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Development of a novel treatment based on PKMYT1 inhibition for cisplatin-resistant bladder cancer with *miR-424-5p*-dependent cyclin E1 amplification

Wataru Fukumoto[1†], Shunsuke Okamura[1†], Motoki Tamai[1†], Junya Arima[1], Ichiro Kawahara[1], Ikumi Fukuda[1], Akihiko Mitsuke[1], Takashi Sakaguchi[1], Satoshi Sugita[1], Ryosuke Matsushita[1], Shuichi Tatarano[1], Yasutoshi Yamada[1], Masayuki Nakagawa[1], Hideki Enokida[1] and Hirofumi Yoshino[1*]

Abstract

Background Chemotherapy including cisplatin is recommended for the treatment of advanced bladder cancer, but its effectiveness is limited due to the acquisition of drug resistance. Although several mechanisms of cisplatin resistance have been reported, there are still many unknowns, and treatment of cisplatin-resistant bladder cancer remains difficult. Accordingly, in this study, we aimed to identify and characterize microRNAs involved in cisplatin resistance.

Methods Small RNA sequencing analysis was performed to search for microRNAs related to cisplatin resistance. The identified microRNAs were then characterized using gain-of-function studies, sensitivity analysis, target gene analysis, and cellular assays.

Results We identified *miR-424-5p* as a candidate microRNA that was downregulated in cisplatin-resistant strains compared with parental strains. Notably, in gain-of-function studies, *miR-424-5p* suppressed the proliferative ability of cisplatin-resistant bladder cancer (CDDP-R BC). Furthermore, *miR-424-5p* restored sensitivity to cisplatin. RNA sequence analysis revealed seven candidate genes targeted by this microRNA. Among them, cyclin E1 (*CCNE1*) was chosen for subsequent analyses because its expression was upregulated in cisplatin-resistant cells compared with parental cells and because recent studies have shown that *CCNE1* amplification is synthetic lethal with PKMYT1 kinase inhibition. Therefore, we performed functional analysis using the PKMYT1 inhibitor RP-6306 and demonstrated that RP-6306 inhibited cell growth through suppression of mitotic entry and restored cisplatin sensitivity in CDDP-R BC.

Conclusions Overall, our findings provided insights into the development of novel therapeutic strategies for CDDP-R BC.

Keywords *miR-424-5p*, Cyclin E1, PKMYT1, Synthetic lethal, Cisplatin-resistant bladder cancer

[†]Wataru Fukumoto, Shunsuke Okamura and Motoki Tamai contributed equally to this work.

*Correspondence:
Hirofumi Yoshino
hyoshino@m3.kufm.kagoshima-u.ac.jp
Full list of author information is available at the end of the article

Background

Bladder cancer (BC) is the tenth most commonly diagnosed cancer worldwide, with 573,000 new cases and 213,000 deaths reported annually [1]. Most BCs are urothelial carcinomas and are classified as non-muscle invasive BC and muscle-invasive BC (MIBC), with approximately 20% presenting with MIBC and 5% with metastatic disease [2]. Moreover, approximately 50% of patients treated with radical cystectomy and pelvic lymph node dissection for MIBC will develop recurrence or distant metastasis [3]. Patients with advanced BC are treated with cisplatin combination chemotherapy [2]. Briefly, cisplatin attaches to the genomic or mitochondrial DNA and creates covalent adducts with them to induce DNA lesions; prevent the production of DNA, mRNA, and proteins; and halt DNA replication, all of which eventually lead to necrosis or death [4]. In addition, cisplatin induces apoptosis mediated by the activation of various signaling pathways, including calcium signaling, death receptor signaling, and activation of mitochondrial pathways [5]. Even with the advent of newer drugs, such as immuno-oncology drugs and antibody–drug conjugates, cisplatin is still employed as a first-line therapy. However, although up to 70% of patients who develop distant metastases or lymph node metastases initially have a good tumor response to systemic chemotherapy, more than 90% of patients will relapse and eventually die from the disease [6]. Therefore, there is an urgent need to elucidate the mechanism of cisplatin resistance.

The mechanisms of cisplatin resistance can be classified into four categories [7]. The first category is pre-target resistance, in which cisplatin binding to DNA is reduced due to decreased cisplatin uptake into the cell. The second category is on-target resistance due to insufficient direct binding between DNA and cisplatin. The third category is post-target resistance due to ineffective cisplatin-mediated DNA damage. The last category is off-target resistance, where the signaling pathway is not triggered by cisplatin. However, much is unknown about cisplatin resistance, and the treatment of patients with BC who have acquired cisplatin resistance is challenging. Our previous report of the relationships between microRNAs and cisplatin resistance in BC showed that *miR-486-5p* restoration significantly inhibited cancer cell proliferation, migration, and invasion in cisplatin-resistant BC (CDDP-R BC) cell lines, and *miR-486-5p* also increased the sensitivity of CDDP-R BC cell lines to cisplatin [8]. We also showed that enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase (EHHADH), a target of *miR-486-5p*, was associated with cisplatin resistance and might contribute to pre-target resistance. However, few studies have reported the association between BC and microRNAs, and further studies and approaches are necessary to overcome cisplatin resistance in BC.

In this study, we further analyzed previous RNA-seq analyses in CDDP-R BC cell lines (CDDP-R BOY and CDDP-R T24) to identify candidate microRNAs that were downregulated in cisplatin-resistant strains. Next, the candidate microRNAs were transfected into a cisplatin-resistant BC strain, and RNA next-generation sequencing was performed to search for candidate genes associated with cisplatin resistance. Because recent studies have reported the development of inhibitors showing synthetic lethality (SL) in cells in which candidate genes are amplified, the inhibitor may have applications in overcoming cisplatin resistance in CDDP-R BC cells [9].

Methods

BC cell lines and culture

We used 2 human BC cell lines: BOY was established in our laboratory from a 66-year-old male patient of Asian descent who was diagnosed with stage IV BC with many lung metastases, and T24 was obtained from American Type Culture Collection (Manassas, VA, USA). These cell lines were cultured in minimum Essential Medium Eagle containing 50 mL of 10% fetal bovine serum (FBS), 50 μg/mL streptomycin, and 50 U/mL penicillin in a humidified atmosphere of 95% air/5% CO_2 at 37 ℃. To establish CDDP-R BC cell lines, we cultured BC cell lines with serial concentrations of cisplatin ranging from 0.01 to 2.0 μg/mL for 6 months. The cells were cultured for 24–36 h in 10 mL medium containing 1 mL cisplatin that had been adjusted to 10 times the target concentration [8, 10].

Transfection with mature microRNAs

BC cells were transfected with the Lipofectamine RNAiMAX transfection reagent (Thermo Fisher Scientific) and Opti-MEM (Thermo Fisher Scientific) with 10 nM miRNA. Mature microRNA (*hsa-miR-424-5p*; product ID: AM17100) and negative-control microRNA (negative control miRNA; product ID: AM 17111) were used in gain-of-function experiments.

MicroRNA and mRNA sequence analysis

To search for microRNAs associated with cisplatin resistance, total RNAs extracted from BOY, CDDP-R BOY, T24, and CDDP-R T24 cell lines were subjected to microRNA sequencing, performed by Riken Genesis Co., LTD. (Tokyo, Japan). We compared parental and CDDP-R cell lines (BOY versus CDDP-R BOY, T24 versus CDDP-R T24) and selected miRNAs with significantly downregulated expression in CDDP-R cell lines (fold-change < −1.0). mRNA sequence analysis was performed by Riken Genesis Co., LTD. to identify the target mRNAs

of *miR-424-5p*. For the samples, a TruSeq Stranded mRNA Library Prep Kit was used to create libraries, and a flow cell manufactured by Illumina Inc. was used for sequencing. The valid read length was 150 bp, and the analysis was performed using a Multiplex method. Candidate target genes were significantly downregulated after transfection with *miR-424-5p* compared with control microRNA (fold-change < −1.0) in CDDP-R BOY and CDDP-R T24 cells.

In silico analysis

To evaluate the clinical relevance of our findings, a The Cancer Genome Atlas (TCGA) cohort database of patients with BC was used. This study followed the criteria for the publication guidelines provided by TCGA. Kaplan–Meier analysis was used to analyze overall survival (OS) using data in the OncoLnc dataset (http://www.oncolnc.org/). To search for the miRNAs associated with cisplatin resistance, we identified miRNAs that showed lower expression in CDDP-R cells than in parental cells for both BOY and T24 lines and that had been reported as tumor-suppressor genes. To identify possible target genes of *miR-424-5p*, we extracted genes that were reduced by transfection with *miR424-5p* in mRNA sequence analysis with genes that may be targeted by *miR-424-5p* based on TargetScan database Release 7.1 (http://www.targetscan.org).

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

To quantify *miR-424-5p* expression, we used Stem-loop RT-PCR (TaqMan MicroRNA Assays; P/N: 4,427,975 for *miR-424-5p*; Applied Biosystems) according to previously published conditions. RNU48 (P/N: 001006; Applied Biosystems) was used as the internal control. For cyclin E1 (*CCNE1*) and PKMYT1, we applied a SYBR-green quantitative PCR-based array approach. The primer set used for determination of mRNA expression was as follows: CCNE1; forward primer, 5′-ACTCAACGTGCAAGC CTCG-3′ and reverse primer, 5′-GCTCAAGAAAGT GCTGATCCC-3, PKMYT1; forward primer 5′-CAT GGCTCCTACGGAGAGGT-3 and reverse primer, 5′-ACATGGAACGCTTTACCGCAT-3. For glucuronidase β (internal control), the set was as follows: forward primer, 5′-CGTCCCACCTAGAATCTGCT-3′ and reverse primer, 5′-TTGCTCACAAAGGTCACAGG-3′. The specificity of amplification was monitored using the dissociation curve of the amplified product.

Cell proliferation assays

To evaluate cell proliferation, we used XTT assays. T24 and BOY cells were seeded in 96-well plates (2×10^3 cells/well) in 100 μL medium containing 10% FBS. Ninety-six hours later, we determined the extent of cell proliferation using a Cell Proliferation Kit II (Roche Diagnostics GmbH, Mannheim, Germany). When using cisplatin, we added 4 or 10 μL adjusted to 10 times the target concentration. RP-6306 was purchased from Selleck.

Cell cycle assays

Cell cycle assays and cell apoptosis assays were were carried out by flow cytometry (CytoFLEX Analyzer; Beckman Coulter, Brea, CA, USA) using a Cycletest PLUS DNA Reagent Kit (BD Biosciences) according to the manufacturer's recommendations, as previously described [11].

Statistical analysis

Relationships between two groups were analyzed using the Mann–Whitney U test; relationships between three or more groups were analyzed using multiple comparison tests with the Bonferroni/Dunn method. All analyses were performed using expert Statview software, version 5.0 (SAS Institute, Inc., Cary, NC, USA). *P* values less than 0.05 were accepted as statistically significant.

Results

miR-424-5p was downregulated in CDDP-R BC

First, RNA-seq analysis was performed using two CDDP-R BC cell lines previously established in our laboratory. The results showed that five microRNAs were downregulated in CDDP-R BC cells compared with the parental strain when considering common microRNAs among both BC cell lines (Fig. 1A). In BOY and T24 cells, RT-qPCR confirmed that *miR-424-5p* was downregulated in the CDDP-R cell line compared to the parental line (Fig. 1B). We then transfected each candidate microRNA into CDDP-R BC cells and evaluated their proliferative potential by XTT assay. As a result, *miR-424-5p*-transfected cells showed the most significant inhibition of proliferative ability (Fig. 1C). *miR-486-5p* was not evaluated in this study because it was previously reported from our laboratory. Based on these results, we focused on *miR-424-5p* because of its potential role in cisplatin resistance.

Function of miR-424-5p in CDDP-R BC cells

Next, we tested the effects of *miR-424-5p* on BC in vitro (S-Fig. 1A). We transfected *miR-424-5p* into BC cells and performed XTT assays. The results showed that transfection with *miR-424-5p* significantly suppressed the proliferative capacity of BOY, CDDP-R BOY, T24, and CDDP-R T24 cells (Fig. 2A, B). In addition, colony formation assays showed results similar to those of XTT assays, indicating inhibition of proliferative capacity (Fig. 2C).

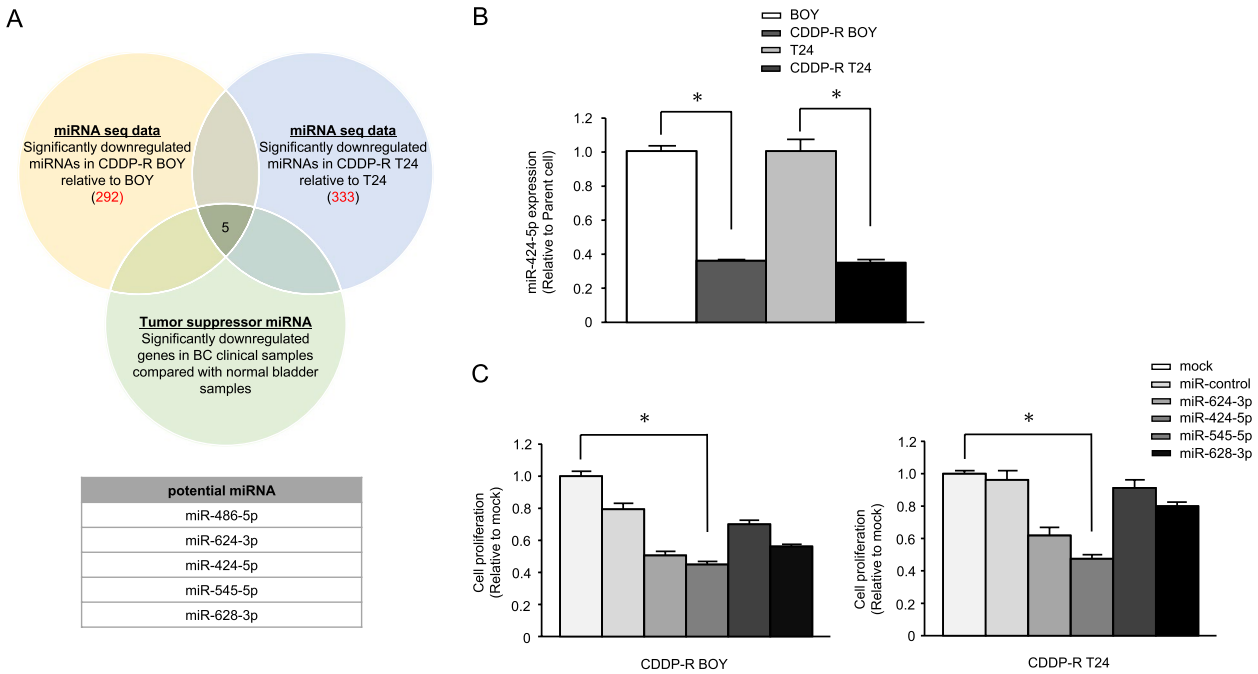


Fig. 1 Targeted genes were extracted and analyzed using TCGA database. **A** RNA-seq analysis of previously established CDDP-R BC was performed to compare parental and CDDP-R BC. Five microRNAs were extracted that were commonly downregulated in two cell lines, BOY and T24 (fold change CDDP-R-BOY /BOY: 0.25, fold change CDDP-R-T24 /T24: 0.74). **B** Comparison of miR-424-5p expression in parental and CDDP-R BC cell lines by RT-qPCR (fold change CDDP-R-BOY /BOY: 0.36, fold change CDDP-R-T24 /T24: 0.35). * $P < 0.05$. **C** Candidate microRNAs were transfected into CDDP-R BC cells, respectively, and their proliferative ability was assessed using the XTT assay. $P = 0.5$

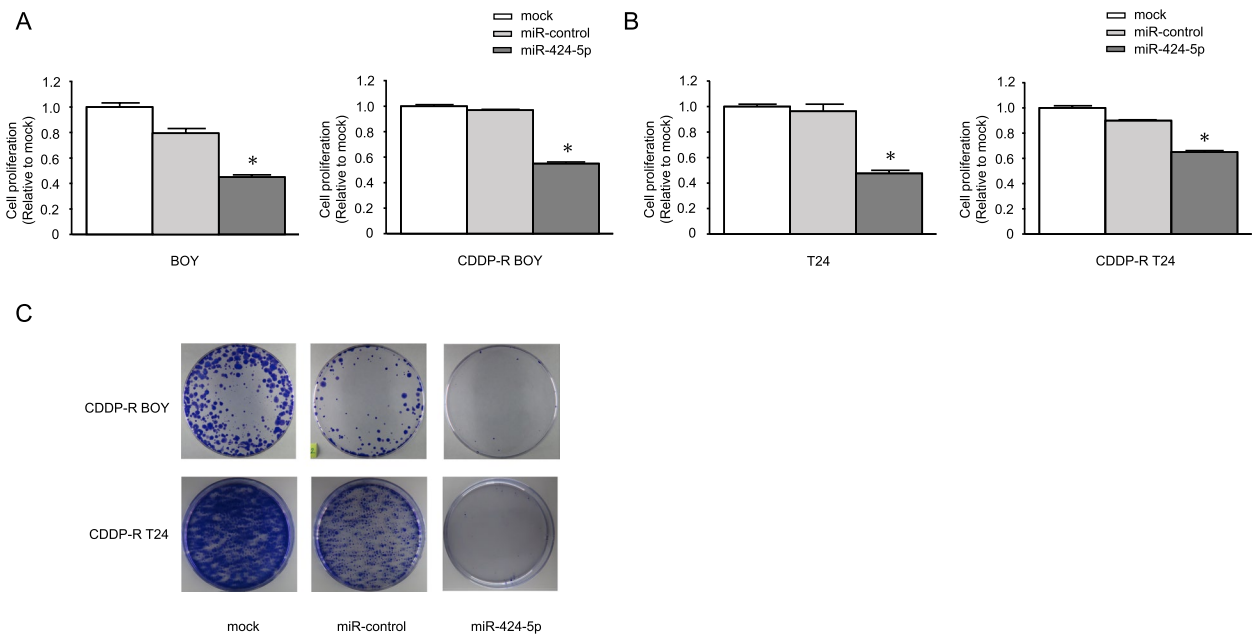


Fig. 2 Effects of transfection with *miR-424-5p*. **A**, **B**, BOY, CDDP-R BOY, T24, and CR-T24 cells were transfected with *miR424-5p*, and proliferative ability was measured using XTT assays. * $P < 0.001$. **C**, Colony formation was performed by transfection of CDDP-R BOY and CDDP-R T24 cells with *miR-424-5p*, and colony-forming ability was measured

Improvement of CDDP sensitivity by transfection with miR-424-5p

Next, XTT assays were performed using 4.0 μM CDDP in combination with *miR-424-5p* to determine whether CDDP resistance was improved. We have previously reported the half-maximal inhibitory concentration (IC_{50}) of BOY, T24, CDDP-R BOY, and CDDP-R T24 cells (BOY IC_{50}: 2.83 μM, CDDP-R BOY IC_{50}: 10.56 μM, T24 IC_{50}: 11.48 μM, CDDP-R T24 IC_{50}: 20.37 μM) [8]. Our current results demonstrated that CDDP-R BOY and CDDP-R T24 cells transfected with *miR-424-5p* exhibited reduced proliferative capacity to the same degree as the parental line (Fig. 3A). Next, administration of *miR-424-5p* in combination with 10 μM cisplatin significantly inhibited proliferative activity compared with CDDP or *miR-424-5p* alone (Fig. 3B). In addition, the XTT assay was performed by combining *miR-424-5p* with CDDP 1 μM, 10 μM, and 20 μM. The results showed that transfection of *miR-424-5p* inhibited the proliferative ability of CDDP-R BC cells in a CDDP concentration-dependent manner (S-Fig. 1B). These suggest that *miR-424-5p* affects CDDP resistance in bladder cancer.

Extraction of the target gene, CCNE1

Next, we further explored the molecular mechanisms regulated by the tumor-suppressor *miR-424-5p*. Therefore, RNA-seq analysis was performed using *miR-424-5p*-transfected cells. We focused on seven genes that were

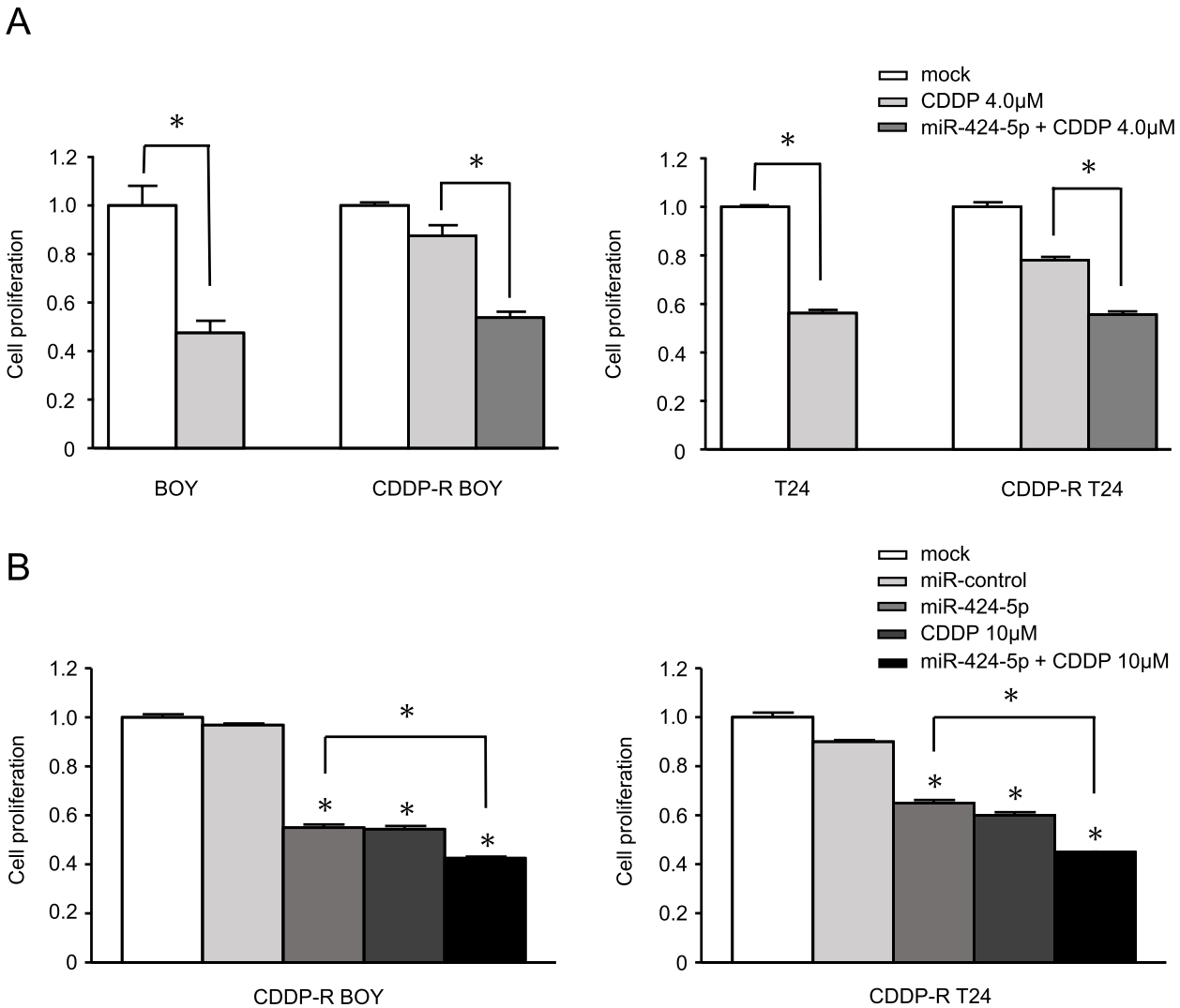
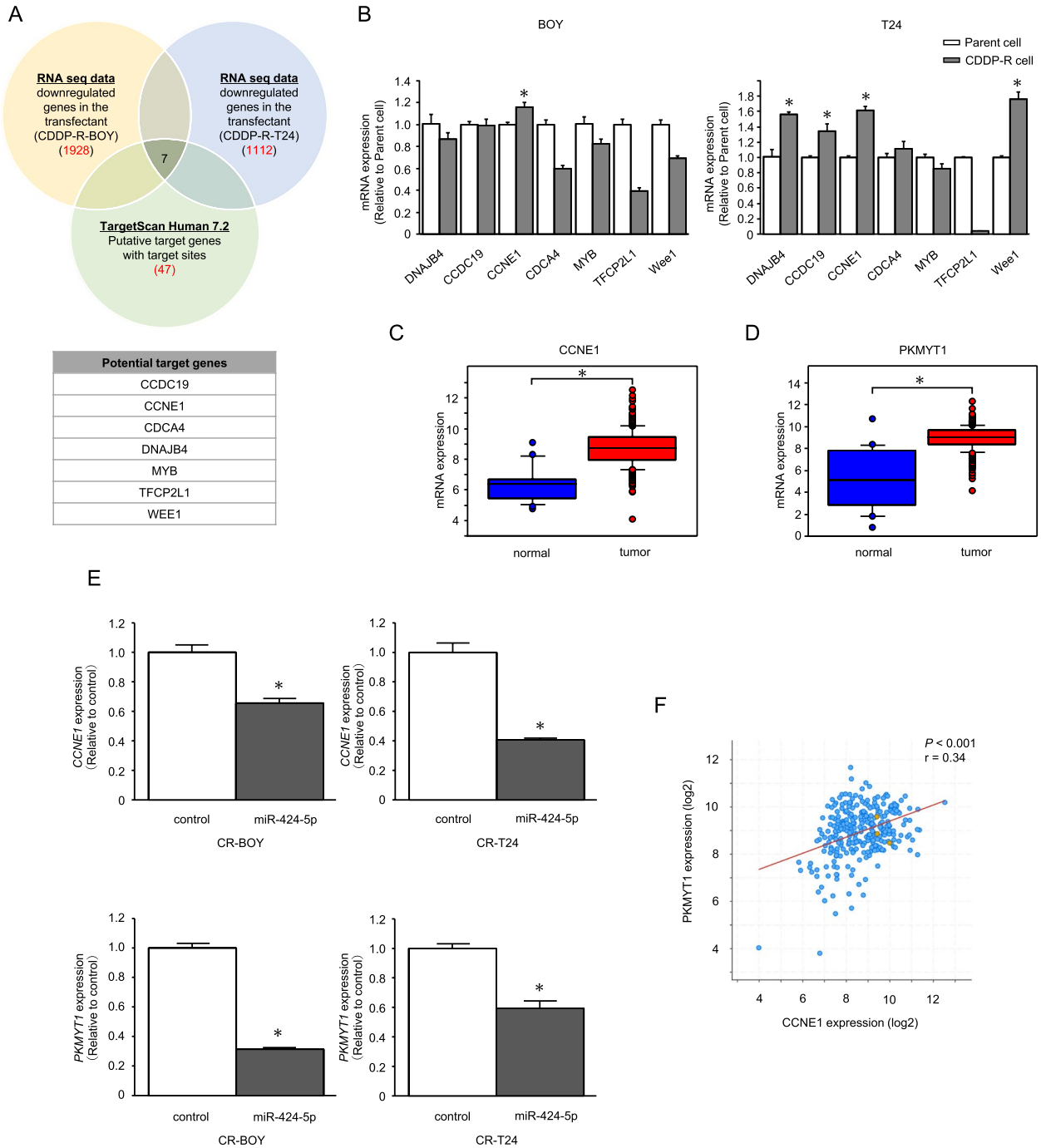


Fig. 3 Improvement of CDDP resistance by *miR-424-5p* and additive effects. **A**, CDDP-R BOY and CDDP-R T24 cells were treated with 4 μM CDDP and transfected with *miR424-5p*, and proliferative ability was measured using XTT assays. * $P < 0.0001$. **B**, CDDP-R BOY and CDDP-R T24 cells were treated with 10 μM CDDP and transfected with *miR-424-5p*, and proliferative ability was measured using XTT assays. * $P < 0.0001$



Potential target genes
CCDC19
CCNE1
CDCA4
DNAJB4
MYB
TFCP2L1
WEE1

Fig. 4 RNA-seq analysis was performed using CDDP-R BOY and CDDP-R T24 cells transfected with *miR-424-5p*. **A**, Seven genes were commonly downregulated in the two cell lines. **B**, Expression of each gene was measured by RT-qPCR in parental and CDDP-R BC cells. * $P < 0.05$. **C**, *CCNE1* expression was compared in normal tissues and BC cells using data from TCGA database (normal: $n = 19$, tumor: $n = 404$). * $P < 0.0001$. **D**, PKMYT1 expression was compared in normal tissues and BC cells using data from TCGA database (normal: $n = 19$, tumor: $n = 404$). * $P < 0.0001$. **E**, Expression of CCNE1 and PKMYT1 in CDDP-R BC cells transfected with *miR-424-5p* was examined by RT-qPCR. * $P < 0.05$. **F**, Analysis of CCNE1 and PKMYT1 correlation using the TCGA database. $P < 0.001$, $r = 0.34$

commonly downregulated in CDDP-R BOY and CDDP-R T24 cells transfected with *miR-424-5p* (Fig. 4A), and RT-qPCR was performed to compare the expression of each gene in parental and CDDP-R BC cells. The results suggested that only CCNE1 was commonly upregulated in CDDP-R BOY and CDDP-R T24 cells (Fig. 4B). Based on these results, we focused on *CCNE1* as a target gene. In silico analysis using TCGA data showed that *CCNE1* was significantly upregulated in BC cells compared with normal cells (Fig. 4C). Recently, PKMYT1 was identified as a gene strongly dependent on *CCNE1* in ovarian cancer [9]. PKMYT1 was significantly upregulated in BC cells compared with normal cells (Fig. 4D). We transfect *miR-424-5p* into CDDP-R BC cells and the expression of CCNE1 and PKMYT1 was reduced (Fig. 4E). Furthermore, we examined the correlation between CCNE1 and PKMYT1, and OS in the TCGA database and found a positive correlation between CCNE1 and PKMYT1 (Fig. 4F and S-Fig. 1C).

Effects of RP-6306 on CDDP-R BC

We used RP-6306, a PKMYT1 inhibitor, to perform XTT assays. In CDDP-R BOY and CDDP-R T24 cells, treatment with RP-6306 resulted in a significant decrease in proliferative capacity. In addition, further inhibition of proliferative potential was observed when the drug was administered in combination with CDDP (Fig. 5A). These results demonstrated the SL of the combined treatment with CDDP and RP-6306 in CDDP-R BC cells. Furthermore, cell cycle assay was performed to elucidate the mechanism of *miR-424-5p* and RP6306 on bladder cancer with high CCNE1. *miR-424-5p* transfection and RP6306 treatment on CDDP-R BOY and CDDP-R T24 cells decreased G2/M ratio in cell cycle assay (Fig. 5B). Furthermore, the combination of RP6306 and CDDP resulted in a further decrease in the G2/M ratio. These results indicate that *miR424-5p* and RP6306 inhibit tumor growth by inducing G1 arrest in high-CCNE1 bladder cancer.

Discussion

MicroRNAs are endogenous small noncoding RNA molecules (19–22 bases long) that regulate genes and genomes at the most critical levels of genome function, including chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability, and

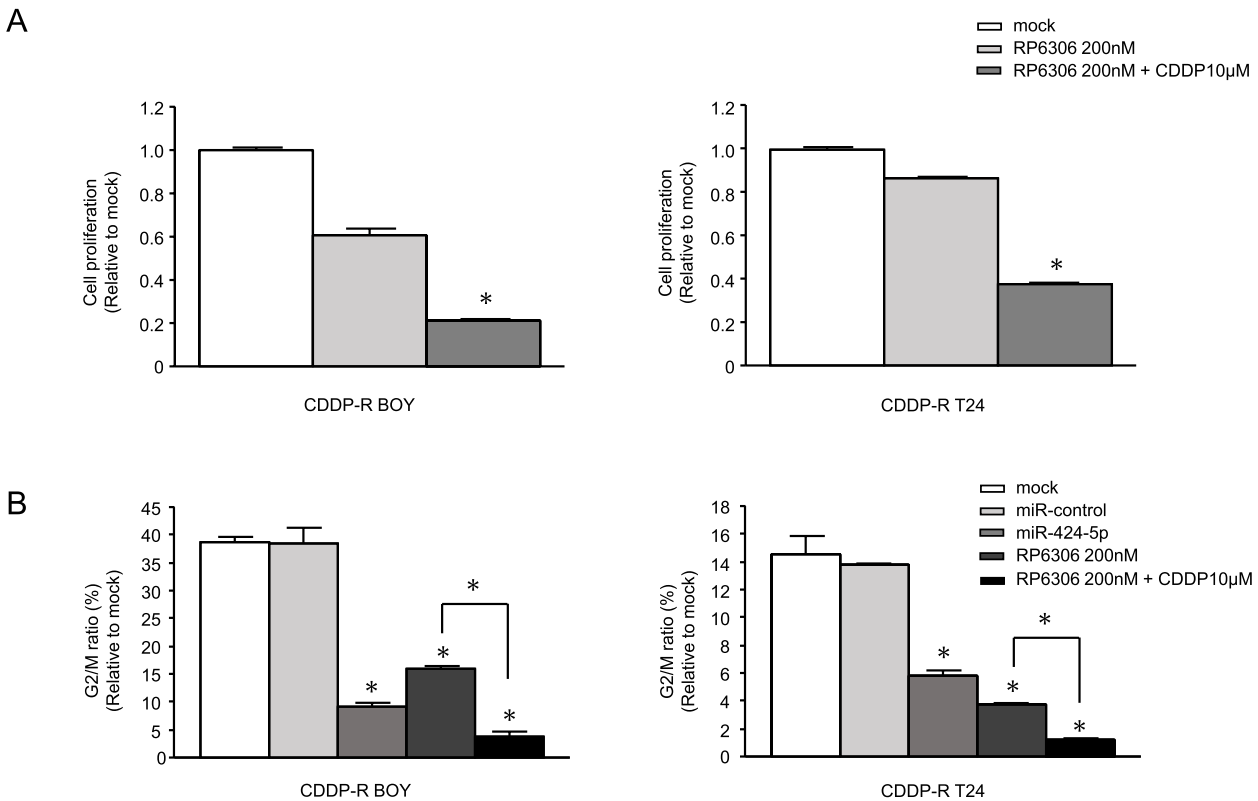


Fig. 5 Effect of RP6306 on CDDP-R BOY and CDDPR-T24 cells. **A**, CDDP-R BOY and CDDP-R T24 cells were treated with RP-6306, and proliferative ability was measured using XTT assays. CDDP (10 μM) was also used in combination. * $P < 0.0001$. **B**, CDDP-R BOY and CDDP-R T24 cells were treated with *miR-424-5p*, RP-6306 and CDDP in cell cycle assay. * $P < 0.05$

translation [12]. Previous studies have shown that microRNAs are important regulators of cell proliferation, differentiation, development, apoptosis, and drug resistance in cancer progression [13, 14]. Associations between microRNAs and CDDP resistance have been reported for various carcinomas. Zhiwen et al. reported that upregulation of *miR-30b* abrogated metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) -induced CDDP resistance in gastric cancer [15]. Moreover, Wang et al. reported that *miR-221* is involved in CDDP resistance by targeting phosphatase and tensin homolog in lung cancer cells [16]. Li et al. reported that *miR-106a* modulates cisplatin sensitivity by targeting programmed cell death 4 in ovarian cancer cells [17]. In this study, based on the results of RNA-seq analysis, we focused on *miR-424-5p*. Previous studies have reported that *miR-424-5p* is downregulated in ovarian, cervical, and hepatocellular carcinomas but upregulated in gastric, oral squamous cell, and thyroid cancers [18–23]. *miR-424-5p* interferes with proliferative and metastatic effects by blocking the G_1/S phase transition in ovarian cancer [18]. Additionally, *miR-424-5p* inhibits cell proliferation and promotes cell apoptosis by targeting the KDM5B-Notch pathway in cervical cancer [19]. Another study showed that *miR-424-5p* downregulates Smad3 expression in gastric cancer by binding to the 3′ untranslated region (UTR) of *Smad3* mRNA [21]. These reports suggest that the distribution of *miR-424-5p* may be tissue-type specific. The association between CDDP and *miR-424-5p* in BC is poorly understood; thus, in this study, we examined this relationship for the first time.

CCNE1 was selected as a target gene because its expression was upregulated in CDDP-R BC cells and downregulated when *miR-424-5p* was transfected into CDDP-R BC cells. Overexpression of *CCNE1* has tumor-promoting effects in various cancers, including colorectal and ovarian cancers [24–26]. *CCNE1* encodes cyclin E1, which binds to cyclin-dependent kinase 2 (CDK2) and has important roles not only in cell cycle progression (the G_1- to S-phase transition) but also in centrosome duplication, a tightly regulated process that maintains genetic stability [27]. Overexpressing CCNE1 results in premature S phase entry, loss or increase of entire chromosomes, DNA damage, and oxidative stress leading to tumorigenesis [28]. The association between microRNA and *CCNE1* has also been reported. In cervical cancer, *miR-16–1* regulates cyclin E1 through the 3′-UTR regulatory region of the *CCNE1* gene [29]. By contrast, our previous study showed that *miR-144-5p* directly regulates *CCNE1* and inhibits cancer cell growth by inducing cell cycle arrest in BC cells [30]. Furthermore, a relationship between *miR-424-5p* and *CCNE1* has been reported in ovarian cancer [18]. These reports support that *CCNE1* is regulated by several downregulated microRNAs and influences cancer cell promotion by activating the cell cycle. *CCNE1* gene amplification is one of the most common molecular genetic alterations that characterize high-grade serous carcinoma, particularly in tumors that develop resistance to platinum-based chemotherapy [27]. However, there are few reports of the relationship between *CCNE1* and CDDP or cisplatin resistance in other cancers, and the mechanism remains unknown. The molecular response to cisplatin in BC cells involves inhibition or induction of genes with specific functions in signal transduction, cell proliferation, cell cycle control, transcriptional and translational regulation, protein degradation, and cellular metabolism, as well as apoptosis and tumor suppression [31]. p53 is repressed in *CCNE1*-rich hepatocytes [28]. These findings suggest that *CCNE1* may protect against cisplatin resistance in cancer cells by suppressing p53 and inhibiting apoptosis. The association of *CCNE1* with cisplatin resistance should be explored more in the future.

SL is a concept introduced nearly a century ago by geneticists to describe situations in which a defect in either one of two genes has little effect on the cell or organism whereas a combination of defects in both genes results in death. In 2005, two groups described the SL interaction between poly (ADP-ribose) polymerase inhibition and BRCA1 or BRCA2 mutation, suggesting a novel strategy for treating patients with BRCA-mutant tumors [32]. In ovarian cancer, *CCNE1* amplification is detected in approximately 20% of tumors, in a manner largely mutually exclusive with homologous recombination deficiency, and is enriched in platinum-refractory tumors. However, *CCNE1* itself is not considered a druggable target, and as an alternative approach, exploiting the vulnerability that SL approaches caused by elevated cyclin E levels may provide a much-needed new therapeutic option for *CCNE1*-amplified tumors [9]. PKMYT1 was identified as the most strongly dependent gene in *CCNE1*-amplified tumor cell lines, and inhibition of PKMYT1 has been shown to have antitumor effects by inducing selective cytotoxicity and DNA damage against ovarian cancers with high *CCNE1* expression [9]. PKMYT1 causes unscheduled selective activation of cyclin-dependent kinase 1 in *CCNE1*-high expressing cells and promotes early mitosis [9]. This kind of oncology drug discovery based on the identification of SL interactions has great potential. However, to the best of our knowledge, there are no reports on the impact of PKMYT1 suppression on BC with high *CCNE1* expression. Therefore, we used BC cells to investigate the effects of RP-6306. Our findings showed that RP-6306 treatment inhibited tumor growth in *CCNE1*-overexpressing cisplatin-resistant BC cells through suppression of mitotic

entry. Furthermore, RP-6306 showed an additive effect with CDDP treatment. Our findings may facilitate the future development of new treatments for CDDP-R BC based on PKMYT1 inhibition.

In this study, we found that *CCNE1* expression was upregulated via *miR-424-5p* in CDDP-R BC. However, the development of *CCNE1* inhibitors has been difficult and has not been applied to clinical practice. In this study, using RP-6306, an inhibitor of PKMYT1, which shows SL against *CCNE1*, we found that inhibition of PKMYT1 in *CCNE1*-high BC had tumor-suppressive effects through suppression of mitotic entry. These findings may lead to a better understanding of CDDP resistance in BC and the development of new therapeutic strategies for CDDP-R BC.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-024-13109-5.

Supplementary Material 1. A, RT-PCR (TaqMan miRNA Assays) to confirm the efficiency of *miR-424-5p* transfection. * $P < 0.0001$. B, *miR-424-5p* was transfected into parental and CDDP-R BC cells, and CDDP sensitivity was assessed by XTT assay. C, Overall survival in BC cohorts based on TCGA data in CCNE1 and PKMYT1. CCNE1: $P = 0.2$, PKMYT1: $P = 0.6$.

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Authors' contributions
WF, SO, MT, and HY designed the study, analyzed data, and finalized the manuscript; WF, SO, MT, JA, IK, IF, AM, SS performed experiments and collected and analyzed data; HY, TS, RY, ST, YY, MN, and HE secured research funding and drafted the article. All authors were involved in writing the manuscript and reviewed and approved the final version.

Declarations

Ethics approval and consent to participate
Approval of the research protocol by an Institutional Reviewer Board: N/A.
Informed Consent: N/A.
Registry and the Registration No. of the study/trial: N/A.
Animal Studies: N/A.

Competing interests
The authors declare no competing interests.

Author details
[1]Department of Urology, Graduate School of Medical and Dental Sciences, Kagoshima University, 8-35-1 Sakuragaoka, Kagoshima 890-8520, Japan.

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