MAD2L1 Promotes Ewing's Sarcoma Progression Through AURKA/MYC Axis

Huishou Chen  
Sun Yat-sen University Cancer Center

Jing Hu  
The Six Affiliated Hospital of Sun Yat-sen University

Juan Wang  
Sun Yat-sen University Cancer Center

Juan Liu  
Sun Yat-sen University Cancer Center

Binbin Chen  
Sun Yat-sen University Cancer Center

Yu Zhang  
Sun Yat-sen University Cancer Center

Mengjia Song  
Sun Yat-sen University Cancer Center

Mengzhen Li  
Sun Yat-sen University Cancer Center

Ye Hong  
Sun Yat-sen University Cancer Center

Feifei Sun  
Sun Yat-sen University Cancer Center

Junting Huang  
Sun Yat-sen University Cancer Center

Jia Zhu  
Sun Yat-sen University Cancer Center

Zijun Zhen  
Sun Yat-sen University Cancer Center

Yi Que  
Sun Yat-sen University Cancer Center

Suying Lu  
Sun Yat-sen University Cancer Center

Yizhuo Zhang (zhangyzh@sysucc.org.cn)  
Sun Yat-sen University Cancer Center
Abstract

Background: Ewing's sarcoma (ES) is a rare and highly aggressive malignant tumor arising from bone and soft tissue. However, driver genes in ES have not been fully identified. It is extremely urgent to identify new tumor markers for ES and transform them into clinical practice.

Methods: Bioinformatics analysis was applied to identify the hub genes in ES. Immunohistochemistry analysis was applied to detect the protein expression levels of potential targets of MAD2L1. ES cell lines and xenograft models were used to investigate protein functions of MAD2L1.

Results: In this study, the expression level of mitotic arrest deficient 2 like 1 (MAD2L1) was found to be significantly upregulated in both ES tissues and cell lines. The expression of MAD2L1 was prominently correlated with event-free survival (EFS) and overall survival (OS). Furthermore, MAD2L1 acted as an oncogene in ES. MAD2L1 inhibition markedly reduced the proliferation and induced the apoptosis of ES cells in vitro and attenuated tumorigenesis in vivo. In terms of underlying mechanisms, we found that MAD2L1 promoted ES progression through the Aurora kinase A (AURKA)/MYC axis.

Conclusion: In summary, MAD2L1 induced cell proliferation and anti-apoptosis capabilities through the AURKA/MYC axis, which provides new insights into the tumorigenesis of ES. Thus, MAD2L1 may be a potential target for clinical intervention in ES patients.

Introduction

Ewing's sarcoma (ES) is a malignant round-cell tumor found in the bone or soft tissue, which predominately affects children, adolescents and young adults, with an estimated incidence rate of approximately 1.5 cases per million globally, and male cases are slightly dominant [1]. With the recent advances in imaging technologies, surgical techniques, radiotherapy and chemotherapy, the 5-year survival rate of patients with ES has reached 60–70% [2]. However, ES is associated with aggressive behaviors with a high propensity for early-onset dissemination, and current treatment strategies are ineffective against metastatic and recurrent diseases. About 25% of ES patients are diagnosed with metastatic disease, and patients with refractory or recurrent disease have poor outcomes, with an event-free survival (EFS) rate lower than 30% [3]. ES is characterized by diverse fusions, involving the Ewing sarcoma breakpoint region 1 (EWSR1) gene and E26 transformation-specific (ETS) transcription factors, with EWSR1-FLI1 as the most common fusion subtype, and the EWSR1-FLI1 fusion protein behaves as an aberrant transcription factor, which modulates the expressions of specific target[4, 5]. EWSR1, EWSR1 fusion protein and EWSR1 interacting molecules can serve as significant therapeutic targets to treat ES or EWSR1-related diseases[6, 7]. However, development of new molecular compounds targeting transcription factors is a challenge[8, 9]. It is extremely urgent to identify new tumor markers for ES and transform them into clinical practice. Since the intricate molecular mechanisms of ES have not yet been entirely explored, accurate diagnosis and treatment of ES are negatively affected. Recently, due to the advances in bioinformatics, the hub genes associated with tumorigenesis and tumor progression have been
screened by microarray-based gene expression analysis[10]. This provides a basis for exploring the molecular mechanisms of ES.

Mitotic arrest deficient 2 like 1 (MAD2L1), a member of the mitotic checkpoint complex (MCC), can bind to and inhibit the anaphase-promoting complex/cyclosome (APC/C) and plays a crucial role in the regulation of mitosis. MCC degradation is a key step in the cell division cycle 20 homolog (CDC20) release and subsequent APC/C activation. Securin and cyclin B1 are subsequently degraded by APC/C-CDC20, causing sister chromatid separation and exit from mitosis[11]. However, aberrant expression of MAD2L1 has been shown to be associated with tumorigenesis and clinical prognosis[12]. A previous study revealed that aberrant expression of MAD2L1 results in aneuploidy and tumorigenesis and leads to lung tumor relapse in mice[13]. MAD2L1 is overexpressed in many human cancers (e.g., rhabdomyosarcoma, gastric cancer, lung cancer, and breast cancer) and is associated with poor prognosis[14–16]. However, the expression and the role of MAD2L1 in ES remain unclear.

According to recent studies, genomic microarrays and high-throughput sequencing technology combined with bioinformatics analysis have gradually become robust approaches for the discovery of novel biomarkers for different diseases. In the present study, several datasets obtained from the Gene Expression Omnibus (GEO) database were analyzed, and the top ten hub genes were extracted. The MAD2L1 gene was identified to be upregulated in both ES tissues and cell lines, and it was significantly associated with the survival of ES patients. The MAD2L1 gene promoted proliferation and reduced apoptosis of ES cells via the Aurora kinase A (AURKA)/MYC axis. Thus, our study shed light on the utilization of MAD2L1 as a novel therapeutic target for the treatment of ES.

**Materials And Methods**

**Data collection**

Data were downloaded from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). A total of 154 samples of ES tissues (GSE12102 and GSE34620) and 39 samples of normal tissues (GSE12474, GSE48574, GSE38417, GSE39454, GSE38680, and GSE13608) were analyzed. All data were processed using the R programming language (https://www.r-project.org/) and were normalized using the limma package.

**Analysis Of Differentially Expressed Genes (Degs)**

DEGs were identified using the limma package, as reported previously. The |log₂ fold-change (FC)| was set to > 1, and the cut-off value was adjusted to a P-value < 0.01, which was considered statistically significant. All the included DEGs had a false discovery rate (FDR) < 0.01. The packages ggplot2 and heatmap were used to plot volcano diagrams and heatmaps of DEGs, respectively.
Gene Ontology (Go) And Kyoto Encyclopedia Of Genes And Genomes (Kegg) Pathway Enrichment Analyses

The clusterProfiler package in the R programming language was used to perform GO functional analysis to identify and compare biological themes among gene clusters. The enriched pathways of DEGs were explored using the KEGG pathway analysis via the clusterProfiler package.

Construction Of Protein-protein Interaction (Ppi) Network

The Search Tool for the Retrieval of Interacting Genes (STRING v10.0) was used for the exploration of potential DEG interactions at the protein level[17]. A PPI score > 0.7 was considered significant. The PPI network was visualized using the Cytoscape plugins cytoHubba and MCODE[18]. The gene expression profiling interactive analysis (GEPIA) web-based tool was utilized for survival analysis.

Patient Tissues And Characteristics

Tissue paraffin sections were collected from the tissue bank at the Sun Yat-sen University Cancer Center (SYSUCC). The patients included in this study were pathologically diagnosed at our center from May 2002 to November 2020. The inclusion criteria were as follows: (1) patients aged ≤ 18 years, (2) those pathologically diagnosed with ES, and (3) those with complete medical records and follow-up data. This study was approved by the SYSUCC Institutional Review Board and the Research Ethics Committee.

Immunohistochemistry (Ihc) Assay

The sections were deparaffinized in xylene and rehydrated through graded concentrations of alcohol, followed by treatment with 3% H₂O₂ in water for 10 min to block endogenous peroxidase activity. Then the sections were blocked by 10% horse serum for 30 min at 37°C and were immunostained with primary antibody (mouse anti-human MAD2L, SANTA) at 4°C overnight. The next day, the sections were incubated with goat anti-mouse secondary antibody for 30 min at 37°C. The expression level of MAD2L1 was scored semi-quantitatively on the basis of staining intensity and distribution, using the immunoreactive score as described elsewhere. The sections with a score of 0–4 points were classified as low expression, whereas those with a score of 5–9 points as high expression.

Cell Culture

The human ES cell lines (A673, RDES, SKNMC and SK-NEP-1 cells) and the human bone marrow mesenchymal stem cell (BMSC) were obtained from Cobioer Biosciences Co., Ltd. (Nanjing, China) and grew in a complete growth medium supplemented with 10% or 20% fetal bovine serum (FBS), as
recommended by the manufacturer. These cells were then incubated in a humidified incubator at 37°C with 5% CO₂.

**Rna Isolation And Quantitative Reverse Transcription-polymerase Chain Reaction (Qrt-pcr)**

Total RNA was extracted from cultured cells with TRIzol reagent (Life Technologies, Inc., Carlsbad, CA, USA). Total mRNA was reversely transcribed into cDNA using an M-MLV Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA, USA). The qRT-PCR was performed using SYBR Premix Ex Taq II (Takara, Shiga, Japan) in an ABI-7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized as an internal control. Three replicates of each sample were amplified in a 10-µL qPCR mixture using the iTaq Universal SYBR Green One-step Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The primers for qRT-PCR were listed in Table S1.

**Cell transfection and viral infection**

For transient knockdown experiments, the small interfering RNAs (siRNAs), MAD2L1 siRNA (siMAD2L1), AURKA siRNA (siAURKA), siRNA (si MYC) and synthetic sequence-scrambled siRNA (si-NC) were obtained from GenePharma Co., Ltd. (Shanghai, China). The short hairpin RNAs (shRNAs) of MAD2L1 were purchased from GeneCopoeia Inc. (Guangzhou, China). The transfection and infection procedures were conducted following the manufacturer's instructions. The sequence of siRNAs was listed in Table S2.

**Cell Proliferation Assay**

To further explore the role of MAD2L1, AURKA and MYC in cell proliferation, the cells transfected with corresponding siRNA or empty vectors (siNC) were seeded into 96-well plates at a density of 6,000 cells/well and then incubated for 0, 24, 48, and 96 h. Cell Counting Kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Inc., Kyushu, Japan) was added to each well of the plate, followed by incubation for 3 h at 37°C in humidified air containing 5% CO₂. After 72 h, the absorbance at 450 nm was measured using a microplate reader.

**Colony Formation Assay**

To examine the effect of MAD2L1 on cell growth, 8 × 10³ cells (A673 and RDES cells) transfected with siNC or corresponding siRNA were seeded into 6-well plates. After being incubated for 7 days at 37°C in 5% CO₂, the cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and counted.

**Cell Cycle Analysis**
ES cells were transfected with siNC and corresponding siRNA, and after 48 h, cells were harvested by trypsinization and washed three times with phosphate-buffered saline (PBS). Cells were fixed with 70% ethanol at 4°C overnight. After that, the cells were washed and resuspended in PBS and stained with 50 mg/mL PI solution containing 2 mg/mL RNase (4A Biotech Co., Ltd.). Next, the cells were analyzed with a flow cytometer (SP6800, Sony Biotechnology Inc., Tokyo, Japan).

**Apoptosis Analysis**

Cell apoptosis analysis was performed with Annexin V-Alexa Fluor 647/7-AAD Apoptosis Detection Kit (4A Biotech Co., Ltd., Beijing, China), following the manufacturer's instructions. In brief, $4 \times 10^5$ cells were harvested, and then, they were washed twice with precooled PBS and resuspended in 400 µL of binding buffer. Afterward, the cells were treated with 5 µL of Annexin V-Alexa Fluor 647 and 7AAD for 5 min in the dark. Apoptosis assay was carried out using flow cytometry (SP6800, Sony, Japan) within 1 h.

**Western Blotting Analysis**

Western blotting analysis

Cells were washed with precooled PBS and lysed for 30 min on ice in RIPA lysis buffer containing protease inhibitor cocktail (KeyGen, Shanghai, China). Subsequently, colorimetric detection and quantitation of total protein were conducted using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was employed to separate proteins, which were then transferred onto polyvinylidene difluoride (PVDF) membranes (Sigma-Aldrich). After that, the membranes were blocked with 5% fat-free milk for 1 h at room temperature. Western blotting was performed with primary antibodies, including rabbit anti-human MAD2L1, rabbit anti-human AURKA, rabbit anti-human MYC, rabbit anti-human MYC p-T58, rabbit anti-human MYC p-S62, and mouse anti-human GAPDH. Goat anti-rabbit/mouse secondary antibodies were used to detect the primary antibodies.

**Co-immunoprecipitation (Co-ip) Assay**

For the Co-IP assay, cells were lysed in lysis buffer 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM DTT, and protease inhibitor cocktail. Then, 1 mg of the cell lysates were incubated with the corresponding MAD2L1/AURKA antibodies overnight at 4°C, and homologous immunoglobulin G (IgG) was used as the negative control. Finally, each lysate was added with an equivalent amount of beads (Thermo Fisher Scientific) and incubated overnight at 4°C.

**An in vivo xenograft mouse model**

To analyze the effect of MAD2L1 on tumor growth in ES, the MAD2L1 inhibitor (M2I1, 25 µM) was added to the cell preparation before injecting into mice. Tumor volume (mm$^3$) was computed using the formula:
0.5 × D1 × D2 × D2, where D1 and D2 are the largest diameter and the smallest diameter of a given tumor, respectively. The growth and volume of the tumor were monitored every three days for up to 4 weeks using a caliper. All the mice were euthanized after 4 weeks of inoculation.

Statistical analysis

Data analysis were carried out using GraphPad Prism 8 or SPSS statistics software (version 22). Continuous variables were expressed as mean ± standard deviation, and differences were compared using Student’s t test or one-way analysis of variance. The differences of categorical variables were compared using the χ2 test. Survival rates were calculated by Kaplan-Meier method and the comparisons were performed using Log-rank test. All tests were two-sided, and differences were considered significant when p < 0.05.

Results

Identification of DEGs in ES and normal samples

In the present study, we detected 1,171 DEGs (555 up-regulated and 616 down-regulated DEGs) in ES samples compared with normal samples (|log2 FC| ≥ 1 and FDR < 0.01). The volcano diagrams of all DEGs are shown in (Fig. 1a), respectively.

Go And Kegg Pathway Enrichment Analyses

The results of GO functional analysis revealed that up-regulated DEGs were particularly enriched in regulation of nuclear division, organelle fission, and mitotic nuclear division, while down-regulated DEGs were particularly enriched in muscle system process, muscle contraction, and muscle cell development (Fig. 1b, 1c).

The results of KEGG pathway enrichment analysis demonstrated that up-regulated DEGs were particularly enriched in cell cycle, hepatitis B virus infection, and human T-cell leukemia virus type 1 infection signaling pathways, while down-regulated DEGs were particularly enriched in adrenergic signaling in cardiomyocytes, calcium signaling pathway, and cardiac muscle contraction signaling pathway (Fig. 1d, 1e, P< 0.05).

Ppi Network Construction And Hub Gene Identification

A total of 751 nodes and 4,280 edges were found in the PPI network (Figure S1). The top ten hub genes were screened by cytoHubba, a plugin in Cytoscape. In this study, the top ten genes with the highest degree values were considered hub genes, including CDK1, CDC20, UBE2C, CCNB1, BUB1B, MAD2L1, KIF11, NDC80, TOPX2, and TOP2A (Fig. 1f). Survival analysis revealed that four genes including MAD2L1 (Fig. 2a), CDK1, BUB1B and KIF11 (Figure S2a-c) were associated with poor prognosis in sarcoma. The
qRT-PCR results manifested that the mRNA expressions of two genes, including MAD2L1 (Fig. 2b) and BUB1B (Figure S2d), were up-regulated in ES cell lines. Zhong et al. suggested that MAD2L1 may have great importance in the occurrence or progression of ES [19], but the specific role of MAD2L1 in ES is unknown.

**Mad2l1 Was Associated With Clinicopathological Characteristics And Prognosis Of Es Individuals**

The results of Western blotting displayed that compared with BMSC, the expression level of MAD2L1 was upregulated in ES cell lines (A673, RDES, SKNMC and SK-NEP-1 cells) (Fig. 2c). Next, the clinical significance of MAD2L1 in ES was evaluated (Table 1). The results implied that the high level of MAD2L1 was correlated with extensive disease. However, there was no significant association of MAD2L1 expression with other clinicopathological characteristics. Survival analysis of follow-up data showed that high expression of MAD2L1 was notably correlated with overall survival (OS) ($P = 0.0209$) and event-free survival (EFS) ($P = 0.0023$) in ES patients (Fig. 2e, 2f). In addition, the univariate analysis exhibited that high expression of MAD2L1, age and extensive disease were remarkably associated with an increased risk of cancer-related death. The multivariate analysis displayed that age, extensive disease and high level of MAD2L1 expression were independent prognostic factors for ES (Table 2). These data indicated that MAD2L1 is significantly associated with extensive disease and is also an independent prognostic factor for ES.
Table 1
The relationship between *MAD2L1* expression and clinical pathological features in 56 ES patients from our department.

<table>
<thead>
<tr>
<th>Clinical Characteristics</th>
<th>NO. of patients</th>
<th>NO. of patients</th>
<th>( \chi^2 )</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lower (n = 31)</td>
<td>Higher (n = 25)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>39</td>
<td>20</td>
<td>19</td>
<td>0.863</td>
</tr>
<tr>
<td>Female</td>
<td>17</td>
<td>11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \leq 10 \text{ years} )</td>
<td>34</td>
<td>14</td>
<td>10</td>
<td>0.151</td>
</tr>
<tr>
<td>10 years</td>
<td>22</td>
<td>14</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Tumor volume</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \leq 200\text{mm}^2 )</td>
<td>31</td>
<td>17</td>
<td>16</td>
<td>0.48</td>
</tr>
<tr>
<td>200mm(^2)</td>
<td>25</td>
<td>14</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>limited disease</td>
<td>50</td>
<td>28</td>
<td>22</td>
<td>8.33</td>
</tr>
<tr>
<td>extensive disease</td>
<td>6</td>
<td>0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>EWSR1 statue</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>non-fusion</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>0.232</td>
</tr>
<tr>
<td>fusion</td>
<td>23</td>
<td>13</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>unknown</td>
<td>27</td>
<td>13</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>
Knockdown of MAD2L1 inhibited proliferation and induced cell cycle arrest and apoptosis of ES cell lines

Next, we investigated the effects of MAD2L1 on the proliferation, cell cycle and apoptosis of ES cells. CCK-8 and colony formation assays revealed that knockdown of MAD2L1 significantly decreased the proliferation of ES cells (A673 and RDES cells) (Fig. 3a-c). With the knockdown of MAD2L1, the number of cells in the G2/M phase apparently increased, while the number of cells in the G1/S phase remarkably decreased (Fig. 3d). In addition, inhibition of MAD2L1 significantly increased the apoptosis rate of A673 and RDES cells (Fig. 3e). These results indicated that inhibition of MAD2L1 impairs proliferation and induces cell cycle arrest and apoptosis of ES cells in vitro.

To investigate the role of MAD2L1 in tumor growth in vivo, A673 cells pretreated with 25 µM M2I1 were subcutaneously injected into 4-5-week-old female nude mice (5 × 10^6 cells per mouse). After one week, tumor growth was observed in all mice. Consistent with the in vitro results, the tumor size and weight in M2I1 group were smaller than those in control group (Fig. 3f-h). Taken together, our data confirmed that MAD2L1 plays a crucial role in proliferation of ES cells in the mouse xenograft model.

MAD2L1 promoted proliferation and reduced apoptosis of ES cells via stabilizing AURKA

In this study, we demonstrated that MAD2L1 promoted proliferation and reduced apoptosis of ES cells. Next, bioinformatics analysis was used to explore the target genes of MAD2L1. MAD2L1 was found to interact with AURKA, with an interaction coefficient of 0.479 (http://www.hitpredict.org/). Consistently, these findings were further confirmed by immunoprecipitation (Fig. 4a, 4b). Knockdown of MAD2L1 markedly decreased AURKA expression in A673 and RDES cells (Fig. 4c, 4d). Interestingly, time-course experiments revealed that knockdown of MAD2L1 significantly reduced the half-life of endogenous AURKA in RDES cells from 33 to 16 min (Fig. 4e). The decrease in AURKA protein abundance was efficiently rescued by the proteasome inhibitor MG132 (Fig. 4f, 4g), suggesting that deletion of MAD2L1 stimulates proteasomal degradation of AURKA. Then the functional experiment demonstrated that
AURKA promoted proliferation (Fig. 5a-c) and cell cycle arrest (Fig. 5d) and reduced apoptosis (Fig. 5e) of ES cell lines.

**Mad2l1 Induced Cell Proliferation And Anti-apoptosis Capabilities Through The Aurka/myc Axis**

Based on the results above, we demonstrated that MAD2L1 promoted proliferation and reduced apoptosis of ES cells via stabilizing AURKA. To further explore the exact mechanism that MAD2L1 promoted tumorigenesis in ES, we searched reported literature and found that MYC acted as a crucial oncogene in ES[20]. In addition, bioinformatics analysis revealed that AURKA interacts with MYC, with an interaction coefficient of 0.702 (http://www.hitpredict.org/). Thus, it was speculated that MYC may be a target gene of AURKA in ES. Consistently, the results of co-immunoprecipitation showed a direct interaction between AURKA and MYC (Fig. 6a, 6b), and knockdown of AURKA significantly decreased MYC expression in A673 and RDES cells (Fig. 6c, 6d). It has been reported that the initial step for degradation of MYC by proteasome is phosphorylation of MYC p-T58 by GS3KB, and subsequently, MYC p-S62 is dephosphorylated[21]. We examined whether AURKA inhibition affected the expressions of MYC p-T58 and p-S62. Time-course analysis showed that treatment with AURKA inhibitor TCS7010 in both A673 and RDES cells resulted in a notable increase in MYC p-T58 and a decrease in MYC p-S62 (Fig. 6e, 6f), suggesting that inactivation of AURKA enhances GSK3b-mediated T58 phosphorylation. In addition, knockdown of MYC significantly decreased proliferation (Fig S3a-c) and induced cell cycle arrest in the G2/M phase (Fig S3d) and apoptosis (Fig S3e) of ES cell lines. Moreover, Western blotting results revealed that knockdown of MAD2L1 apparently decreased the expression of MYC, while overexpression of AURKA can markedly reverse the down-regulation of MYC expression induced by MAD2L1 knockdown (Fig. 7a,b), indicating that MAD2L1 promotes MYC expression through AURKA. What’s more, MAD2L1 inhibition impaired proliferation and induced apoptosis of ES cells, which were markedly attenuated after AURKA overexpression (Fig. 7c-f). The results indicated that MAD2L1 induces cell proliferation and anti-apoptosis capabilities through the AURKA/MYC axis.

**Discussion**

ES is the second most frequent bone or soft tissue tumor occurring in childhood and adolescence, following osteosarcoma. Despite the presentation of multimodal treatment regimens, including surgery and radiotherapy, as well as multi-agent chemotherapy, the long-term survival of patients with recurrent or metastatic ES remains unacceptably low. Considering the adverse outcomes of ES, detection of specific biomarkers for this disease is highly vital for early diagnosis and treatment to improve survival outcomes.

In the present study, we detected a high expression level of MAD2L1 in ES patients with a poor prognosis. We also found that knockdown of MAD2L1 significantly decreased proliferation and induced cell cycle arrest in the G2/M phase and apoptosis of ES cells *in vitro*. In terms of mechanisms, MAD2L1 promoted
cell proliferation and anti-apoptosis capabilities through the AURKA/MYC axis. The results indicated that MAD2L1 may be a therapeutic target of ES.

MAD2L1 is a component of the mitotic spindle assembly checkpoint that plays an important role in mitotic progression\[22\]. However, abnormally expressed MAD2L1 may act as an oncogene in diverse types of cancers [23–25]. The expression level and role of MAD2L1 in ES remain elusive. In this study, the expression level of MAD2L1 was found to be upregulated in both ES tissues and cell lines. This is consistent with the results of previous studies, in which the expression level of MAD2L1 is upregulated in numerous types of cancers, including lung cancer, breast cancer, and gastric cancer\[25–27\]. In our previous study, we noted that MAD2L1 is associated with poor outcomes in patients with rhabdomyosarcoma. [28] It inspires us to speculate whether MAD2L1 plays a crucial role in ES. As expected, MAD2L1 expression is significantly associated with the EFS and OS of ES individuals. Moreover, MAD2L1 is also an independent prognostic factor for ES. As far as we know, this is the first time to confirm that MAD2L1 is related to ES clinically. However, the sample size needs to be enlarged to further confirm this conclusion.

After analyzing clinical samples, the experimental model was utilized to search for further support. We found that knockdown of MAD2L1 significantly decreased proliferation and promoted apoptosis of ES cells in vitro and in vivo, in line with previous reports that MAD2L1 acts as an oncogene in various cancers, including gastric cancer, rhabdomyosarcoma, and colorectal cancer\[25, 29, 30\]. Xia et al. revealed that MAD2L1 promotes the proliferation and migration of rhabdomyosarcoma cells\[14\]. Li et al. demonstrated that MAD2L1 promotes the proliferation and invasion of lung adenocarcinoma cells\[31\]. However, there are few studies on the cancer-promoting mechanisms of MAD2L1. Pajuelo-Loza et al. revealed that MAD2L1 modulates stemness and tumorigenesis in human gastric cancer cell lines through CXCR4-SNAI2-MMP1\[12\]. Nonetheless, the mechanism by which MAD2L1 exerts its tumor-promoting effect in ES remains elusive. AURKA belongs to the highly conserved Aurora family of mitotic kinases, whose activation is crucial for the mitotic process. However, abnormal expression of AURKA in various cancers promotes tumorigenesis by participating in cancer cell growth, metastasis, anti-apoptosis, and drug resistance\[32, 33\]. In the present study, we demonstrated that MAD2L1 promoted proliferation and reduced apoptosis of ES cells via stabilizing AURKA. As far as we know, this is the first time to report that MAD2L1 inhibits the ubiquitination degradation of AURKA. Carol et al. demonstrated that the AURKA inhibitor MLN8237 induces cell growth arrest in ES, but the tumor-promoting mechanism of AURKA in ES was not further explored\[34\]. In this study, for the first time, we demonstrated that AURKA induces cell proliferation and anti-apoptosis capabilities by stabilizing MYC in ES.

Inevitably, there were also some limitations. First, the exact mechanisms underlying how MAD2L1 could interact with AURKA remained unclear. The detailed mechanism needs to be further studied. Second, the sample size for survival analysis was relatively small, which might cause some bias. Third, the animal model used in this study was the cell line-derived xenograft, so the conclusions need to be further validated by patient-derived xenografts.
Conclusions

In summary, the expression level of MAD2L1 is upregulated in both ES tissues and cell lines. Besides, MAD2L1 promotes proliferation and reduces apoptosis of ES cells. In terms of underlying mechanisms, MAD2L1 induces cell proliferation and anti-apoptosis capabilities through the AURKA/MYC axis. Therefore, MAD2L1 may be a potential target for the treatment of ES.

Abbreviations

ES: Ewing’s Sarcoma; MAD2L1: mitotic arrest deficient 2 like 1; AURKA: aurora kinase A; MYC: MYC proto-oncogene; CDK1: cyclin dependent kinase 1; BUB1B: BUB1 mitotic checkpoint serine/threonine kinase B; KIF11: kinesin family member 11; DEGs: differentially expressed genes; PPI: protein-protein interaction; STRING: The Search Tool for the Retrieval of Interacting Genes; GO, Gene ontology. KEGG, Kyoto Encyclopedia of Genes and Genomes.

WB: Western blotting; BCA: bicinchoninic acid; GEO: Gene Expression Omnibus; EFS: event-free survival; OS: overall survival; SD: standard deviation; SEM: standard error of the mean; IHC: immunohistochemistry.

Declarations

Acknowledgements

We would like to thank the patients that collaborated for the realization of this study providing their samples and clinical information for research use

Conflicts of Interest

All authors declare no conflicts of interest.

Author Contribution Statement


Ethics approval and consent to participate

This study protocol was approved by the SYSUCC Institutional Review Board and the Research Ethics Committee (IRB: B2021-472-01). Animal researches were executed in compliance with the Ethics Committee of Sun Yat-sen University Cancer Center.

Consent for publication
Not applicable.

Availability of data and materials

Additional experimental data are available upon request to the corresponding author.

Funding Statement

This work was supported by the National Natural Science Foundation of China [grant number 81570201].

References


Figures
Figure 1

Bioinformatics analysis identified DGEs in ES and performed functional enrichment.

a Volcano plots of (GSE12102, GSE34620, GSE12474, GSE48574, GSE38417, GSE39454, GSE38680 and GSE13608), where red dots, green dots, and black dots indicate upregulated, downregulated, and not differentially expressed genes, respectively (| log₂ FC | ≥ 1 and false discovery rate (FDR) < 0.01). b, c GO
analysis of the upregulated genes and downregulated genes in DGEs (FDR < 0.01). d, e KEGG analysis of the upregulated genes and downregulated genes in DGEs (FDR < 0.01). f The top 10 hub genes of DGEs.

**Figure 2**

MAD2L1 was upregulated in ES and associated with poor prognosis of patients.

a K-M Plot analysis of MAD2L1 based on TCGA sarcoma database. b mRNA expression levels of MAD2L1 in BMSC and 4 ES cell lines. c Protein expression levels of MAD2L1 in BMSC and 4 ES cell lines. d Representative images of the expression of MAD2L1 protein in patients with RMS detected by IHC (×100). e, f Overall survival (OS) curve and event-free survival (EFS) curve of ES patients expressing high-levels or low-levels of MAD2L1.
Figure 3

MAD2L1 regulated ES cell proliferation, cell cycle and apoptosis.

a, b CCK8 assay in A673 and RDES after genetic knockdown of MAD2L1 by siRNAs. c Colony formation assay of A673 and RDES cells after MAD2L1 knockdown. d Cell cycle analysis in A673 and RDES after MAD2L1 knockdown. e Apoptosis analysis in A673 and RDES after MAD2L1 knockdown. f Photographs
of harvested tumors of A673 tumor-bearing mice. Tumor volumes of A673 xenograft models in controls group and M2I1 group. Tumor weight of the two independent groups was recorded at the endpoint of the experiment. Data are shown as mean ± SD of six mice in each group.

**Figure 4**

**MAD2L1 promoted proliferation and reduced apoptosis of ES via stabilizing AURKA**

a, b Results of immunoprecipitation in A673 and RDES to verify the interaction of MAD2L1 and AURKA. c, d Protein expression levels of AURKA in A673 and RDES with MAD2L1 knockdown. e Time-course analysis of AURKA protein levels in MAD2L1-depleted RDES cells. f, e MAD2L1-depleted A673 and RDES cells were treated with MG132 (10 μM) for 10 h before harvest. MAD2L1 and AURKA were analyzed by immunoblot, with GAPDH as a loading control. *P < 0.05, **P < 0.01, ***P < 0.001. Abbreviation: ES, Ewing’s sarcoma.
Figure 5

AURKA regulated ES cell proliferation, cell cycle and apoptosis in vitro.

a, b CCK8 assay in A673 and RDES after genetic knockdown of AURKA by siRNAs. c Colony formation assay of A673 and RDES cells after AURKA knockdown. d Cell cycle analysis in A673 and RDES after
AURKA knockdown. e Apoptosis analysis in A673 and RDES after AURKA knockdown. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 6**

AURKA promoted proliferation and reduced apoptosis of ES via stabilizing MYC

a, b Results of immunoprecipitation in A673 and RDES to verify the interaction of AURKA and MYC. c, d Protein expression levels of MYC in A673 and RDES with AURKA knockdown. e Time-course analysis of MYC, MYC p-T58, MYC p-S62 protein levels in ES cells were treated with TCS 7010 (1 μM).
Figure 7

**MAD2L1 induced cell proliferation and anti-apoptosis capabilities through AURKA/MYC axis**

a, b Western blot analysis of the protein expression in siMAD2L1 Ewing sarcoma cells treated with or without oeAURKA. c, d CCK8 assay in A673 and RDES after genetic knockdown of MAD2L1 alone or combined with AURKA overexpression. e Colony formation assay of A673 and RDES after genetic knockdown of MAD2L1 alone or combined with AURKA overexpression. f Apoptosis analysis in A673 and RDES after genetic knockdown of MAD2L1 alone or combined with AURKA overexpression. ns, no significance, *P < 0.05, **P < 0.01, ***P < 0.001.
Supplementary Files

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

- FigureS1.tif
- FigureS2.tif
- FigureS3.tif
- TableS1.docx
- TableS2.docx