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LETTER TO THE JOURNAL

The mechanism of resistance to CDK4/6 inhibition and novel combination therapy with RNR inhibition for chemo-resistant bladder cancer

Bladder cancer (BCa) is the most prevalent urological cancer worldwide [1]. A significant proportion of BCa (89%) exhibits molecular alterations in the cell cycle pathway, and targeting cyclin-dependent kinases 4 and 6 (CDK4/6) is deemed as a promising therapeutic strategy [2]. Selective inhibitors of CDK4/6 (CDK4/6is) have been approved by the US Food and Drug Administration (FDA) [3]. They could induce cell cycle arrest in BCa immediately, and after this "sensitive stage", unknown compensatory mechanism may cause acquired resistance [4, 5]. To address this issue, our study employed multi-omics and identified ribonucleotide reductase regulatory subunit M2 (RRM2), a crucial component of the ribonucleotide reductase (RNR) complex [6], as a key mediator in conferring acquired resistance. We further investigated whether Palbociclib activates proteolysis of RRM2 by the ubiquitin-proteasome system (UPS) and the ubiquitin-like proteins (UBLs) during the sensitive stage. Additionally, we explored whether RRM2 is controlled by E2F transcription factor 3 (E2F3) when acquired resistance is established. Interestingly, upregulation of RRM2 may also cause chemotherapy resistance [7]. Thus, we verified if concurrent inhibition of RNR and CDK4/6 holds promise as a novel therapeutic strategy for BCa patients, especially those exhibit resistance to chemotherapy. All the study designs and methods are described in the Supplementary file.

List of abbreviations: BCa, bladder cancer; CAM, chicken chorioallantoic membrane; CDK4/6, cyclin dependent kinases 4 and 6; CDK4/6is, CDK4/6 inhibitors; CPDB, consensus path database; DFS, disease-free survival; E2F1, E2F Transcription Factor 1; E2F3, E2F Transcription Factor 3; FDA, U.S. Food and Drug Administration; GEPIA, gene expression profiling interactive analysis; RB, retinoblastoma; RNR, ribonucleotide reductase; RRM1/2, ribonucleotide reductase regulatory subunit m1/2; RRM2, ribonucleotide reductase regulatory subunit m2; SAM, synergistic activation mediator; SODB, spatial omics database; STRING, search tool for the retrieval of interacting genes/proteins; TCGA, the cancer genome atlas; UBLs, ubiquitin-like proteins; UPS, ubiquitin-proteasome system.

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Retinoblastoma (RB)-positive BCa elicits a sequential progression from sensitivity to resistance to Palbociclib [8]. We utilized multi-omics to identify key regulators of this process (Figure 1A, Supplementary Tables S1-S5). The only candidate matching all three high-throughput screening approaches was RRM2, and pathway analysis further demonstrated related mechanisms (Supplementary Figures S1-S2). To validate this finding, we examined the cell cycle distribution and expression levels of the other RNR subunit RRM1 and RRM2 in a time kinetic (Figure 1B-C, Supplementary Figure S3A-D). Transcript levels were initially downregulated, followed by a partial recovery, while the decline and recovery pattern of proteins mirrored this. We then transduced single-guide RNAs of RRM1 and RRM2 into T24 synergistic activation mediator (SAM) cells and confirmed partial resistance (Figure 1D-E, Supplementary Figure S3E). However, degradation of RRM2 was still observed at early time points (Supplementary Figure S3F), indicating that proteolysis might be essential for therapy response. We then applied the proteasome inhibitors Epoxomicin/MG-132 in combination with Palbociclib. As shown, protein degradation of RRM2 was effectively blocked, but only partially for RRM1 (Figure 1F, Supplementary Figure S4A-B). We next tested the combination of Palbociclib with ubiquitin-like proteins (UBLs) inhibitor MLN4924 and proved that the initial degradation of RRM1/2 was UPS-dependent but UBL-independent (Figure 1G, Supplementary Figure S4C-E).

To further investigate the regulation network of RRM2, we interrogated RRM2 in The Cancer Genome Atlas (TCGA) bladder carcinoma study for mRNA co-expression (Supplementary Table S6) and performed network analysis using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) (Supplementary Figure S5A). Additionally, by analyzing spatial transcriptomics data on Spatial Omics DataBase (SODB) and ConsensusPathDB (CPDB), we found that E2F1 was spatially co-expressed with RRM2 (Supplementary Figure S5B-C). We confirmed

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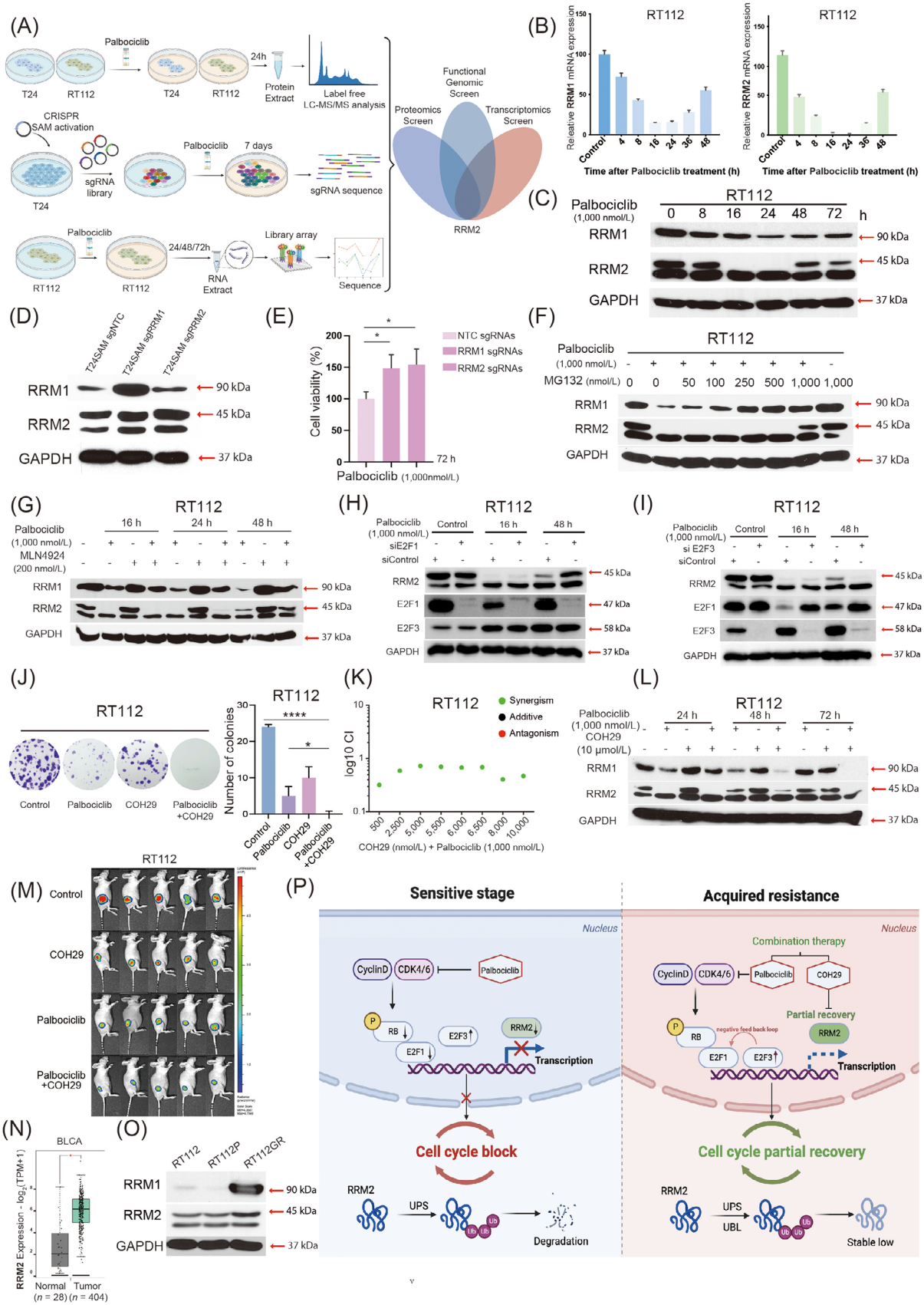

FIGURE 1 Identification of the mechanism of resistance to Palbociclib and novel overcoming combination therapy with RNR inhibition for chemo-resistant bladder cancer. (A) Schematic of a multi-omics approach including proteomics, transcriptomics, and Clustered Regularly Interspaced Short Palindromic Repeats activation (CRISPRa) functional genomics screens for candidates that are correlated to sensitivity and

that Palbociclib mainly affected the expression levels of E2F1 and E2F3, and notably, E2F3 was consistently upregulated (Supplementary Figure S5D-E). By knocking down E2F1, we observed a significant re-expression of RRM2, whereas blocking E2F3 maintained its downregulation (Figure 1H-I). Surprisingly, after long-term suppression of RRM2 expression by siE2F3, we observed an upregulation of E2F1 and blocking of cell cycle re-entry (Figure 1I, Supplementary Figure S5F). Double knockdown of E2F1 and E2F3 slightly prevented the recovery of RRM2 expression levels (Supplementary Figure S5G). These findings collectively suggest that E2F1 might have a suppressive function, while E2F3 drives the RRM2 re-expression.

We conducted comprehensive tests with the novel validated RNR inhibitor COH29 [9] in combination with Palbociclib to overcome acquired resistance. The combination successfully blocked long-term colony formation (Figure 1J, Supplementary Figure S6A). Additionally, cell viability assays confirmed synergism (Figure 1K, Supplementary Figure S6B). It is worth noting that the combination partially blocked the recovery of RRM2 in RT112 and UM-UC-3 cells, but not in T24 and 253J cells (Figure 1L, Supplementary Figure S6C). These suggested that the synergism is independent of RRM2 expression level, as COH29 blocked the function of RNR (Supplementary Figure S6D). Furthermore, synergism and senescent morphology were also observed using a living cell monitoring system in the combination groups (Supplementary Figure S7A). However, apoptosis through Caspase3/7 could not be observed (Supplementary Figure S7B). Subsequently, the combination was tested in two models: the chicken chorioallantoic membrane (CAM) and the murine subcutaneous xenograft model. Both monotherapies suppressed tumor growth, while the combination induced more potent effect (Figure 1M, Supplementary Figure S8A-E). This effect was attributed to proliferation as indicated by a significant decrease in Ki-67 expression (Supplementary Figure S8F).

acquired resistance to Palbociclib in bladder cancer cell lines. Created with Biorender.com. (B) QRT-PCR (QPCR) analysis of RRM1 and RRM2 in bladder cancer cell lines with Palbociclib at different time points, genes expression was compared to cells with control ($n = 3$; mean ± SD). (C) Western blot analysis of bladder cancer cell lines treated with Palbociclib at indicated time points, cell extracts were analyzed with antibodies against RRM1/2 and GAPDH. (D) Western blot analysis of RRM1/2 and GAPDH in T24SAM sgRRM1, T24SAM sgRRM2 and T24SAM NTC cell lines with antibodies against RRM1/2 and GAPDH. (E) Cell viability detected by Cell titer blue assay of the above cell lines was normalized to T24SAM NTC cells after 3 days of treatment with 1000nM Palbociclib ($n = 3$; mean ± SD). (F) Bladder cancer cell lines were treated with Palbociclib together with increased concentration of different proteasome inhibitors MG-132, and cell extracts were analyzed with antibodies of RRM1/2 and GAPDH 24 h later with Western blot. (G) Western blot of RRM1/2 and GAPDH in bladder cancer cells treated with Palbociclib (1,000 nmol/L) and UBL inhibitor MLN4924 (200 nmol/L) respectively, or in combination for 16, 24, and 48 h. (H-I) siPools against E2F1 or E2F3 was applied alone or in combination with Palbociclib (1,000 nmol/L), cells were extracted at 16 and 48 h, expression of RRM2, E2F1 and E2F3 was evaluated by Western blot. (J) RB-positive bladder cancer cell line RT112 was treated with half-maximal inhibitory concentration (IC50) COH29 and Palbociclib (1,000 nmol/L) mono or in combination therapy, clonogenic assay was started with 200 cell seeds and last for 21 days, with staining and monitoring, more than 50 cells may be counted as a colony and quantitative data. (K) Combination index (CI) analysis of the combined treatment of COH29 and Palbociclib on bladder cancer cell line, Combination Index was calculated with normalized cell viability results of 72 h treatment with dose kinetics of COH29 and 1,000 nmol/L Palbociclib, dot plot stands for Log10 CI value and green means synergism, black means additive and red means antagonism under different concentration of COH29. (L) Bladder cancer cell line was treated with COH29 with or without Palbociclib up to 72 h, RRM1/2 and GAPDH expression was detected by Western blot. (M) Representative bioluminescence images from xenografts-bearing Balb/c mice divided into four groups ($n = 5$) with different treatments as shown. (N) RRM2 expression in bladder cancer from TCGA RNA-seq. Tumor ($n = 404$), Normal ($n = 28$), ($^*P < 0.05$). (O) RRM1/2 and GAPDH protein expression was detected in RT112 Gemcitabine-Resistant (GR), parental, and wildtype cell lines by Western blot. (P) Schematic representation of the mechanistic model by which RRM2 dominates Palbociclib resistance. Response of bladder cancer cells to Palbociclib could be divided into sensitive stage characterized by the full block of cell cycle progression and acquired resistance stage with partial recovery of cell cycle progression. In the sensitive stage, the transcription of RRM2 is blocked and its protein gets degraded through the UPS system, meanwhile, its suppressor E2F1 gets downregulated, and its activator E2F3 gets upregulated. Resulting in the cell cycle being blocked in the S phase for around 24 h. With the accumulation of E2F3 and released suppression of E2F1, the RRM2 gets partially re-expressed and maintains at a sufficient level under the regulation of both UPS and UBL. As a major mechanism of acquired resistance to Palbociclib, re-expression of RRM2 accompanies partial recovery of cell cycle progression. With the RNR inhibitor, COH29, we have proven the combination therapy could overcome acquired resistance and this combination could be a potential therapeutic strategy for bladder cancer. Created with Biorender.com. Abbreviations: CDK4/6, cyclin dependent kinases 4 and 6; LC-MS/MS, liquid chromatography with tandem mass spectrometry; CRISPR, clustered regularly interspaced short palindromic repeats; SAM, synergistic activation mediator; sgRNA, single guide RNA; RRM2, ribonucleotide reductase regulatory subunit m2; RRM1, ribonucleotide reductase regulatory subunit m1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BLCA, bladder cancer; RT112P, RT112 parent; RT112GR, RT112 gemcitabine resistance; UPS, ubiquitin-proteasome system; E2F3, E2F Transcription Factor 3; E2F1, E2F Transcription Factor 1; RB, retinoblastoma; UBLs, ubiquitin-like proteins; CRISPRa, regularly interspaced short palindromic repeats activation; NTC, negative control; IC50, half-maximal inhibitory concentration; CI, Combination index; GR, Gemcitabine-Resistant.

By utilizing Gene Expression Profiling Interactive Analysis (GEPIA), we found that RRM2 was significantly upregulated in tumor tissues, while RRM1 and RB showed no difference (Figure 1N, Supplementary Figure S9A). Higher expression of them was related with significantly worse disease-free survival (DFS) (Supplementary Figure S9B). We also identified 4 patients from the Cornell/Trento cohort [10] who had primary and paired advanced tumors and were treated with chemotherapy. Among them, 2 patients (WCM088 and WCM117) showed amplified RRM2 only in the metastasis (Supplementary Figure S9C), suggesting that amplification of RRM2 may contribute to chemo-resistance. In addition, we retrospectively collected 20 muscle-invasive BCa specimens from patients who underwent radical cystectomy (Supplementary Table S7). These specimens were divided into three subgroups according to their response to chemotherapy. We observed lower RRM2 levels in the chemo-responded group compared to chemotherapy-naïve patients, but an increase in the chemo-resistant group (Supplementary Figure S9D-E). We tested the combination therapy in chemo-resistant BCa cell lines and found that they responded synergistically (Figure 1O, Supplementary Figure S9F-L).

In conclusion, we identified RRM2 as key mediator in conferring acquired resistance to Palbociclib in BCa (Figure 1P). Dual inhibition of RNR and CDK4/6 could effectively overcome this acquired resistance and holds promise as a potential therapeutic strategy for chemo-resistant BCa.

DECLARATIONS

AUTHOR CONTRIBUTIONS

Zhichao Tong, Wanhai Xu, Roman Nawroth and Jürgen E. Gschwend designed the study and wrote the manuscript, Zhichao Tong, Yubo Zhao, Lou Lienhard, Shiyu Bai, Ziqi Wang, Yuling Zhao, Thilo Bracht, Barbara Sitek performed the experiments, Qi Pan, Pengyu Guo, Benedikt Ebner analyzed the data.

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The authors have nothing to report.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

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DATA AVAILABILITY STATEMENT

Additional information is available from the corresponding author upon reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The research procedures were approved by the Committees for Ethical Review of Harbin Medical University (2022-DWSYLLCZ-38; KY2023-62).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.