Relationship of N6-Methyladenosine-Related lncRNAs With Tumor Microenvironment and Clinical Prognosis in Osteosarcoma

Wen-Kang Chen
The First Clinical Affiliated Hospital of Guangxi Medical University
Han-Jing Zhang
University of South China
Yong-Hui Jiang
University of South China
An-Song Liu
University of South China
Ke Yin
University of South China
Pei-Han Xie
University of South China
Ying-Ying Chen
The First Clinical Affiliated Hospital of Guangxi Medical University
Zhu Dai (oliverdai@hotmail.com)
University of South China
Xin-Li Zhan
The First Clinical Affiliated Hospital of Guangxi Medical University

Research Article

Keywords: N6-methyladenosine, long non-coding RNAs, prognosis, tumor microenvironment, osteosarcoma

Posted Date: January 17th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1206956/v1

Abstract

Background: The aberrant expression of long non-coding RNAs (lncRNAs) can affect the occurrence and progression of various cancers, including osteosarcoma (OS), by regulation of N6-methyladenosine (m6A) modification. However, their interaction involved in the prognostic value of OS and tumor microenvironment remains unclear.

Methods: We collected data from TCGA and GEO datasets. Using Pearson correlation, univariate, multivariate, LASSO Cox regression analyses, we identified the prognostic m6A-related lncRNAs and conducted the prognostic cluster and model. Survival analyses were used to analyze different clusters and models, respectively. The receiver operating characteristic (ROC) curve and independent prognosis analysis was used to evaluate the performance of the risk model. We also examined the relationship of the risk model and cluster with immune checkpoint (PD1 and CTLA-4) and tumor microenvironment (TME).

Results: A total of 43 m6A-related lncRNAs have significant prognostic significance for OS patients. Patients of cluster 2 have a longer survival time than cluster 1. CD8+ T cells, resting NK cells, and Monocytes were highly inflamed tissues in cluster 2, while gamma delta T cells and M0 macrophages were lowly in cluster 1. Meantime, OS patients with lower prognosis scores had better overall survival status, and ROC curves proved that the risk model had better prognostic abilities with the area under the curve (AUC) of 0.938 and 0.924 in train and test datasets, respectively. The risk score of 17 m6A-related lncRNAs was identified as an independent risk factor for OS. Both PD1 and CTLA-4 were closely associated with m6A-related lncRNAs and prognostic scores. The risk score was higher in cluster 2, while cluster 1 had a lower purity of tumor cells and a higher density of immune-related cells in the TME.

Conclusions: We established a new m6A-related lncRNAs prognostic biomarker that can predict the prognostic risk of OS was significantly linked with the OS tumor microenvironment.

Introduction

Osteosarcoma (OS) is a common malignant bone tumor predominantly occurring in children and adolescents[1]. Following the development of diagnosis, chemotherapy, surgery, and immunotherapy, the clinical prognosis of OS patients has significantly improved. Unfortunately, the 5-year survival rate for patients with metastases and relapses is less than 20%[2]. Therefore, advances in new therapeutic alternatives and the identification of predictive biomarkers are essential for the development of individualized treatment for OS patients.

N6-methyladenosine (m6A) modification is a common and abundant RNA modification that is pivotal in almost all cellular functions. As a reversible epigenetic modification, m6A regulators can bind to specific nuclear readers and affect the whole process of gene expression by regulatory proteins known as methylases (“writers”), demethylases (“erasers”), and recognizing the chemical signatures (“readers”). Lots of investigations have described that the dysregulated expression of m6A-related regulators may be

significantly associated with patient response to chemoradiotherapy, metastasis, and poor prognosis of OS[3]. A recent study confirmed that m6A writer METTL3 and METTL14 were significantly related to the poor clinical prognosis of OS[4]. Additionally, the METTL3 expression level is high in human OS tissues and overexpression of it can promote cell proliferation, migration, and invasion via increasing the m6A methylation level of LEF1[5]. Conversely, overexpression of METTL14 can decrease the proliferation, migration, invasion, and increased apoptosis in human OS cells by activating caspase-3[6]. The function of m6A modification in OS is becoming increasingly clear, but the underlying molecular mechanism of m6A modification in OS is still in its infancy.

Long non-coding RNAs (lncRNAs) are non-coding transcripts with larger than 200 nucleotides in length and play a critical role in the progression of OS via affecting virtually all biological functions, including regulation of transcriptional and post-transcriptional levels, or directly forming RNA-protein complexes[7]. It has been demonstrated that aberrant expression of lncRNAs can participate in the regulation of m6A modification and affect the occurrence and progression of various cancers, such as OS, prostate cancer, hepatocellular carcinoma, and so on[8]. Studies by Chen et al. revealed that m6A eraser ALKBH5 overexpression is involved in the regulation of chemoresistance and predicts poor prognosis in patients with OS[9]. In addition, ALKBH5 can also enhance the stability of lncRNA Plasmacytoma Variant Translocation 1 (PVT1) and thus promote OS growth[10]. However, the specific role of m6A-related lncRNAs remains unclear; therefore, illustrating the mechanism of these regulators in the development of OS may be useful for prognostic targets.

The tumor microenvironment (TME) is mainly composed of three cell styles: tumor cells, stromal cells, and tumor-infiltrating immune cells. Meantime, the ESTIMATE score of TME could estimate the abundance of infiltrating immune cells and stromal cells based on gene expression. A good understanding of the tumor immune microenvironment is crucial for the occurrence and progression of cancers to improve immunotherapy[11]. Research shows that targeting stromal cells and immunological checkpoint (programmed cell death-1 (PD-1) and cytotoxic T-lymphocyte-associated antigen-4 (CTLA-4)) can be a new way to improve the therapeutic effect of osteosarcoma effectively[12, 13]. Thus, it is important to clarify the relationship of TME with the clinicopathological parameters of OS.

In this context, clinical information and gene expression data were abstracted from The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) public databases. Based on bioinformatics and statistical analysis of the data from 88 OS patients, we identified 43 out of 535 m6A-related lncRNAs had prognostic value. Furthermore, we constructed an m6A-related lncRNA prognostic signature according to the ability of 17 m6A-related lncRNAs to predict the clinical outcomes of OS patients. In the meanwhile, a novel high-low risk prognostic model and consensus cluster model of m6A-related lncRNAs were identified to forecast the clinical prognosis of OS. Moreover, we also explored the relationship between the model and TME as well as immune cells.

Materials And Methods

Patients and samples

Data acquisition.

The TARGET-OS RNA-sequencing dataset and corresponding clinical characteristics were downloaded from TCGA (https://portal.gdc.cancer.gov/projects/TARGET-OS). The inclusion criteria were as follows: 1) patients with a diagnosis of OS; and 2) clinical data including age, gender, survival time, and metastasis There was no prognostic information, and clinical characteristics of some patients were also enrolled. In summary, 86 patients with OS were included. GSE21257, GSE28424, and GSE19276 were downloaded from the GEO (www. ncbi.nlm.nih.gov/gds) were downloaded to validate the differential expression of m6A-related lncRNA genes in OS.

Selection and consensus clustering of m6A-related lncRNAs

We obtained 23 m6A genes were from the TCGA database, which compose of the writer (METTL14, METTL3, METTL16, VIRMA, RBM15B, ZC3H13, WTAP, and RBM15), eraser (FTO and ALKBH5), and reader (HNRNPA2B1, YTHDF1, YTHDF2, YTHDF3, HNRNPC, YTHDC1, YTHDC2, FMR1, IGFBP1, IGFBP2, IGFBP3, RBMX, and LRPPRC). Pearson's correlation analysis was used to screened m6A-related lncRNAs, and 535 m6A-related lncRNAs were identified. The criteria of the process are |Pearson R| >0.4 and p <0.001. The expression of m6A genes and prognostic lncRNAs were obtained using the "limma" package in R software.

The identification of 43 prognostic m6A-related lncRNAs was carried out using univariate Cox regression analysis with a cut-off of $p < 0.05$. Cluster analysis was performed to divide patients into two clusters (cluster 1 and cluster 2) using the "ConsensusClusterPlus" R package.

Gene set enrichment analysis

To explore the difference in the underlying molecular signaling mechanisms between the two cluster groups, GSEA-4.1.0 software from the website (https://www.gsea-msigdb.org/gsea/index.jsp) was performed to investigate enriched items. The Gene sets with P adjusted value < 0.05 were selected as statistically significant enrichment.

Tumor Microenvironment Assessment

To evaluate tumor microenvironment and prognostic m6a-related lncRNAs, the ESTIMATE algorithm was employed to calculate the TME-related score of each OS patient based on the "estimate" R package. The infiltration of 22 immune cells was also analyzed via the CIBERSORT algorithm. Pearson correlation analysis was used to investigate the relationship between the cluster and immune cell infiltration. Finally, we preserved the statistically significant immune cell differences in OS *($P < 0.05$)* for subsequent analyses. The correlations of the immune checkpoint with the risk score and prognostic m6A-related lncRNAs were also demonstrated by the R package.

Construction and validation of risk model and independent prognostic analysis

A total of 43 m6A-related lncRNA prognostic factors in the LASSO regression were analyzed by multivariate analysis. The risk score was generated as follows: risk score = sum(each gene's expression levels × corresponding coefficient) / sum(each gene's mean expression levels × the corresponding coefficient). Using "caret" and "glmnet" R package, 17 m6A-related lncRNA prognostic model was constructed and patients are included in train (construction of risk model) and test sets (validation of risk model), respectively. The risk score was calculated as follows: risk score = coe1*exp1+ coe2*exp2+……+. Then, the OS patients or high-risk or low-risk groups were identified based on the median risk score. The Kaplan–Meier survival curve was employed to evaluate the prognostic ability of the prognostic risk model. The AUC value of the ROC curve was used to examine the sensitivity and specificity of the risk model.

The univariate and multivariate Cox regression analyses were used to assess the independent prognostic role of risk score and some clinical parameters (including gender, metastases, and age). $P < 0.05$ was considered to be statistically significant.

Survival and clinical information Analysis

To explore the differences in the prognosis of OS, the Kaplan–Meier survival curve was carried out. Firstly, we employed the overall survival in different clusters. Subsequently, the survival time of patients in high- and low-risk groups was also compared with Kaplan–Meier survival curves. In addition, and clinical features in each subgroup, including gender, age, and metastases, were also evaluated using the Kaplan–Meier survival curve. The distributions of the risk model, clusters, gender, age, metastases, and prognostic m6A-related lncRNA were visualized by the "heatmap" R package.

Results

Identified M6A-related LncRNAs in Patients with OS

The detailed flow diagram for the prognostic model is shown in Figure 1. Depending on the downloaded data file from the TCGA database, the matrix expression of 23 m6A genes and 14086 lncRNAs was screened. LncRNAs that were significantly related to greater than or equal to one of the 23 m6A genes (|Pearson R|≥0.4; P-value<0.001) were defined as m6 A-related lncRNAs. Then, 535 m6A-related lncRNAs were obtained via Pearson correlation analysis and validated by the GEO database (GSE21257, GSE28424, and GSE19276). Finally, the m6A-lncRNA coexpression network was visualized using the network chart (Figure 2A). Given that lncRNAs exert a decisively favorable influence on tumorigenesis and development, univariate Cox regression was further performed to uncover the underlying relations between m6A-related lncRNAs and prognosis value (P value<0.05). Results demonstrated that 43 m6A-related prognostic lncRNAs were significantly correlated with prognosis (Figure 2B). As well, the co-expression relationships between every m6A-related prognostic lncRNAs and immune checkpoints were also analyzed. Accordingly, SNHG12, LINC01357, SNHG7, GAS5, OLMALINC, ADAMTS9-AS2, SNHG6,

AC079089.1, SNHG1, and SNHG4 were positively correlated with PD1 and CTLA-4, while FGD5-AS1, MSC-AS1, AC090559.1, and AC008074.2, were negatively correlated with them (Figure 2C and D). In summary, the results suggest that there are significant associations between immune checkpoints and m6A-related lncRNAs in OS.

Consensus Clustering of M6A-Related Prognostic LncRNAs in OS Patients

To uncover the underlying role of prognosis-associated m6A-related lncRNAs in the development of OS, consensus clustering was used to group OS patients, cluster1 and cluster2, based on the expression of 14 m6A-related prognostic lncRNAs. The matrix heat map for $k = 2$, also called CM plots, reveals the classification effect between the two clusters (Figure 3A). To find out the k for which the distribution reaches an approximate maximum, indicating maximum stability, the common clusters for $k = 2$ through 9 were reflected by the empirical cumulative distribution function (CDF) (Figure 3B). The score of the delta area plot illustrates the relative increase in cluster stability (Figure 3C). Together, a total of 85 patients with OS were divided into clusters 1 (n = 36) and 2 (n = 49).

To evaluate the different prognoses of patients between the two clusters, a Kaplan-Meier survival analysis was conducted. Results demonstrated that patients of cluster 2 had a longer survival time than cluster 1 (Figure 3D, P = 0.001). To figure out the possible associations between the m6a prognosis-related lncRNAs and clinical characteristics (metastasis, gender, and age), a heat map A heatmap was used to analyze their relationship (Figure 3E). Results demonstrated these clinical features were no significant difference in the two clusters. Meantime, the expression of most m6A-related lncRNAs such as AC110015.1, LINC01549, and AC010609.1 was highly expressed in cluster 1, while others such as PAXIP1−AS2 and MSC−AS1 were highly expressed in cluster 2.

Potential Biological Functions of the Two Clusters

To further clarify the potential biological process and pathway relating to the specific molecular differences between two subgroups, Gene Set Enrichment Analysis (GSEA) including Gene Ontology (GO), KEGG pathway, and tumor hallmark analysis was carried out from the TCGA database in different clusters (Table S1). In terms of GO cell component (CC), the gene sets were enriched in the basement membrane and collagen-containing extracellular matrix (ECM) (Figure 4A). The ECM structural constituent and receptor serine-threonine kinase binding are significantly enriched categories in GO molecular function (MF) (Figure 4B). The GO biological processes (BP) were mainly enriched in vitamin biosynthetic process, pigment granule localization, prostaglandin, and astrocyte development relative to cluster 2, while negative regulation of cell division and replacement ossification were enriched in cluster 1 (Figure 4C). Furthermore, KEGG analysis showed that the gene sets were mainly enriched in focal adhesion, mitogen-activated protein kinase (MAPK) signaling pathway, Hedgehog (Hh) signaling pathway, and so on, compared with cluster 1 (Figure 4D). Additionally, Hallmark analysis revealed that the gene sets were mainly enriched in apical surface, coagulation, apical junction, Hh signaling, epithelial-mesenchymal transition, and cholesterol homeostasis (Figure 4E). The outcome revealed that the cellular biological effects related to the m6A-related lncRNAs in OS.

M6A-related lncRNAs and TME in OS Patients

Herein, we further explore the immune infiltration ratio of different tumor-infiltrating immune cells in OS tissues. The CIBERSORT algorithm was used to analyze 22 different immune cell types in two clusters. Results revealed that infiltration fraction of CD8+ T cells, gamma delta T cells, resting NK cells, Monocytes, and M0 Macrophages showed obvious differences in two clusters (Figure 5A). Compared with cluster 2, cluster 1 has the lower levels of all immune cell types including CD8+ T cells, resting NK cells, and Monocytes, and higher levels of gamma delta T cells and M0 macrophages (Figure 5B). By the important roles of TME in tumorigenesis, the ESTIMATE evaluation is performed to examine stromal and immune scores for all OS samples. Here, there was no significant difference in immune scores between the two clusters. Furthermore, the ESTIMATE and stromal scores of cluster 2 were higher than that of cluster1 (Figure 5C). These results may give us some insights into the m6A-related lncRNA patterns that may remarkably influence specific immune cell types and TME, thus potentially suppressing or strengthening the response to immunotherapy.

Prognostic Analysis of Risk Model in M6A related lncRNAs

LASSO Cox regression analysis is extensively applied to heighten the forecast accuracy and interpretability of the statistical model. The application of this method is available to select the best prognostic markers to predict clinical results[14]. Consequently, 17 out of 43 m6A-related lncRNAs were discerned for the subsequent analysis (Figures 6A). To develop a signature for prognosis prediction of OS, patients (n=85) were divided into train set (n=44) and internal test set (n=41). Based on the median value of the prognostic risk grade, all sets were used to construct a high-low risk group to assess the prognostic risk of OS patients. The distribution of risk grade and the survival status of patients between the low-risk and high-risk groups are depicted in Figure 6B. Results revealed that the number of deaths is positively related to the risk score. Meanwhile, the relative expression standards of the 17 m6A-related lncRNAs for each patient are shown in Figure 6C. The expression of FGD5-AS1, SRP14-AS1, AC106771.1, ZFPM2-AS1, AC090559.1, AC009113.1, AC008074.2 were associated with the low-risk group, while BCAR3-AS1, AC110015.1, AC104461.1, AL121957.1, GAS5, MIR210HG, AC010609.1, AC025917.1, L161729.1, and SNHG4 were associated with the high-risk group. Kaplan-Meier survival analysis demonstrated that OS patients with the overall survival of higher risk scores were worse than lower risk scores (P 0.001) (Figure 6D).

Validation of Risk Model in M6A related lncRNAs

To validate the predictive capability of this established model, the distribution of risk grade, survival status, and expression of the m6A-related lncRNAs were assessed in the test set (Figure 7A and B). Kaplan-Meier Survival analyses showed the overall survival of the low-risk group was longer than that of the high-risk group (P 0.001) (Figure 7C). Results were consistent with the train set. Subsequently, we further analyzed the ROC curves in train and test sets (Figure 7D) and proved that candidate m6A-related lncRNAs had excellent predictive abilities for OS (AUC = 0.938 and 0.924, respectively). Finally, a heatmap for the correlated analysis was used among 17 m6A-related lncRNAs and the clinic characteristics

(gender, age, and metastasis), clusters, and immune scores, respectively (Figure 7E and F). The expression of m6a prognosis-related lncRNAs in high-low risk groups was not different, while risk score was related to metastasis, immune scores, and clusters (P<0.05).

Independent prognostic analysis of Risk Model

Based on univariate and multivariate Cox regression analyses, the independent prognostic analysis of clinical features was performed to evaluate the risk model. We compared the risk scores and clinicopathological parameters including age, gender, and metastasis of OS in the train and test model, respectively (Figure 8). We found that risk score was an independent prognostic risk factor for OS (P<0.05). Furthermore, the subgroup analysis was applied to validate whether our model is useful to patients with different clinical parameters such as age, sex, and metastasis (Figure 9). Results revealed that our model could be used to the following different clinical characteristics: age (<=15 or >15), sex, and metastasis (P<0.05). These results have shown that the prognostic risk model of the 17 m6A-related lncRNAs for OS was comparatively dependable.

Evaluation of Risk Model with Immune cell substyles and immune checkpoint

To assess the relationship of immune cell substyles and immune checkpoints with risk scores, a scatter plot was depicted to validate this nexus. we found a positive correlation between risk scores and numbers of M0 macrophages, and negative correlations between risk scores and numbers of CD8 T cells and activated memory CD4 T cells (Figures 10A-C). The immune checkpoint expression of PD-1 and CTLA-4 were negatively correlated with the risk score (Figures 10D and E).

Discussion

Numerous recent studies have shown that dysregulated m6A modification is pivotal in tumorigenesis, invasion, metastasis, and innate immune of OS, which may bring breakthroughs in the diagnosis and treatment of OS[15]. Furthermore, aberrant expression of lncRNA is strongly related to the tumor initiation and progression in the tumor via targeting m6A regulators[16]. However, the clinical relevance of the m6A-related lncRNAs in OS remains unclear. Therefore, we conducted an m6A-related lncRNA prognostic marker to explore the possible therapeutic targets in OS patients.

With the development of next-generation sequencing and microarray technologies at the genome level, the public databases including GEO and TCGA enable investigators worldwide to identify differentially expressed genes (DEGs) involved in the onset and development of OS and other diseases[17, 18]. In the study, we investigated the roles of m6A-related lncRNAs and constructed a novel m6A-related lncRNA-based risk signature in OS based on the GEO and TCGA database. First of all, we identified 535 lncRNAs that were closely related to 22 m6A-related regulators. 43 out of 535 m6A-related lncRNA were further analyzed to have significant prognostic significance for OS patients. Thereafter, we identified two clusters of OS patients using consensus clustering to clarify the relationship of m6A-related lncRNAs with the prognosis and immune landscape of OS. Based on a Kaplan-Meier survival analysis, the survival time of

cluster 2 is longer than cluster 1, indicating that m6A-related lncRNAs were related to the prognosis of patients with OS. Furthermore, potential biological functions were examined by GSEA in two clusters. The GSEA results for the GO and Hallmark revealed that most gene sets were related to tumor prognosis. Meanwhile, the GSEA results for KEEN also showed different expression gene sets were significantly associated with OS prognosis-related signaling pathways such as focal adhesion, MAPK signaling pathway, and Hh signaling pathway[19–21]. Additionally, the fraction of some immune infiltration cells, ESTIMATE score, and stromal score had shown obvious differences in different clusters, indicating m6A-related lncRNAs might be closely associated with TME of OS. Consequently, these findings impetus us to whether the m6A-related lncRNAs can be served as an available prognosis biomarker in OS.

We further explore the role of m6A-related biomarkers and therapeutic targets in OS. Base on the expression and coefficient of candidate 17 m6A-related lncRNAs, we established a novel risk signature. Among these 17 m6A-related lncRNA biomarkers, BCAR3-AS1, AC110015.1, AC104461.1, AL121957.1, GAS5, MIR210HG, AC010609.1, AC025917.1, AL161729.1, and SNHG4 were high-risk associated, whereas FGD5-AS1, SRP14-AS1, AC106771.1, ZFPM2-AS1, AC090559.1, AC009113.1, and AC008074.2 were protective (Table 1). Among them, overexpression of miR210HG is positively associated with the poor prognosis in OS patients[22]. Additionally, patients with the low-risk signature of m6A-related lncRNAs are beneficial to the survival time and closely associated with the survival rate of patients with OS. The result of our study is consistent with these researches of m6a-related biomarkers involved in the prognosis in OS[4, 23]. The risk score of m6A-related lncRNAs was identified as an independent risk factor for OS. More importantly, the risk score for cluster 1 with poor survival time was higher than that for cluster 2 with longer survival time, thus confirming the consistency of the two methods for predicting prognosis in OS patients. The AUC values in both the train and test set for overall survival were greater than 0.9, suggesting that the risk model had excellent stability and performance in predicting the prognosis of OS patients.

Infiltrating immune cells in the TME are closely connected with the prognosis of OS patients[24]. In our study, we attempted to evaluate the relationship between the risk signature and the immune microenvironment in OS. Based on the results, patients with high immune scores had lower risk scores than those with low immune scores. It has been demonstrated that high immune scores have a better prognosis in OS patients[25], which is consistent with the result of our m6A-related lncRNA risk model. In addition, a positive relationship was found between the risk score and the infiltrating proportion of M0 macrophages, whereas negative correlations were found between the risk score and the infiltration of activated memory CD4+ T cells and CD8+ T cells. Previous studies also revealed dysfunctional or exhausted T cells that existed in osteosarcoma patients are critically involved in OS initiation and progression[26]. As well, our result coincided with the research of the high infiltration of M0 macrophages is related to worse clinical outcomes in OS[27]. Therefore, m6A-regulated lncRNAs might serve as critical modulators in the immune infiltration response of OS.

Recently, immunotherapy related to immune checkpoint inhibitors has received considerable attention in many cancers, including OS[28]. Common immune checkpoint PD-1 and CTLA-4 are known to be related

to the OS progress and prognosis. Interestingly, an anti-PD-1-antibody-treated mouse model of OS obtains significantly fewer pulmonary metastasis[29]. In this study, we demonstrate that most m6A-related lncRNAs were related to the expression of PD1 and CTLA-4 in OS and the risk score had a significantly negative correlation with them. Hence, these m6A-related lncRNA-based risk signatures may function as promising predictors, and targeting these lncRNAs may be a possible method to enhance immunotherapy in OS.

There are some limitations of the study. We extracted a novel m6A-related lncRNA prognosis signature, but their specific relationship with m6A regulators should be verified. Meanwhile, our findings are based on public databases, and external validation by relevant clinical datasets needs to be emphasized. Finally, the potential functional roles and mechanisms of m6A-related biomarkers have not been fully investigated. As a result of these restrictions, future work in OS should include an illustration of the potential role of m6A-related lncRNAs in vivo and in vitro experiments.

In summary, we have constructed a novel predictive model based on 17 m6A-related lncRNAs using comprehensive bioinformatics analysis. Meanwhile, we established a unique prognostic model that has better prognostic abilities to predict the prognosis of OS patients and prognostic m6A-related lncRNAs were closely associated with TME. Our findings could motivate researchers to concentrate on the potential roles and detailed mechanisms of m6A-related lncRNAs in OS, allowing for the discovery of effective therapeutic targets for OS treatment and an improvement in OS patient prognosis.

Abbreviations

LncRNAs: long non-coding RNAs; OS: osteosarcoma; N6-methyladenosine: m6A; TME: tumor microenvironment; AUC: the area under the curve; ROC: receiver operating characteristic; PVT1: Plasmacytoma Variant Translocation 1; PD-1: programmed cell death-1; CTLA-4: cytotoxic T-lymphocyte-associated antigen-4; TCGA: The Cancer Genome Atlas; GEO: Gene Expression Omnibus; CDF: cumulative distribution function; GO: Gene Ontology; GSEA: Gene Set Enrichment Analysis; CC: cell component; ECM: extracellular matrix; MF: molecular function; BP: biological processes; MAPK: mitogen-activated protein kinase; Hh: Hedgehog; DEGs: differentially expressed genes.

Declarations

Data Availability Statement:

The dataset from the TCGA database generated and/or analyzed during the current study are available in the TCGA dataset repository (https://tcga-data.nci.nih.gov/tcga/) and Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/). And all data and materials for this study shall be availed whenever requested by the editorial team, reviewers, and other users.

Author Contributions:

Xin-li Zhan and Zhu Dai conceived the study and performed the bioinformatics analyses. Ying-Ying Chen and Pei-Han Xie downloaded and organized the clinical and gene expression data. Liu An-Song and Ke Yin performed the statistical analyses. Wen-Kang and Han-jing Zhang wrote the manuscript. Yong-Hui Jiang critically revised the article for essential intellectual content and administrative support. All authors reviewed and approved the final manuscript.

Acknowledgments

We are grateful to Dr. Xin-li Zhan (Spine and Osteopathy Ward, The First Affiliated Hospital of Guangxi Medical University) and Dr. Zhu Dai (the First Affiliated Hospital, Department of Orthopaedics) for his kindly assistance in all stages of the present study.

Funding

This work was supported by grants from the Health Commission of Hunan Province (20201907) and the Natural Science Foundation of Hunan Province (2021JJ40497).

a) Ethics approval and consent to participate

Not applicable. All data in this study are publicly available.

b) Consent for publication

Not applicable.

c) Competing interest:

We declare that we do not have any competing interests.

References

1. Chen C, Xie L, Ren T, Huang Y, Xu J, Guo W: **Immunotherapy for osteosarcoma: Fundamental mechanism, rationale, and recent breakthroughs**. *Cancer Lett* 2021, **500**:1–10.
2. Meyers PA, Healey JH, Chou AJ, Wexler LH, Merola PR, Morris CD, Laquaglia MP, Kellick MG, Abramson SJ, Gorlick R: **Addition of pamidronate to chemotherapy for the treatment of osteosarcoma**. *Cancer* 2011, **117**(8):1736–1744.
3. Zhang Y, Wang Y, Ying L, Tao S, Shi M, Lin P, Wang Y, Han B: **Regulatory Role of N6-methyladenosine (m(6)A) Modification in Osteosarcoma**. *Front Oncol* 2021, **11**:683768.
4. Li J, Rao B, Yang J, Liu L, Huang M, Liu X, Cui G, Li C, Han Q, Yang H *et al*: **Dysregulated m6A-Related Regulators Are Associated With Tumor Metastasis and Poor Prognosis in Osteosarcoma**. *Front Oncol* 2020, **10**:769.
5. Miao W, Chen J, Jia L, Ma J, Song D: **The m6A methyltransferase METTL3 promotes osteosarcoma progression by regulating the m6A level of LEF1**. *Biochem Biophys Res Commun* 2019, **516**(3):719–

725.

6. Liu Z, Liu N, Huang Z, Wang W: **METTL14 Overexpression Promotes Osteosarcoma Cell Apoptosis and Slows Tumor Progression via Caspase 3 Activation**. *Cancer Manag Res* 2020, **12**:12759–12767.
7. Ghafouri-Fard S, Shirvani-Farsani Z, Hussein BM, Taheri M: **The critical roles of lncRNAs in the development of osteosarcoma**. *Biomed Pharmacother* 2021, **135**:111217.
8. Dai F, Wu Y, Lu Y, An C, Zheng X, Dai L, Guo Y, Zhang L, Li H, Xu W *et al*: **Crosstalk between RNA m(6)A Modification and Non-coding RNA Contributes to Cancer Growth and Progression**. *Mol Ther Nucleic Acids* 2020, **22**:62–71.
9. Wang Y, Zeng L, Liang C, Zan R, Ji W, Zhang Z, Wei Y, Tu S, Dong Y: **Integrated analysis of transcriptome-wide m(6)A methylome of osteosarcoma stem cells enriched by chemotherapy**. *Epigenomics* 2019, **11**(15):1693–1715.
10. Chen S, Zhou L, Wang Y: **ALKBH5-mediated m(6)A demethylation of lncRNA PVT1 plays an oncogenic role in osteosarcoma**. *Cancer Cell Int* 2020, **20**:34.
11. Wang Q, Hu B, Hu X, Kim H, Squatrito M, Scarpace L, de Carvalho AC, Lyu S, Li P, Li Y *et al*: **Tumor Evolution of Glioma-Intrinsic Gene Expression Subtypes Associates with Immunological Changes in the Microenvironment**. *Cancer Cell* 2018, **33**(1):152.
12. Shi X, Li X, Wang H, Yu Z, Zhu Y, Gao Y: **Specific inhibition of PI3Kdelta/gamma enhances the efficacy of anti-PD1 against osteosarcoma cancer**. *J Bone Oncol* 2019, **16**:100206.
13. Zhang K, Dong C, Chen M, Yang T, Wang X, Gao Y, Wang L, Wen Y, Chen G, Wang X, *et al*: **Extracellular vesicle-mediated delivery of miR-101 inhibits lung metastasis in osteosarcoma**. *Theranostics* 2020, **10**(1):411–425.
14. Xu F, Huang X, Li Y, Chen Y, Lin L: **m(6)A-related lncRNAs are potential biomarkers for predicting prognoses and immune responses in patients with LUAD**. *Mol Ther Nucleic Acids* 2021, **24**:780–791.
15. Hu Y, Zhao X: **Role of m6A in osteoporosis, arthritis, and osteosarcoma (Review)**. *Exp Ther Med* 2021, **22**(3):926.
16. Yi YC, Chen XY, Zhang J, Zhu JS: **Novel insights into the interplay between m(6)A modification and non-coding RNAs in cancer**. *Mol Cancer* 2020, **19**(1):121.
17. Wu G, Zhang M: **A novel risk score model based on eight genes and a nomogram for predicting overall survival of patients with osteosarcoma**. *BMC Cancer* 2020, **20**(1):456.
18. Blum A, Wang P, Zenklusen JC: **SnapShot: TCGA-Analyzed Tumors**. *Cell* 2018, **173**(2):530.
19. Weber M, Soder S, Sander J, Ries J, Geppert C, Kesting M, Wehrhan F: **Craniofacial Osteosarcoma-Pilot Study on the Expression of Osteobiologic Characteristics and Hypothesis on Metastasis**. *Front Oncol* 2020, **10**:745.
20. Huang Z, Barker D, Gibbins JM, Dash PR: **Talin is a substrate for SUMOylation in migrating cancer cells**. *Exp Cell Res* 2018, **370**(2):417–425.
21. Han J, Shen X: **Long noncoding RNAs in osteosarcoma via various signaling pathways**. *J Clin Lab Anal* 2020, **34**(6):e23317.

22. Li J, Wu QM, Wang XQ, Zhang CQ: **Long Noncoding RNA miR210HG Sponges miR-503 to Facilitate Osteosarcoma Cell Invasion and Metastasis**. *DNA Cell Biol* 2017, **36**(12):1117–1125.
23. Zhang P, Xu K, Wang J, Zhang J, Quan H: **Identification of N6-methyladenosine related LncRNAs biomarkers associated with the overall survival of osteosarcoma**. *BMC Cancer* 2021, **21**(1):1285.
24. Heymann MF, Lezot F, Heymann D: **The contribution of immune infiltrates and the local microenvironment in the pathogenesis of osteosarcoma**. *Cell Immunol* 2019, **343**:103711.
25. Hong W, Yuan H, Gu Y, Liu M, Ji Y, Huang Z, Yang J, Ma L: **Immune-related prognosis biomarkers associated with osteosarcoma microenvironment**. *Cancer Cell Int* 2020, **20**:83.
26. Liu MX, Liu QY, Liu Y, Cheng ZM, Liu L, Zhang L, Sun DH: **Interleukin-35 suppresses the antitumor activity of circulating CD8(+) T cells in osteosarcoma patients**. *Connect Tissue Res* 2019, **60**(4):367–375.
27. Le T, Su S, Shahriyari L: **Immune classification of osteosarcoma**. *Math Biosci Eng* 2021, **18**(2):1879–1897.
28. Thanindratarn P, Dean DC, Nelson SD, Hornicek FJ, Duan Z: **Advances in immune checkpoint inhibitors for bone sarcoma therapy**. *J Bone Oncol* 2019, **15**:100221.
29. Zheng B, Ren T, Huang Y, Sun K, Wang S, Bao X, Liu K, Guo W: **PD-1 axis expression in musculoskeletal tumors and antitumor effect of nivolumab in osteosarcoma model of the humanized mouse**. *J Hematol Oncol* 2018, **11**(1):16.

Tables

Table1.The m6A related-lncRNAs in the prognostic classifier associated with

osteosarcoma in the TCGA data set.

Figures

Figure 1

Flow chart involved in data analysis of m6A-targeted lncRNAs in OS. LncRNAs: long non-coding RNAs; OS: osteosarcoma; N6-methyladenosine: m6A;TCGA: The Cancer Genome Atlas;GSEA: Gene Set Enrichment Analysis.

Figure 2

M6A-related lncRNAs and immunological checkpoint in OS patients. (A) Sankey relational diagram for 22 m6A regulators and m6A-related lncRNAs. (B) Univariate Cox regression was preformed to identify m6A-

lncRNAs	LASSO Coeffificient	Univariate cox regression analysis			
		HR	HR.95L	HR.95H	P-value
BCAR3-AS1	1.36	7.64	1.28	45.53	0.03
FGD5-AS1	-0.69	0.45	0.25	0.81	0.01
AC110015.1	0.04	2.08	1.31	3.30	0.00
AC104461.1	0.41	2.34	1.11	4.92	0.03
AL121957.1	1.22	2.36	1.24	4.47	0.01
GAS5	0.06	1.96	1.34	2.88	0.00
MIR210HG	0.13	1.57	1.07	2.31	0.02
SRP14-AS1	-0.15	0.33	0.13	0.85	0.02
AC106771.1	-0.94	0.11	0.01	0.83	0.03
ZFPM2-AS1	-0.21	0.45	0.23	0.88	0.02
AC010609.1	0.20	1.52	1.20	1.94	0.00
AC090559.1	-0.31	0.33	0.17	0.65	0.00
AC009113.1	-0.47	0.47	0.25	0.91	0.02
AC008074.2	-0.68	0.04	0.00	0.65	0.02
AC025917.1	0.08	2.45	1.02	5.92	0.05
AL161729.1	0.70	2.72	1.23	5.99	0.01
SNHG4	0.17	2.09	1.32	3.33	0.00

related lncRNAs with prognostic value. (C and D) The correlations of the 43 prognostic m6A-related lncRNAs with PD1 and CTLA-4, respectively. *P < 0.05, **P < 0.01.

Figure 4

The function analysis of different gene sets in OS. Part of GSEA results are listed as above. m6A related lncRNAs were enriched in multiple cancer-related functions and pathways, P < 0.05.

Figure 5

Immune characteristics in two m6A related lncRNA patterns. (A) Differences in the levels of infiltration of the 22 immune cells in m6A related lncRNA patterns. (B) Different expression of ESTIMATE score, immune score and stromal scorein three m6A Patterns.

Figure 6

Construction of prognostic risk model based on 17 m6A-related lncRNAs. (A) LASSO2 regression was performed to select 17 prognostic m6A-related lncRNAs to calculate the minimum criteria and coefficients. (B and C) Risk related curve, heatmap and spot plot of train set in high and low-risk groups. (D) Kaplan-Meier survival curve of train set was constructed for comparing the survival time between high and low-risk groups.

calculating the minimum criteria (A,B) and coefficients

Figure 7

Validation of risk model in m6A-related lncRNAs. (A and B) Risk related curve, heatmap and spot plot of test set in high and low-risk groups. (C) Kaplan-Meier survival curve of test set was constructed for comparing the survival time between high and low-risk groups. (D) ROC curve to evaluate the accuracy of risk model to predict the survival time of OS. Left is train group, while right is train group, AUC>0.7. (E) Heatmap of clinical features, immune score, cluster and prognostic lncRNAs in high/low-risk group. * *P*<0.05, ** *P*<0.01. (F) Comparison of risk score in the different sample classification of Cluster 1/2, high/low immune score, and metastasis, *P*<0.05.

Figure 8

Independent prognostic analysis in train and test sets. Multivariate and univariate analysis presented risk scores were risk factors for the prognosis of OS, *P<0.05.*

Figure 10

The relationship between risk score and immune cells and immune checkpoint

(A-C) Scatter plots of immune cells showed M0 Macrophages is positive related with risk score, R>0 and *P<0.05*. CD8 T cells and activated memory CD4 T cells are negative related with risk score, R□0 and *P<0.05*. (D and E) The gene expression of PD1 and CTLA-4 are higher in low risk group *(P□0.05)*.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.doc