Low expression of MYCN promotes cisplatin resistance by suppressing cisplatin-induced apoptosis in epithelial ovarian cancer

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Abstract. Epithelial ovarian cancer (EOC) is the most common cause of gynecological cancer-associated mortality. Cisplatin is one of the most effective chemotherapeutic drugs used in EOC; however, its use can lead to relapse due to cisplatin resistance. MYCN sensitizes neuroblastoma to undergo cisplatin-induced apoptosis. However, to the best of our knowledge, there have been no studies to date on the association between MYCN and cisplatin resistance in EOC. Therefore, the present study assessed this association. Datasets from The Cancer Genome Atlas database were used. The overall survival (OS) of patients receiving platin-based therapy was analyzed using Kaplan-Meier Plotter software. RNA sequencing data of 300 patients with EOC were downloaded from cBioportal. The co-expressed genes were subjected to ‘Kyoto Encyclopedia of Genes and Genomes’ analysis using DAVID software. For gene set enrichment analysis, the expression matrix was separated according to the median expression of MYCN, which was selected for hallmark gene set enrichment. Immunohistochemistry was used to assess MYCN expression in EOC tissue. Western blotting was used to evaluate MYCN, p53, Bax and Bcl-2 protein expression levels in EOC cells. Cell viability and apoptosis were assessed using Cell Counting Kit-8 and flow cytometry, respectively. The results demonstrated that MYCN upregulation was associated with increased cisplatin sensitivity and prolonged OS of patients with EOC and patients receiving platin-based therapy. Cisplatin downregulated MYCN expression in cisplatin-sensitive, but not resistant, EOC cells. The genes co-expressed with MYCN were primarily involved in pathways involved in ‘chemotherapeutic resistance’ and ‘apoptosis’. MYCN enriched the apoptosis and p53 signaling pathways in hallmark gene sets. Cells in which MYCN was knocked down demonstrated significantly increased cisplatin resistance; however, MYCN overexpression in cisplatin-resistant cells restored cisplatin sensitivity. Collectively, the present study demonstrated that MYCN downregulation promoted cisplatin resistance by suppressing cisplatin-induced apoptosis in EOC.

Introduction

Ovarian cancer (OC) is a gynecological cancer which contributes to a large number of deaths each year in industrialized countries; in 2020, it was estimated that there were 21,750 new cases and 13,940 associated deaths in the United States (1). It is estimated that 80% of patients with OC are eligible for the gold standard treatment of aggressive surgical debulking and platinum-based chemotherapy (2) and that 70% of these will develop platinum resistance and fatal disease following the long-term use of platinum (3). The outcomes of patients with platinum-resistant OC are poor, with a median overall survival (OS) rate of <12 months (4).

Resistance to platinum-based chemotherapy is a major clinical challenge in the treatment of OC, which results in a high mortality-to-incidence ratio (5). Epithelial OC (EOC) presents at an advanced stage globally and is the most common cause of gynecological cancer-associated mortality (6). In recent years, there have been notable achievements in the development of treatments of EOC, which have been validated by landmark clinical trials, such as a combination of surgery and systemic therapy, targeted therapy, chemotherapy and maximal surgical effort, of which the latter remains the mainstay (7). Therefore, overcoming platinum resistance is key to improving the prognosis of patients with EOC.

Numerous mechanisms and biological pathways underlying platinum resistance are being investigated. It has been reported that cisplatin functions by covalently binding to the DNA of tumor cells to form platinum-DNA adducts and induces cell apoptosis (8,9). One established mechanism for cisplatin resistance is evasion of cell apoptosis following long-term use of cisplatin (10), which results in resistance to cisplatin (11,12). Integrated genomic analysis of EOC
reported one of the most common focal amplifications to be in the 8q24 region containing MYC (13). MYCC, MYCN and MYCL nuclear proteins are members of the MYC family that bind to and control ~15% of the human genome (14). It has been reported that downregulation of MYCN does not influence other MYC members such as MYCC and MYCL (15). MYC belongs to the Myc proto-oncogene family, which encodes basic helix-loop-helix/leucine zipper transcription factors, and functions in numerous types of human malignancy (14), including lung cancer (16) and mammary adenocarcinomas (17). Moreover, the Myc signaling pathway is one of the most commonly activated oncogenic pathways in human malignancy (18). Furthermore, previous studies have reported that Myc-mediated transcriptional networks are under tight regulation in normal cells and control numerous cellular processes, such as metabolic processes and cell proliferation, differentiation and apoptosis (14,19,20). As a member of the MYC family, MYCN is a type of short-lived transcription factor that is dysregulated in numerous types of human cancer (21), serves as a therapeutic target (22) and is associated with poor clinical outcome in multiple types of cancer (23). It has been reported that amplification of MYCN is associated with an aggressive phenotype and a poor prognosis in neuroblastoma; relapse of platinum-resistant neuroblastoma is the primary cause of mortality in patients with MYCN amplification (24). MYCN overexpression is significantly associated with poor outcomes in breast cancer (25). However, MYCN contributes to cisplatin sensitization in acute myelogenous leukemia (26).

The function of MYCN in EOC and chemotherapeutic resistance remains unclear. Therefore, the present study assessed the role of MYCN in EOC chemotherapeutic resistance.

Materials and methods

Bioinformatics analysis. The datasets used in the present study are available from The Cancer Genome Atlas (TCGA) database (tcga-data.nci.nih.gov/tcga) under TCGA-OV project (13). The analysis of the OS of patients receiving platin-based therapy was performed using Kaplan-Meier Plotter software (kmplot.com/index.php?p=service&cancer=ovar) with auto select best cut-off.

The GSE114206 dataset (27), which contains mRNA expression profiles of 12 patients with EOC (cisplatin-resistant patients, n=6; cisplatin-sensitive patients, n=6) was obtained from Gene Expression Omnibus (GEO) database (ncbi.nlm.nih.gov/geo/).

RNA sequencing data of 300 patients with OC (TCGA pan-cancer project) was downloaded from cBioportal (cbioportal.org/) (13). The co-expressed genes, assessed using Spearman's correlation analysis, were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg/) analysis using DAVID software version 2.0 (https://david.ncifcrf.gov/). For gene set enrichment analysis (GSEA), the expression matrix was separated according to the median expression of MYCN. The expression matrix was used for Hallmark gene set enrichment using GSEA software (V.4.1.0) (gsea-msigdb.org/gsea/index.jsp).

Tissue samples. In total, 26 female patients with EOC who underwent primary surgery followed by cisplatin-based chemotherapy at the First Affiliated Hospital of Chongqing Medical University (Chongqing, China) between 2015 and 2019 were enrolled in the present study. None of these patients received radiotherapy before the surgery. The subtypes were assessed using histological examination performed by pathologists. A total of 22 patients (84.6%) were assessed as being serous and 4 (15.4%) were assessed as having mucinous EOC. Following primary chemotherapy, patients who relapsed within 6 months were assigned to the cisplatin-resistant group (n=13; mean age, 56 years; range, 37-70 years) and those who relapsed after 6 months or did not relapse were assigned to the cisplatin-sensitive group (n=13; mean age, 51 years; age range, 42-68 years). All patients provided written informed consent prior to inclusion in the study. The present study was approved by the Institutional Ethics Committee of The First Affiliated Hospital of Chongqing Medical University (Approval No. T FAHCQMU-2021-010). The characteristics of patients with EOC are presented in Table I.

Immunohistochemical analysis. Samples were fixed by 4% PFA solution, embedded by paraffin at 4˚C overnight, sliced into 4 µm sections, and incubated at 60˚C for 30 min. Following deparaffinization by xylene I and xylene II (each for 20 min) at room temperature, rehydration by alcohol series (100, 95%, 85%, and 75%), antigen retrieval by citric acid repair solution at oven for 5 min and endogenous peroxidase inhibition by 3%H2O2 at room temperature for 10 min, serous EOC sample slides were incubated with anti-MYCN antibody (1:100; cat. no. 10159-2-AP; ProteinTech Group, Inc.) at 4˚C overnight. Slides were incubated with goat-anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:50; PR30009; ProteinTech Group, Inc.) for 1 h at room temperature, followed by assessment of peroxidase activity using diaminobenzidine for 10 min at room temperature. The tissue sections were visualized using a light microscope (40x). The statistical analysis was performed using histochemistry score (H-score) as previously reported (28).

Cells and cell culture. The human EOC SK-OV-3 cell line, which is commonly used in the study of cisplatin-resistant in serous EOC (29,30), was purchased from Jiangsu KeyGEN BioTech Co., Ltd. The human EOC cisplatin-resistant SK-OV-3/DDP cell line was purchased from Shanghai Chuan Qiu Biotechnology Co., Ltd. Cells were cultured in RPMI-1640 medium ( Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (PAN-Biotech GmbH) and 1% penicillin/streptomycin (Beyotime Institute of Biotechnology) in an incubator at 37˚C with 5% CO2.

Cisplatin treatment. The SK-OV-3 and SK-OV-3/DDP cells were treated with a range of concentrations of cisplatin (0, 5 and 10 µM) for 24 h at 37˚C.

Lentivirus construction and infection. The short hairpin (sh)RNA MYCN (LV-sh-MYC; 5'-GCAGAAACCACA ACATCCG-3'), negative control (LV-sh-NC; 5'-TTC TCCGACGTGTCAGT-3'), MYCN-overexpressing (LV-MYC) and NC lentivirus (LV-NC with a scrambled
sequence) were purchased from Shanghai GenePharma Co., Ltd. The sequences were ligated into plko.1-puro plasmid. The lentivirus was packaged by transfection of 2 µg plko.1-puro, 1 µg psPAX2 and 2 µg pMD2.G into 293 cells for 24 h. The supernatant was collected for harvesting lentivirus particles. All lentiviruses contained GFP and puromycin resistance genes. At 72 h post-transduction, cells (MOI=10) were selected using puromycin (2 µg/ml) and maintained using puromycin (1 µg/ml) (Beyotime Institute of Biotechnology). The transfection efficiency in SK-OV-3 cells and SK-OV-3/DDP cells was assessed using western blotting.

Western blotting. SK-OV-3 and SK-OV-3/DDP cells were treated with cisplatin (0, 5 and 10 µM) for 24 h at 37˚C and LV-sh-MYCN SK-OV-3 cells and LV-MYCN SK-OV-3/DDP cells were treated with cisplatin (0 and 10 µM) for 24 h at 37˚C, then harvested using PBS and lysed using RIPA buffer [Roche Diagnostics (Shanghai) Co., Ltd.] with protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The protein concentration was evaluated using the BCA method (Beyotime Institute of Biotechnology). The transfection efficiency in SK-OV-3 cells and SK-OV-3/DDP cells was assessed using western blotting.

Table I. Characteristics of patients with epithelial ovarian cancer.

| Characteristic          | Sensitive, n=13.0 | Resistant, n=13 | P-value |
|-------------------------|-------------------|----------------|---------|
| Median age, years (range) | 51.0 (42.0-68.0) | 56.0 (37.0-70.0) | 0.198   |
| Histology               |                   |                |         |
| Serous (%)              | 10.0 (76.9)       | 12.0 (92.3)    | 0.296   |
| Mucinous (%)            | 3.0 (23.1)        | 1.0 (7.7)      |         |
| FIGO stage              |                   |                | 0.187   |
| I (%)                   | 2.0 (15.4)        | 0.0 (0.0)      |         |
| II (%)                  | 3.0 (23.1)        | 1.0 (7.7)      |         |
| III (%)                 | 6.0 (46.1)        | 11.0 (84.6)    |         |
| IV (%)                  | 2.0 (15.4)        | 1.0 (7.7)      |         |
| Grade                   |                   |                | 0.500   |
| 1/2 (%)                 | 5.0 (38.5%)       | 6.0 (46.2%)    |         |
| 3 (%)                   | 8.0 (61.5%)       | 7.0 (53.8%)    |         |
| Median CA125 at diagnosis, U/ml (range) | 474.0 (46.0-1,483.0) | 884.0 (28.5-3949) | 0.215   |

FIGO, The International Federation of Gynecology and Obstetrics; CA125, cancer antigen 125.

Cell Counting Kit-8 (CCK-8) assay. LV-sh-MYCN SK-OV-3 and LV-MYCN SK-OV-3/DDP cells (1x10^4 cells/well) were seeded into a 96-well plate and treated with 0, 2, 4, 6, 8, 10 and 15 µM cisplatin (Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C. Cell viability was determined using CCK-8 assay (Abcam), for which the cells were incubated at 37˚C for 1 h. The absorbance was measured at 450 nm using an Infinite M200 PRO spectrophotometer (Tecan Group, Ltd.).

Aptosis analysis. The number of apoptotic cells was quantified using Annexin V-FITC/propidium iodide (PI) staining. LV-sh-MYCN SK-OV-3 and LV-MYCN SK-OV-3/DDP cells (1x10^4 cells/well) were incubated in 6-well plates overnight at 37˚C and treated with cisplatin (0 and 10 µM) for 24 h at 37˚C. Cell viability was determined using CCK-8 assay (Abcam), for which the cells were incubated at 37˚C for 1 h. The absorbance was measured at 450 nm using an Infinite M200 PRO spectrophotometer (Tecan Group, Ltd.).
Statistical analysis. Statistical analysis was performed using GraphPad Prism 8.0.1 (GraphPad Software, Inc.). Fisher's exact test was used for the analysis of histology, FIGO stage and grade. Unpaired Student's t test was used for analysis of age and CA125. H scores are presented as the median + interquartile range; all other data are from at least 3 independent experimental repeats, presented as the mean ± standard deviation. Comparisons between 2 groups were performed using Mann-Whitney test; for comparisons of ≥3 groups, Kruskal-Wallis followed by Dunn's post hoc test was used. P<0.05 was considered to indicate a statistically significant difference.

Results

High MYCN expression is positively associated with greater OS in patients receiving platin-based therapy. The data of 424 patients (low MYCN group, n=212; high MYCN group, n=212) obtained from TCGA database were assessed using OS analysis, which demonstrated that high expression of MYCN was associated with greater OS (Fig. 1A). A heatmap of the top 50 differentially expressed genes demonstrated that MYCN was increased in cisplatin-sensitive patients compared with cisplatin-resistant patients (Fig. 1C). The immunohistochemistry assessment of the tumor tissue collected in the present study demonstrated that MYCN protein expression in patients with cisplatin-sensitive EOC was significantly higher than that in cisplatin-resistant EOC (Fig. 1D and E).

Low MYCN protein expression levels are positively associated with cisplatin-resistance. Western blotting demonstrated that MYCN expression was significantly lower in SK-OV-3/DDP compared with SK-OV-3 cells (Fig. 2A). Moreover, cisplatin (0, 5 and 10 µM) significantly decreased MYCN protein expression in a dose-dependent manner in SK-OV-3 cells but not in SK-OV-3/DDP cells (Fig. 2B).

MYCN downregulation promotes cisplatin resistance in EOC cells. Western blotting demonstrated notable knockdown of MYCN by LV-sh-MYCN compared with LV-sh-NC in SK-OV-3.
cells (Fig. 3A). The present study assessed the effect of MYCN knockdown on MYCC and MYCL protein expression levels, which demonstrated that LV-sh-MYCN did not affect MYCC and MYCL expression compared with LV-sh-NC in SK-OV-3 cells. CCK-8 assay demonstrated that cisplatin markedly decreased SK-OV-3 cell viability in a dose-dependent manner; furthermore, compared with LV-sh-NC, cell viability was significantly higher in the LV-sh-MYCN group following cisplatin treatment (Fig. 3B). Flow cytometry of SK-OV-3 cells demonstrated that cisplatin induced significant cell apoptosis in the LV-sh-NC group compared with the untreated LV-sh-NC group, but there was no significant difference between cisplatin treated group and cisplatin untreated group in the LV-sh-MYCN group. Furthermore, compared with LV-sh-NC,
cisplatin-induced cell apoptosis was significantly decreased in the LV-sh-MYCN group. In groups without cisplatin treatment, there was significantly decreased cell apoptosis in the LV-sh-MYCN group compared with the LV-sh-NC group (Fig. 3C).

**MYCN upregulation reverses cisplatin resistance of EOC cells.** Western blotting demonstrated significant overexpression of MYCN in LV-MYCN SK-OV-3/DDP cells compared with LV-NC in SK-OV-3/DDP cells (Fig. 4A). CCK-8 assay in the SK-OV-3/DDP cells demonstrated that cisplatin markedly decreased cell viability in a dose-dependent manner; moreover, compared with LV-NC, cell viability was significantly decreased by cisplatin (≥4 µM) in the LV-MYCN group (Fig. 4B). Flow cytometry demonstrated that in the SK-OV-3/DDP cells, cisplatin induced significantly increased apoptosis in the LV-MYCN group compared with the LV-NC group; furthermore, compared with LV-NC, cisplatin-induced cell apoptosis was significantly increased in the LV-MYCN group. In the groups without cisplatin treatment, there was significantly increased cell apoptosis in the LV-MYCN group compared with the LV-NC group (Fig. 4C).

**MYCN downregulation promotes cisplatin resistance by decreasing cisplatin-induced apoptosis.** KEGG enrichment analysis of co-expressed genes of MYCN in the TCGA-OC dataset demonstrated that they were primarily involved in pathways that contributed to chemotherapeutic resistance and participated in cell apoptosis, such as 'MAPK signaling pathway', 'PI3K/AKT signaling pathway' and 'p53 signaling pathway'.
pathway’ (Fig. 5A). Globally, GSEA demonstrated that MYCN was enriched in ‘apoptosis’ and ‘p53 pathway’ in the hallmark gene set (Fig. 5B-D).

To evaluate these results, p53, BAX and Bcl-2 protein expression levels were assessed using western blotting. In SK-OV-3 cells, treatment with cisplatin induced the significant upregulation of p53 protein expression levels and significantly increased the Bax/Bcl2 ratio in the LV-sh-NC group compared with the untreated group; however no significant difference was demonstrated in the LV-sh-MYCN group compared with the untreated group (Fig. 6A). Furthermore, compared with LV-sh-NC, cisplatin treatment induced significant upregulation of p53 protein expression levels and significantly increased Bax/Bcl2 ratio in LV-sh-MYCN group; moreover, in the groups without cisplatin treatment, there were significantly higher p53 protein expression levels and Bax/Bcl2 ratio in the LV-sh-MYCN group compared with the LV-sh-NC group (Fig. 6B).

Discussion
The present study was based on bioinformatics analysis, which demonstrated that patients with high MYCN expression had greater OS. High MYCN expression was associated with increased OS of patients receiving platin-based therapy; immunohistochemistry of tumor tissue collected in the present study demonstrated that there was significantly higher MYCN protein expression levels in cisplatin-sensitive EOC than cisplatin-resistant EOC.
Therefore, it was hypothesized that MYCN was inhibited cisplatin resistance in EOC.

A previous study of expression profile of EOC reported that MYCN is overexpressed in C5 subtype tumors compared with three other molecular subtypes (C1, C2 and C4) of high-grade serous EOC (31), which indicated its aggressive role in high-grade serous EOC. Furthermore, MYCN overexpression has been reported to be predictive of an aggressive phenotype and poor prognosis in neuroblastoma (23), breast cancer (24) and spinal ependymoma (32); however, MYCN overexpression has been reported to be predictive of an aggressive phenotype and poor prognosis in neuroblastoma (23), breast cancer (24) and spinal ependymoma (32); however, MYCN contributes to cisplatin sensitization in acute myelogenous leukemia (25). Consistent with this, the present study demonstrated that MYCN protein expression in SK-OV-3/DDP cells was significantly lower compared with that in SK-OV-3 cells and cisplatin significantly decreased MYCN protein expression levels in SK-OV-3 cells, but not in SK-OV-3/DDP cells. These results indicated that cisplatin functioned by suppressing expression of MYCN in EOC. However, the association between MYCN protein expression and cisplatin-induced cell behavior in EOC is unknown.

Apoptosis serves a key role in tissue homeostasis in response to numerous stimuli (33); decreased apoptosis associated with occurrence, development and drug resistance of tumors (34). Cisplatin functions by covalently binding to the DNA of tumor cells to form platinum-DNA adducts and induces cell apoptosis (8,9). Once the cisplatin-induced apoptotic pathway is blocked, tumor cells acquire resistance to the proapoptotic effects of cisplatin, thus decreasing its antitumor efficacy (35). In the present study, viability and apoptosis of SK-OV-3 and SK-OV-3/DDP cells following treatment with cisplatin was assessed, which demonstrated that the SK-OV-3 cells in which MYCN was knocked down exhibited a significantly decreased sensitivity to cisplatin-induced cell apoptosis compared with NC. Furthermore, SK-OV-3/DDP cells with MYCN overexpression exhibited a significantly increased sensitivity to cisplatin-induced cell apoptosis. Collectively, these results demonstrated that MYCN increased cisplatin-induced apoptosis and that apoptosis may be the primary mechanism by which MYCN inhibits cisplatin resistance in EOC. However, the molecules that mediate the role of MYCN in EOC remain to be elucidated.

The genes co-expressed with MYCN were primarily involved in pathways which contributed to chemotherapeutic resistance and participated in cell apoptosis, including ‘MAPK signaling pathway’, ‘PI3K/AKT signaling pathway’ and ‘p53 signaling pathway’. Globally, GSEA demonstrated that MYCN was enriched in ‘apoptosis’ and ‘p53 pathway’ in the hallmark gene sets. The tumor suppressor p53 is a transcription factor that regulates molecules in extrinsic (Bcl2 family) and intrinsic (mitochondrial) apoptotic pathways (36-38). Balance of Bcl2 family members determines whether a cell undergoes apoptosis or survival (39). Cisplatin increases p53 levels and facilitates the apoptotic response in tumor cells (40); moreover, cisplatin activates Bax, decreases expression of Bcl2 and shifts the Bax/Bcl2 ratio in a pro-apoptotic direction in tumor cells (41). Furthermore, the emergence of p53 mutant cisplatin-resistant OC cells has been demonstrated following drug exposure (42) and patients with OC who have p53 mutations are more resistant to cisplatin-based therapy (43). In the present study, p53, Bax and Bcl-2 protein expression levels were assessed using western blotting, which demonstrated that following treatment with cisplatin, SK-OV-3 cells in which MYCN was knocked down exhibited significantly decreased p53 protein expression.
levels and Bax/Bcl2 ratio, whereas SK-OV-3/DDP cells with overexpressed MYCN exhibited significantly increased p53 protein expression levels and Bax/Bcl2 ratio. Therefore, it was hypothesized that MYCN affected cisplatin resistance by regulating p53 expression and ratio of Bax/Bcl2.

In conclusion, the present study suggested that MYCN served as a potential marker for cisplatin treatment in EOC. Specifically, the present study demonstrated that patients with high expression of MYCN were more sensitive to cisplatin, whereas patients with low expression of MYCN may be resistant to cisplatin. Furthermore, it may be hypothesized that the findings for cisplatin may be analogous to other chemotherapy drugs that lead to cell apoptosis. However, one weakness in current study is the use of only one EOC cell line and experiments should be replicated using another EOC cell line.

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Availability of data and materials
The datasets generated and/or analyzed during the current study are available in the GEO repository, accession number GSE114206.

Authors' contributions
RY, HZ and RW performed experiments and data analysis. LX conceived and supervised the study and wrote the manuscript. RY and LX confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate
All patients provided signed consent prior to their inclusion in the present study. The present study was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Chongqing Medical University (approval No. TFAHCQM U-2021-010).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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