | qPCR   | U6       | 2−ΔΔCt                                | MiR-542-3p                    | No                                | 0.841   | 77.8% | 93.6% |
| Liu, Zhao et al. [32] | 2018 | Asian     | 95    | N/I          | 95    | 63M 32F     | <20:69≥20:26  | 37 yes, 58 no| serum  | qPCR   | U6       | 2−ΔΔCt                                | MiR-375                      | No                                | 0.89    | 82.1% | 74.7% |
| Monterde-Cruz et al. [2] | 2018 | Mexican   | 15    | 9M 6F        | 20    | 15 9M6F     | 13 yes, 2 no  | serum  | qPCR   | RNU6   | 2−ΔΔCt                                | miR-215-5p                    | No                                | miR-215-5p: 0.8667, miR-642a-5p: 0.8413, 2-miRNAs: 0.8520 | N/I     | N/I   | N/I   |
| Author                  | Year  | Ethnicity | N  | Sex   | Mean Age (y) | N  | Sex   | Mean Age (y) | Metast Specim | Det Met | Normaliz. | Method for Expression Level Calculation | Differentially Expressed MicroRNAs | Up- or Down-regulation Description Only | AUC      | SEN    | SPE    |
|------------------------|-------|-----------|----|-------|--------------|----|-------|--------------|---------------|---------|-----------|----------------------------------------|--------------------------------|----------------------------------|----------|--------|--------|
| Tian et al. [33]       | 2018  | Asian     | 30 | N/I   | ≤12:35      | 65 | 30M   | ≤12:30       | No serum qPCR  | U6      | N/I       | MiR-337-5p                              | No                               | N/C                              | 0.7761   | N/I    | N/I    |
| Xu et al. [34]         | 2018  | Asian     | 30 | N/I   | ≤12:30      | 30 | N/I   | ≤12:30       | serum qPCR     | U6      | N/I       | MiR-411                                | Yes                              | N/C                              | N/C      | N/C    | N/C    |
| Yao et al. [35]        | 2018  | Asian     | 70 | N/I   | ≤55: 84     | 152 | 8M    | ≤55: 68      | serum qPCR     | U6      | 2−ΔΔCt  | MiR-101                                | No                               | 0.850                           | 78.95%   | 82.86% |
| Zhou et al. [36]       | 2018  | Asian     | N/I| N/I   | N/I         | N/I| N/I   | N/I          | serum qPCR     | N/I     | N/I       | MiR-95-3p                               | Yes                              | N/C                              | N/C      | N/C    | N/C    |
| Zhou et al. [37]       | 2018  | Asian     | 50 | N/I   | ≤19: 47     | 98 | 62M   | ≤19: 51      | serum qPCR     | cel-MiR-39 | 2−ΔΔCt  | MiR-139-5p                              | No                               | 0.846                           | 76.5%    | 80%    |
| Zhou et al. [38]       | 2018  | Asian     | 7  | 4M    | ≤19: 24     | 7  | 4M    | ≤19: 20      | serum qPCR     | U6      | 2−ΔΔCt  | MiR-22                                 | Yes                              | N/C                              | N/C      | N/C    | N/C    |
| Cuscino et al. [9]     | 2019  | Italian   | 3  | N/I   | ≤14: 16.8   | 5  | M     | ≤14: 16.8   | plasma Digital PCR | U6      | 2−ΔΔCt  | 5 new microRNA candidates                | Yes                              | N/I                              | N/I      | N/I    | N/I    |
| Huang et al. [4]       | 2019  | Asian     | 30 | 22M   | ≤16: 26     | 50 | 22M   | ≤16: 24      | serum qPCR     | U6 and cel-MiR-39 | ΔCt | MiR-487-a, miR-493-5p, miR-501-3p, miR-502-5p | No                               | miR-487a: 0.83, miR-493-5p: 0.79, miR-501-3p: 0.82, miR-502-5p: 0.83, 4-miRNAs: 0.89 | N/I      | N/I    | N/I    |
| Huang, Sun et al. [39] | 2019  | Asian     | 50 | 32M   | ≤14: 30     | 50 | 3M    | ≤14: 20      | plasma qPCR    | U6, cel-MiR-39 | ΔCt | MiR-663a                             | No                               | 0.86                             | 67.35%   | 89.8%  |
| Shi et al. [40]        | 2020  | Asian     | 60 | N/I   | ≤50: 72     | 124 | 79H   | ≤50: 52      | serum qPCR     | cel-miR-39 | 2−ΔΔCt  | MiR-194                                | No                               | 0.855                           | 84.2%    | 79.1%  |
| Zhang et al. [41]      | 2020  | Asian     | 20 | 12M   | ≤18.5       | 41 | 27F   | ≤18.5       | Extrac. Vesicul. qPCR | U6, Cel-miR-39 e let-7i-5p | 2−ΔΔCt  | MiR-101                                | No                               | 0.7957                          | N/I      | N/I    | N/I    |
4. Discussion

Although perioperative management, surgery and multiagent chemotherapy have greatly evolved in recent years, OS is still the most common malignant bone tumor in children and adolescents [42], with an incidence rate of 4.5/million/year [43], and with a very high morbidity and mortality rate [44]. Besides this, in recent years, no great progressions in OS diagnosis or early detection have been accomplished for clinical application, despite efforts to identify more tumor-related regulators and molecules involved in the growth and metastasis of this tumor [45,46].

MicroRNAs are described as small non-coding RNAs, composed of 22 to 24 nucleotides, which regulate gene expression in several cellular processes. The deregulated expressions of these microRNAs interfere with the cell cycle, potentially causing abnormal cells. This deregulation is closely associated with the development of several pathologies, including cancer [3]. MicroRNAs can be found not only in cells and tissues, but also circulating freely in body fluids, such as serum, plasma and urine, among others. These circulating microRNAs have remarkable stability in body fluids, allowing us, by analyzing their concentrations and compositions, to diagnose diseases, including OS. Thus, circulating microRNAs can act as potential biomarkers in the diagnosis of OS, being minimally invasive and effective in the early detection of the disease [20].

In this context, it is imperative to develop a rigorous strategy for microRNA quantitation experiments to avoid the potential biases introduced by differences in the amounts of starting material, sample collection and quality, RNA isolation, and PCR efficiency. These issues are especially relevant for the reverse transcription quantitative polymerase chain reaction (RT-qPCR) method, which is most commonly used to quantify circulating microRNAs. The use of microRNAs as cancer biomarkers in serum or plasma samples might be compromised if these issues are not taken into consideration.

In this systematic review, we identified a total number of 60 microRNAs from 35 studies evaluating the diagnostic potential of circulating microRNAs for osteosarcoma detection. Interestingly, a large number of studies selected microRNA candidates for expression evaluation by analyzing similar studies involving different types of cancer from the literature. However, their results might be compromised for the reasons mentioned above, and there is often limited overlap between them due to the varied sample sources or analysis means. In this context, miRNA signatures that consist of a variety of different miRNAs provided by large-scale studies are still missing, and would help to provide additional important information and to improve differentiation between pathologies, given that some microRNAs, such as MiR-21 and MiR-20a, are frequently not disease-specific.

Despite the considerable number of studies, the available data are not sufficient for a specific microRNA or group of microRNAs to be established as an OS diagnostic biomarker. First, the sample size was found to be very small in a large number of the included studies, and most of them also did not offer important information about the characterization of the OS and control samples evaluated. The description of the participant’s age, sex and other characteristics, including tumor location, subtype and the presence of metastasis, are important when clarifying to whom the study findings are applicable, allowing them to be generalized or showing their limitations. It would be helpful if the studies included broader population samples from different ethnic groups, especially from high-OS incidence countries, in order to investigate if microRNA expression is ancestry-specific. The inclusion of metastatic patients should also be re-evaluated. While only 1 study did not enroll patients with metastasis, 10 out of the 35 studies did not mention whether metastatic patients were included. Studies performing separate analyses of patients with or without metastasis could help to reveal both metastasis-specific and non-metastasis-specific miRNA biomarkers.

Chemotherapy and surgery may also affect the expression of circulating microRNAs [4,20,35] once antineoplastic drugs, for example, are demonstrated to regulate cell proliferation and angiogenesis, which may have a big impact on microRNAs expression profiling. So, in order to avoid the effect of treatment on microRNA expression, only
pre-therapy OS samples should be included. In 14 of the studies included, however, this information was not available, and patients undergoing chemotherapy treatment were included in 6 of them.

Differences in the execution of methods applied to identify differentially expressed circulating microRNAs can also influence the screening of circulating microRNAs for OS detection, and thus require attention. Specimen types and their preservation [47,48], and microRNA isolation protocols [49], are some examples. MicroRNAs’ expressions and the final quantitative results can also be highly influenced by different normalization methods [50,51]. Quantitative PCR methods of microRNA expression are not currently universal, and there is no consensus about the ideal endogenous reference gene to be used for the normalization of microRNA expression data from patients with OS and other types of cancer. The most used endogenous reference genes in the included studies are RNU6B, cel-miR-39 and U6 snRNA, but other unusual microRNAs, such as MiR-320a, MiR-15a-5p and let-7i-5p, were also found. It is important to reinforce that the expressions of microRNAs used as the endogenous reference should be consistent among all samples and groups, as they play an instrumental role in the evaluation of circulating microRNA expression; as such, the development of a rigorous normalization strategy should be considered to avoid measurement errors.

Although several microRNAs showed excellent diagnostic performances for osteosarcoma, and a large number of microRNAs had their expressions evaluated, the overlapping rates of OS-specific circulating microRNAs were low in the analyzed literature. Only two microRNAs, MiR-320a and MiR-95-5p, were evaluated in three different studies each, and their expressions in different studies were sometimes inconsistent [22,36]. Consequently, future studies should perhaps focus on combining the different microRNA markers already evaluated in a diagnostic model for the early detection of OS, in addition to the identification of more circulating microRNAs, thus enhancing diagnostic power.

5. Conclusions

In conclusion, this systematic review suggests that, although circulating microRNAs hold great potential to be used as diagnostic markers for OS, future studies should consider a more stringent standardization of sample characterization and microRNA quantitation protocols. Verification of these OS-specific microRNAs in large-scale screening studies would also be helpful to determine their diagnostic efficiency for the early detection of OS. Our study highlights that it is imperative to develop a rigorous strategy for microRNA quantitation experiments that allows the use of microRNAs as cancer biomarkers in serum or plasma samples.

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