Transcriptome-based Stemness Indices Analysis Reveals Platinum-based Chemotherapeutic Response Indicators in Advanced-stage Serous Ovarian Cancer

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Research

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Abstract

Background: Serous ovarian cancer (SOC) is a main histological subtype of ovarian cancer (OC). Cancer stem cells (CSCs), with self-renewal and differentiation potential, are considered to be the cause of chemoresistance in SOC. However, the underlying modulation mechanisms of chemoresistance led by cancer stemness are still undefined.

Results: We found that mRNAsi and corrected mRNAsi (by tumor purity) scores were both greater in tumors of Grade 3 and 4 than that of Grade 1 and 2, indicating a stronger stemness in cancer cells of higher grades. A total of 42 key genes were obtained from the most significant mRNAsi-related gene module. Functional annotation revealed that these key genes were mainly involved in mitotic division and were closely related to cell proliferation. A total of 13 potential platinum response indicators were selected from the genes enriched to platinum-response associated pathways. Among the 13 key genes, we identified 11 genes with prognostic value of progression-free survival (PFS) in advanced-stage SOC patients treated with chemotherapy containing platinum and 7 prognostic genes in patients treated with the combination of platinum and taxol. The expression of the 13 key genes was also validated between platinum-resistant and sensitive SOC samples of advanced stages in two Gene Expression Omnibus (GEO) datasets.

Conclusion: The results revealed that CDC20 was a potential platinum-based chemotherapeutic response indicator in advanced-stage SOC and the findings may provide new insight into the prediction of drug response thus to guiding the use of chemotherapies in patients of advanced-stage SOC in the clinic.

Background

OC is the leading lethal malignancy occurred in female reproductive organs. Annually, 230,000 women will be diagnosed and 150,000 will die worldwide [1]. Serous ovarian cancer (SOC) was a distinct histological subtype of OC and often diagnosed at advanced stages, with a disappointing prognosis [2, 3].

In recent years, the hypothesis of a subpopulation of tumor cells, cancer stem cells (CSCs), has been widely accepted. CSCs possess the potential of self-renewal and uncontrolled growth. The subpopulation of CSCs has persistently maintained the competence of self-perpetuation and simultaneously giving rise to differentiated types of progeny tumor cells through asymmetrical division. The stemness of CSCs is an important cause of tumor chemoresistance and also a potential target of anticancer strategies [4–6]. Investigating key genes among the stemness-associated genes in advanced-stage SOC might be a feasible way to find drug response indicators.

In the last decade, high-throughput technology has achieved a considerable amount of data storage in public databases, which provides feasibilities to conduct high-quality data mining by utilizing the underlying data. Therefore, machine learning has been successfully applied to the medical fields, particularly in oncological research. To summarize the features of stem cells, researchers have performed
analyses with machine learning methods. Malta et al. [7] used a one-class logistic regression (OCLR) machine learning algorithm to extract the transcriptomic and epigenetic feature sets from normal tissue-derived pluripotent stem cells and their differentiated progeny, which have different degrees of stemness. They identified stem cell signatures and quantified stemness by using transcriptome and methylome data. Ultimately, two stemness indexes, mRNAsi and mDNA-based stemness indices (mDNAsi) were proposed in their study. The researchers further analyzed cancer stemness in 33 tumor types in TCGA to verify the two stemness indices. Based on the study, we obtained the stemness index of each SOC sample in TCGA to further utilize in our study.

In the present study, we aimed to investigate the clinical significance of mRNAsi and identify gene modules as well as key genes closely correlated to the stemness of advanced-stage SOC according to mRNAsi with the method of WGCNA. Potential drug response indicators would be selected from the stemness-associated key genes by OS and PFS analyses on advanced-stage SOC patients and validated in multiple datasets. In summary, we investigated the cancer stemness-associated key genes by bioinformatic methods and further selected potential drug response indicators among them by prognostic value and expressional validation. The results of this study may provide new clues for drug response indication to guide the use of chemotherapy in the treatment of advanced-stage SOC.

**Results**

**The prognostic roles and clinical characteristics of mRNAsi/corrected in SOC**

mRNAsi is a novel stemness index for evaluating the similarity between tumor cells and pluripotent stem cells. It could describe the dedifferentiation potential of tumor cells and be considered a quantitative marker of CSCs stemness. The mRNAsi index was reported to be derived from normal cells and cells with different degrees of stemness via the OCLR algorithm calculating on the TCGA transcriptomic data [7]. Tumor tissues were composed of different kinds of cells, including tumor cells and other types of cells, such as stromal and immune cells. Tumor purity was considered a an interference factor affecting the evaluating of the mRNAsi score. A previous study had reported the tumor purity score of multiple cancers in TCGA evaluated by the ESTIMATE method and we obtained the tumor purity score of OC from the study [8]. The mRNAsi was corrected as previously reported (mRNAsi/tumor purity) [9]. To explore the relevance of mRNAsi/corrected mRNAsi and the clinical features, we performed analyses on SOC patients in TCGA. Because of the extremely small sample size of Grade 1 and Grade 4, as well as stage I and stage IV in the TCGA database, we divided the SOC samples into two groups according to histopathological grades (G1+G2 and G3+G4) and clinical stages (stage I-II and stage III-IV), respectively.

As shown in **Figure 1A and 1B**, SOC samples of higher grades (Grade 3 and Grade 4) had greater mRNAsi and corrected mRNAsi score than those tumor samples of lower grades (Grade 1 and Grade 2) with significance. mRNAsi and corrected mRNAsi scores were not significantly associated with clinical stages (**Figure 1C,1D**). These results indicated that SOC samples of higher histopathological grades had greater stemness than those of lower grades.
As reported, CSCs were the cause of tumor chemoresistance and recurrence. PFS was used as a surrogate of chemotherapy response in OC [10]. To reveal a potential drug response indicator of stage III-IV SOC by using mRNAsi, we first conducted the PFS analyses on mRNAsi and corrected mRNAsi. In the study, SOC cases of stage III-IV with complete available information of progression-free survival time were divided into low and high score groups based on the mRNAsi or corrected mRNAsi scores. We observed that there was no significant difference in PFS between low and high-mRNAsi/corrected mRNAsi groups (Figure 1E,1F). More unexpectedly, advanced-stage SOC patients with greater mRNAsi score, which indicated stronger stemness of the tumor cells, had an even higher PFS rate within approximately 4 years showed in the survival curve. Similar results were observed in the analysis of corrected mRNAsi. This finding was very different from our understanding that greater stemness of tumor cells would indicate decreased progression-free survival [11, 12]. The above results indicated that the mRNAsi score in SOC samples had a close correlation with the histopathological grades. The corrected procedure didn't impact the results of clinical correlation analyses. Therefore, mRNAsi, instead of corrected mRNAsi, was used in the subsequent analyses.

DEGs between normal ovarian and SOC tissues

To reveal the potential key genes associated with stemness characteristics of SOC cells according to mRNAsi, we first screened DEGs between normal ovarian samples and SOC samples of all stages (N=379). Thus, we identified 7255 DEGs, including 3790 upregulated ones and 3465 downregulated ones (Figure 2A,2B). The top GO categories were shown in Figure 2C. Among the GO categories, “mitotic nuclear division” was the most enriched biological process (BP) of the DEGs between normal and SOC samples.

Identification of mRNAsi-related modules and key genes by WGCNA

To identify mRNAsi-associated modules and key genes, WGCNA was used to construct a co-expression network of DEGs to cluster the samples and genes into biological modules. In the study, outlier samples were first eliminated from the analysis (Figure S1A) and the remained samples of stage III-IV (N=337) were clustered according to mRNAsi and EREG-mRNAsi score (Figure S1B). β=8 (scale-free $R^2=0.950$) was selected as the soft threshold to ensure the scale-free network (Figure S1C). Thus, we obtained 9 biological gene modules (Figure 2D).

To explore the relationship between gene modules and the mRNAsi scores of the advanced-stage SOC samples, we used MS to quantify the correlation between the overall gene expression level of the corresponding module and mRNAsi. As shown in Figure 2E, the upper row of each module was the $R^2$ value, representing the degree of correlation between gene expression and mRNAsi or EREG-mRNAsi in the corresponding module. According to the $R^2$ value, the green module was most positively associated with mRNAsi (with a correlation coefficient close to 0.80, p value=1.8e-68) and the blue module was most negatively associated with mRNAsi (with a correlation coefficient of 0.71, p value=7.9e-62) (Figure 2F,2G).

To study the key genes positively correlated to stemness characteristics of advanced-stage SOC, we
chose the green module for further analyses. To avoid missing some crucial enriched pathways caused by a lack of genes, the criteria were defined as cor. MM>0.80 and cor.GS>0.40 (not 0.50 as reported [9]) to obtain more potential key genes thus we could acquire full information of enriched pathways. Finally, we obtained 42 key genes including AURKA, AURKB, BIRC5, BUB1, CCNA2, CCNB2, CDC20, CDC6, CDCA5, CDK1, CENPA, CKAP2L, DEPDC1, DLGAP5, ECT2, ERCC6L, EXO1, FAM83D, HJURP, KIF15, KIF18B, KIF23, KIF2C, KIF4A, MCM10, MELK, MYBL2, NCAF, NCAH, NDC80, NEK2, NUF2, NUSAP1, ORC1, PLK1, RACGAP1, RRM2, SGO1, TOP2A, TPX2, TTK, and UBE2C.

**Function annotation and pathway enrichment of the selected key genes**

To elucidate the biological process and signaling pathways the selected key genes involved in, GO and KEGG analyses were performed. The results revealed that the top 5 BP of the green module was “mitotic nuclear division”, “chromosome segregation”, “nuclear division”, “nuclear chromosome segregation”, “mitotic sister chromatid segregation” (Figure 3A). These key genes were mainly involved in the pathways of the cell cycle (Figure 3B). Interestingly, the selected key genes were also enriched to the platinum response-associated pathways “cellular senescence” [13-15], “p53 signaling pathway” [16, 17], and “Platinum drug resistance” (Figure 3B). Cell cycle arrest was also the main molecular mechanism of the platinum anticancer effect [18, 19]. To explore the potential predictor of platinum-based therapeutic response, we selected a total of 13 key genes enriched to the four platinum response-associated pathways to be further analyzed in advanced-stage SOC.

**Correlation between key genes at mRNA and protein level**

To explore the mutual correlation of the key genes and their protein products, we used the Pearson correlation method and STRING online tool to perform the analysis. As shown in the Figure 3C, each node represented a protein in the network. The PPI network constructing with the encoded proteins of the selected key genes showed a wide and strong relationship among the proteins. We calculated the edge connecting nodes in the network (Figure 3D) and found every node in the network was connected to the rest 12 proteins, indicating having a mutual correlation with all of the rest proteins. We observed dense and balanced correlations among all the paired key genes. At the mRNA level, The relationship between PLK1 and CDC20 had the highest correlation coefficient of 0.78. The relationship between BUB1 and TOP2A, as well as BUB1 and CDC20, had a lower correlation coefficient of 0.77 and 0.76. AURKA and ORC1 had the lowest correlation coefficient of 0.58 (Figure 3E). These results demonstrated that the selected key genes composed a strong and dense interaction network with each other.

**Expressional validation of key genes in multiple datasets**

As shown in Figure 4A and 4B, the 13 selected key genes all had significantly higher expression levels in SOC samples of stage III-IV than in normal ovarian samples (p<0.001). To verify the overexpression of the key genes in SOC samples at the bulk RNA level, we selected two GEO datasets for validation. We first compared the expression of the key genes between normal ovarian samples (N=10) and advanced stage, high-grade SOC samples (N=53) in the GSE18520 dataset, and confirmed that all the key genes were
significantly overexpressed (p<0.001) in SOC tissue (Figure 5A). Moreover, we also evaluated the differential expression of the selected key genes between paired normal oviducts and HGSOC samples in GSE69428. As shown in Figure 5B, all of the 13 key genes were significantly upregulated in SOC tissue compared to normal oviducts (p<0.001). These results further confirmed that the overexpression of the screened key genes in HGSOC.

To further understand the expression levels of the key genes in multiple cancer types, we used Oncomine to perform the pan-cancer analyses. Except ORC1 was not in the top 10% gene rank of DEGs, the other 12 key genes were all ranked in the top 10% gene within at least 1 OC dataset (Figure 5C). The results strongly indicated that these key genes might be consistent genes of oncogenes or even consistent stemness biomarkers widely overexpressed in multiple cancer types.

In contrast to traditional bulk RNA-seq, where gene expression was measured by average method across thousands of cells, the technology of scRNA-seq focused on the gene expression in individual cells and could provide more information on tumor heterogeneities. In the analyses with PanglaoDB, we first explored the expression level and positive distribution of the key genes within a scRNA-seq dataset of HGSOC. The 13 key genes had different expression levels and different positive-cell distribution among all the OC cells in the analysis (Figure S2). The positive expression rate of the key genes was very different from each other. However, we observed that the positive-expression cells were distributed in the cell population with a quite uniform pattern. Moreover, we also evaluated the expression pattern of the key genes in another scRNA-seq dataset of ESCs. Interestingly, except for MYBL2 and ORC1, the positive-expression ESCs of the rest 11 key genes tended to be gathered in a small subpopulation at the upright in the t-SNE plots (Figure S3). On one hand, the above results suggested that each of the key genes was expressed in a certain portion of the ovarian cancer cells but not every single cell in the cell population, indicating a presence of intra-tumor stemness heterogeneities in SOC. On the other hand, the expression level and distribution pattern of the key genes in the ESCs further confirmed that the key genes were closely associated with the stemness of cells.

The Prognostic Value and platinum-based chemotherapeutic response of the Key Genes

First, we explored the OS prognostic role of the key genes in SOC patients of stage III-IV by Kaplan-Meier plotter. The results revealed that AURKA, MYBL2, ORC1, and PLK1 were associated with the OS of advanced-stage SOC patients with statistical significance. The higher expression level of AURKA, MYBL2, and ORC1 could predict shorter OS while the higher expression level of PLK1 could predict a longer OS (Figure 6, Table1).

To explore potential indicators of platinum-based chemotherapeutic response among the stemness-associated key genes, we conducted OS and PFS analyses on the key genes among stage III-IV patients of SOC treated with chemotherapy containing platinum or the combination of platinum and taxol. Among the 13 key genes, the expression level of only 4 genes, AURKA, CCNA2, MYBL2, and ORC1 significantly affected the OS of advanced stage SOC patients treated with chemotherapy containing platinum (Figure S4). The higher expression level of AURKA, MYBL2, and ORC1 could predict shorter OS while the higher
expression level of CCNA2 could predict longer OS. The expression level of 11 key genes (AURKA, BIRC5, CCNA2, CCNB2, CDC20, CDK1, ORC1, PLK1, RRM2, TOP2A, and TTK) were significantly associated with PFS (Figure 7). Among the prognostic genes of PFS, except for ORC1, the higher expression level of the other 10 genes could predict significantly longer PFS (Table 2).

We further explore potential response indicators of chemotherapy containing both platin and taxol by OS and PFS analyses. The expression level of CCNA2, CDK1, ORC1, TOP2A, and TTK were significantly associated with the OS of advanced-stage SOC patients receiving platin combined with taxol and the higher expression level of the 5 genes could predict shorter OS (Figure S5, Table 1). The higher expression level of 7 PFS-associated genes, BIRC5, CCNB2, CDC20, MYBL2, PLK1, TOP2A, and TTK, could predict longer PFS (Figure 8, Table 2).

The differential expression of key genes between platinum-resistant and sensitive SOC samples

As shown above, the 13 selected key genes were closely associated with the drug response to platinum-based chemotherapy. Thus, we selected two GEO datasets to perform validation to illuminate whether the key genes played roles in the modulation of platinum sensitivity. The analysis on GSE131978 revealed that BIRC5, BUB1, CDC20, CDK1, and ORC1 had statistically significant differential expression between platinum-resistant and sensitive samples of stage III-IV SOC. And the expression level of the 5 genes was higher in platinum-sensitive samples than in platinum-resistant samples. In the analysis on GSE51373, we found that BUB1, CDC20, PLK1, and TOP2A had differential expression between chemotherapy (contains platinum) resistant and sensitive samples of advanced-stage SOC. Consistent with the results of GSE131978, the 4 genes were also downregulated in chemotherapy-resistant samples compared to the chemotherapy-sensitive ones. The two datasets had only one overlapped DEG CDC20 among the 13 selected genes. Consider the results of survival analyses, the higher expression level of CDC20 could predict longer PFS of advanced-stage SOC patients treated with chemotherapy containing platinum or a combination of platinum and taxol. These results indicated the higher expression level of CDC20 could predict a higher sensitivity to platinum-based chemotherapy.

Discussion

SOC is a main histological subtype of OC with a poor prognosis. Debulking surgery combined with platinum-based chemotherapy is the primary therapy of SOC. Although platinum-based therapy continued to be the first-line option of advanced-stage SOC, platinum is not the best approach for the part of patients with limited platinum sensitivity (a platinum-treatment free interval of 6–12 months) [20]. Nowadays, poly (ADP-ribose) polymerase (PARP) inhibitors have provided great therapeutic benefits to OC patients. Platinum sensitivity is also prospective biomarkers for predicting the response to PARP inhibitors (PARPi) thus to guiding the drug choice for OC patients [21].

CSCs are a subpopulation of cancer cells closely correlated to survival, tumor recurrence, and therapeutic resistance of OC patients [22, 23]. As the stemness of CSCs is an important cause of the generation of
chemoresistance [5, 24], investigating reliable drug response indicators, especially the response indicator of a platinum-based chemotherapy among stemness-associated genes is feasible and essential.

In the study, we identified platinum-based chemotherapeutic response indicator among key genes associated with CSCs stemness characteristics by multi-step bioinformatics methods. Before all analyses, we obtained another stemness index, corrected mRNAsi, with the score of tumor purity computed in a previous study [8]. In the analyses of correlation between mRNAsi and histopathological grades, we divided patients into two groups, G1 + G2, and G3 + G4, because of the extremely small sample size of patients with tumors at Grade 1 and Grade 4. We found that the mRNAsi was significantly higher in the high-grade group than the low-grade group. Malta et al had reported that a higher value of stemness indices was associated with greater tumor dedifferentiation, which was reflected by a higher histopathological grade [7]. Our results were very consistent with the concept proposed by Malta.

As PFS is a surrogate of drug response, we focused on the correlation between mRNAsi/corrected mRNAsi with PFS rather than OS time among SOC patients of advanced stages. In the PFS curves of mRNAsi/corrected mRNAsi, we observed a tendency without statistical significance that within approximately 4 years, the PFS rate of high mRNAsi/corrected mRNAsi group was higher than that of low mRNAsi/corrected mRNAsi group. This was very different from our understanding that greater stemness of cancer cells would eventually lead to a shorter PFS [11, 12]. The results might be explained by three possible causes. First, the absence of statistical significance might be caused by quite a small sample size included in the analysis. Second, the mRNAsi score of tumors was computed according to the transcriptomic characteristics of pluripotent stem cells and their differentiated progeny. Perhaps only a small part of genes involved in mRNAsi have a significant effect on the PFS of advanced-stage SOC patients. Thus, the analysis of the correlation between mRNAsi score and survival time could be influenced by a lot of confounding factors. Third, the overexpression of some stemness-associated key genes involved in mRNAsi led to longer PFS of advanced-stage SOC patients. The third potential cause needed to be validated in the subsequent analyses.

Another important question was the choice between mRNAsi and corrected mRNAsi in the subsequent analyses. The purity variation of different tumor samples was caused by two main factors, the intrinsic and the extrinsic ones. Intrinsic factors implied that purity levels were a tumor-specific characteristic and extrinsic factors imply that the purity was dependent on the way how a sample is collected. The purity variation caused by the intrinsic factors was mainly due to the presence of non-cancerous cells, such as immune cells, fibroblasts, and endothelial cells in the intra-tumor microenvironment and immune cells were the most important component [8]. Malta et al, who newly computed the stemness indices mRNAsi and mDNAsi, had reported that immune microenvironment content was closely associated with stemness indices [7]. And the tumor-infiltrating lymphocytes (TILs) were also an independent prognostic factor of OC. Therefore, TILs might be an important factor that should be considered in the analysis of the correlation between mRNAsi and survival time of patients with OC. On the other hand, TCGA argued that 60% of purity is sufficient to distinguish the tumors’ signals from other non-cancerous cells [8]. Within the 262 samples with tumor purity data in our analysis, the purity score of 106 samples was over 0.9 and
that of 258 samples was over 0.6, which probably due to the lack of para cancer tissue of the ovarian tumor. It indicated the purity score was enough for the analyses and the correcting procedure maybe not a necessity for OC tissues. Moreover, the correcting procedure might remove the effect of non-cancerous stemness in tumor microenvironment on mRNAsi. Therefore, we tended to conduct analyses with mRNAsi but not corrected mRNAsi in this study.

To identify key genes with a strong connection to mRNAsi, we used the WGCNA method, with which a weighted gene co-expression network was constructed and the DEGs were classified into different gene modules by different gene expression patterns [25]. The correlation strength between gene modules and certain clinical features were scored and modules with the strongest correlation to clinical features could be discovered by the score. We chose the module most positively correlated to mRNAsi to investigate stemness-associated biomarkers which possibly governed the stemness of SOC.

Inappropriate criteria for screening genes often leads to information loss. When using GS > 0.50 instead of 0.40 in the preliminary analyses, we found that the pathway “Cellular senescence”, “p53 signaling pathway”, and “Platinum drug resistance” were missed in the results because of the small number of genes. Using a suitable score of MM and GS, we obtained 42 key genes from the selected green module. The functional annotation of the key genes revealed that the genes were most enriched to the biological process of mitotic nuclear division, which was consistent with the top GO category of DEGs between OC and normal samples. This confirmed the uncontrolled cell proliferation was a core characteristic of OC cells as well as the ovarian cancer stem cells (OVSCSs). In the results of the KEGG pathway, we found that the pathway “Cell cycle” [18, 19], “Cellular senescence” [13–15], “p53 signaling pathway” [16, 17], and “Platinum drug resistance” were closely associated with platinum sensitivity according to previous reports. A recent review had summarized that an insufficient dose of platinum might lead to a cytostatic response, named dormancy, rather than cytotoxic response, through inducing cell cycle arrest and cellular senescence [26]. It had been reported that dormancy was a quiescent state of CSC and the slow-cycling properties of the state were the main cause of therapy resistance and tumor relapse [27, 28]. In addition, the generation of platinum resistance was depending in part on the p53 status of the cancer cells [26]. Therefore, investigating platinum-response indicators among the key genes enriched to the platinum-response associated pathways could be reliable.

The differential expression of the 13 selected key genes was validated by datasets of GEO and Oncomine, the data of which were based on bulk RNA of samples. As expected, the expression level of the 13 key genes were all up-regulated in the advanced-stage HGSOC samples compared to the normal ovarian tissue included in the GSE18520 dataset. However, the origin of HGSOC is located in the fallopian tube rather than the ovarian epithelium [29]. Therefore, we further analyzed the GSE69428 dataset and found that the 13 key genes all had a higher expression level than the paired normal oviducts. This validation confirmed the upregulation of the key genes in SOC tissue from the perspective of in-situ growth and tumorigenesis. The analysis of Oncomine demonstrated that the 13 key genes might be stemness-associated in multiple cancers. Moreover, we utilized the PanglaoDB database to explore the cell distribution with positive expression of the key genes at a single-cell transcriptome level. The gene
expression profiling on bulk mRNA reflected the average expression level of a crowd of cells while the results obtained from scRNA-seq reflected the expression level on an individual cell. The results revealed that the key genes were not expressed on every cell in the HGSOC sample. Each key gene had a different density of distribution, indicating that the potential stemness-associated key genes might govern the stemness of different tumor cell subpopulations. This could be a possible cause leading to the heterogeneity of cancer cell stemness and might explain why only a part of CSCs transformed themselves into a quiescent state to preserve self-perpetuation and show the status of drug resistance. In the scRNA-seq dataset of ESCs, a kind of pluripotent stem cells, we observed that the positive expression cells of the 13 key genes tended to gather in a small subpopulation. This results further confirmed that the key genes were associated with cell stemness of pluripotent stem cells.

To explore the prognostic value of the key genes, we found 4 genes (AURKA, MYBL2, ORC1, and PLK1) were significantly correlated with the OS of advanced-stage SOC patients. Among the 4 genes, the median OS difference between low expression group and high expression group of AURKA, MYBL2, and ORC1 was longer than 6 months, indicating the three genes were significant prognostic biomarkers of advanced-stage SOC. AURKA [30] had been reported to govern the self-renewal capacity of glioma-initiating cells. PLK1 was also reported to promote Epithelial-Mesenchymal Transition (EMT), a biological process that was closely associated with cell stemness, in gastric carcinoma cells [31, 32]. These reports confirmed that AURKA and PLK1 were potential biomarkers modulating the stemness of SOC. However, MYBL2 and ORC1 hadn't been reported whether associated with cancer stemness. Among the total of 11 key genes associated with platinum-based therapy response investigated by the PFS analyses, higher expression of all these genes but ORC1 could predict longer PFS of advanced-stage SOC. It was very interesting that higher expression of AURKA could predict longer PFS and shorter OS. Similar results were also obtained on the gene TOP2A and TTK in patients receiving platin and taxol. This indicated that these genes could promote tumor progression as oncogenes while improving PFS as a drug sensitivity biomarker. However, the underlying mechanism of how the genes impacted OS and PFS in the reverse pattern still needed to be further illuminated. Among the PFS-associated genes, AURKA [33], CDK1 [34], and RRM2 [35] had been validated correlating to sensitivity to platinum-based chemotherapy by experimental methods. These reports partially verified that our study identifying platinum-response indicators among stemness-related key genes according to mRNAsi was reliable and worthwhile. However, the function of the genes on maintaining stemness and modulating platinum sensitivity of ovarian cancer stem cells (OVCSCs) still needs to be verified by experimental methods.

Moreover, the differential expression level of the 13 potential platinum-response indicators was validated between platinum-resistant and platinum-sensitive SOC samples. The results revealed that only CDC20 had a higher expression level in platinum-sensitive samples than that of platinum-resistant in both two GEO dataset. This results further confirmed that CDC20 was a potential response indicator to chemotherapy based on platinum. However, the generation of chemoresistance was a complex process involving networks of genes and pathways. A single biomarker would be not precise enough to predict drug response. Investigating drug response indicators by multiple methods, constructing a drug-response
prediction model with multiple genes, and validating the effectiveness of the drug response indicators by experimental methods would be carried out in our next task.

In conclusion, we investigated platinum-response indicators among stemness-related key genes in advanced-stage SOC according to mRNAsi in this study. By evaluation of prognostic value and expressional validation, CDC20 was identified as a stemness biomarker and platinum-response indicator in advanced-stage SOC. This conclusion would provide clues to guide drug use in clinic and still needs to be further validated by experimental methods.

Materials And Methods

Data acquisition and pre-processing

The RNA sequencing (RNA-seq) expression data used in this study were downloaded from the UCSC Xena project (https://xena.ucsc.edu/) babased on February 2020. The datasets included tumor tissue samples from TCGA (N = 379 for SOC) and normal ovarian tissue (N = 88) from GTEx. Both datasets have been previously recomputed to minimize differences from distinct sources based on a standard pipeline. The corresponding clinical information of the OC dataset was downloaded from the TCGA database (https://portal.gdc.cancer.gov/). The mRNAsi indices of 273 SOC samples in TCGA were obtained from a previous study [7]. A script in Perl language (https://www.perl.org/) was used to convert gene IDs to gene symbols. The RNA-seq data of 379 SOC samples and 88 normal ovarian tissue samples were combined into a matrix file by using a script in the R language.

Clinical Feature Correlation Analysis Of Mrnasi And Corrected Mrnasi

The tumor purity score of the SOC samples in TCGA was obtained from a previous study [8]. Corrected mRNAsi was calculated by the mRNAsi score/tumor purity score. A total of 262 tumor samples with available information of mRNAsi, histopathological grades, and clinical stages were included in the analyses of correlation between mRNAsi and clinical characteristics. The same analyses on corrected mRNAsi and clinical characteristics were conducted among 254 patients of SOC. The above analyses were conducted by the beeswarm package in R. Wilcoxon test was used to determine the significant difference between the two groups. Removed the samples with incomplete information of survival time and samples of stage I-II, progression-free survival analyses were conducted on both mRNAsi and corrected mRNAsi. A total of 240 SOC patients of stage III-IV were included in the analyses. The prognostic significance of mRNAsi and corrected mRNAsi was explored by survival and surviminer packages in R.

Differentially Expressed Genes (degs) Analysis
The DEGs were screened by using the RNA-seq data of OC datasets in TCGA and normal samples in GTEx downloaded from the UCSC Xena project. The limma package in R [36] was used to perform the differential expression analysis and the Wilcoxon test was used to determine the significant difference in the processing. \(|\text{Log}_2 \text{ Fold change (FC)}| > 1\) and False Discovery Rate (FDR) < 0.05 were set as the criteria to screen the DEGs between normal and tumor samples. The heatmap and volcano plot was drawn with the heat map package. The Gene Ontology (GO) terms were visualized by the GOplot package in R.

**Identification Of Key Genes By Wgcna**

The co-expression network of DEGs according to mRNAsi was constructed by the WGCNA package in R [25]. The R packages “matrixStats”, “foreach”, “Hmisc”, “doParallel”, “fastcluster”, “dynamicTreeCut” and “survival” were also used in this process. The input data was first prepared by removing normal samples and SOC samples of stage I-II. Thus 352 SOC samples were remained to be analyzed subsequently. Genes with incomplete expression data or samples without available clinical information were deleted. The corresponding mRNAsi score was also deleted. Then the samples were clustered with the average method according to the gene expression level. The cut-height was set at 100 and the minimum size of gene groups was set as 10 to exclude the outlier. In this procedure, 15 outlier samples were removed and 337 samples were included in the subsequent analyses. The pre-processed data was intersected with the mRNAsi data and analyzed.

The optimal power-value was selected to construct a scale-free network according to the Pearson correlation coefficient among genes. The power-value was then determined as 8 by calculating the correlated genes between the scale-free R^2 and mean connectivity. A GeneTree was constructed per the power-value and the dynamic module was identified with a minimum gene size of 50. Adjacent modules were merged with the criteria MEDiss Thres < 0.25. The module-trait correlations with mRNAsi and EREG-mRNAsi were plotted.

After selecting modules of our interest, we calculated the gene significance (GS, a correlation between gene expression levels and sample traits) and module membership (MM, a correlation between genes in a certain module and gene expression profiles for each gene). To obtain more possible enriched pathways, we defined cor. gene MM > 0.80 and cor.gene GS > 0.4 instead of 0.50 [9] as the thresholds to obtain more potential key genes.

**Function annotation and pathway enrichment analysis of key genes**

The org.Hs.eg.db package in R was utilized to convert gene symbols of the key genes into Ensemble ID. Then the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were performed by using the R packages cluster Profiler, enrichplot, and ggplot2. The top terms
of enrichment results were visualized in bubble plots. The candidate key genes were eventually selected according to the enriched pathways.

The PPI network was constructed by using STRING (Version 11.0, https://string-db.org/) [37]. The minimum required interaction score was set as medium confidence (0.4). We calculated the number of adjacent nodes of each gene in the PPI network by using the corresponding output file downloaded from STRING. The number of adjacent nodes was presented in a barplot to show the connectivity of each gene in the PPI network. The Pearson's correlation coefficient between the paired key genes was computed according to the gene expression levels and visualized by the corrplot package in R. The results with a correlation coefficient > 0.4 was considered presenting a strong correlation between the paired genes.

**Data validation**

The significant differential expression of the selected key genes was showed by R packages heatmap and ggpubr. We further selected two datasets GSE18520 [38] and GSE69428 [29], from the GEO database (https://www.ncbi.nlm.nih.gov/geo/), to validate the differential expression of the selected key genes at a bulk mRNA level. The GSE18520 dataset included 10 normal ovarian surface epithelium samples and 53 advanced-stage, high-grade SOC samples. The GSE69428 dataset included 10 high grade serous ovarian cancer (HGSOC) samples and 10 paired normal oviducts samples. The limma package was used to process the data of gene expression profiling and conduct the DEG analyses. Oncomine (https://www.oncomine.org/) was utilized to investigate the mRNA expression levels of the key genes at a pan-cancer level. The PanglaoDB online database (https://panglaodb.se/) was used to validate the key gene expression at the single-cell transcriptome level [39]. We selected two human datasets of single-cell RNA sequencing (scRNA-seq) which had been pre-processed according to the protocol of 10× chromium. The ovarian tumor dataset (SRA634975: SRS2724911) included 2547 single cells of high grade serous ovarian cancer. Another dataset (SRA553822: SRS2119548) included 6501 human embryonic stem cells (ESCs). Dimensionality reduction for visualization was performed by t-distributed stochastic neighbor embedding (t-SNE).

**Prognostic and chemotherapeutic response predict the value of key genes**

Survival analyses of key genes were conducted with the Kaplan Miere Plotter (https://kmplot.com/). The prognostic value of the key genes on OS was examined in total patients of SOC included in the database. The impact of the selected key genes on the OS and PFS of patients treated with chemotherapy containing platin or treated with the combination of platin and taxol was examined on SOC patients of stage III-IV. A p value < 0.05 was considered representing statistical significance. In addition, GSE131978 [40] and GSE51373 [10] datasets were selected to validate whether the key genes were associated with platinum sensitivity in advanced-stage SOC patients. In the study, 7 platinum-based chemotherapy-resistant SOC samples of stage III-IV and 4 platinum-based chemotherapy-sensitive samples selected from GSE131978 (platform GPL570) were used in the analysis. 10 platinum-resistant SOC samples and
13 platinum-sensitive SOC samples of stage III-IV from the GSE51373 dataset were also utilized in the validation.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data used and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

The authors declare that they have no competing interests.

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Author's contributions

Zhiqing Liang conceived and designed the project. Qingyu Liu acquired and pro-processed the data. Xinwei Sun, Jie Huang, and Ge Diao conducted the data analysis. Xinwei Sun prepared the initial manuscript. Zhiqing Liang revised the manuscript and had the primary responsibility for the final content. All authors had read and approved the final manuscript.

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Tables

**Table 1** The significant impact of the key genes on the overall survival time of the advanced-stage, SOC patients as well as patients treated with different strategy of chemotherapy.
| Gene expression | Low  | High | Low  | High  | Difference |
|-----------------|------|------|------|-------|------------|
| **Stage III-IV, SOC** |      |      |      |       |            |
| AURKA           | 678  | 345  | 45.13| 36.73 | 8.40       |
| MYBL2           | 425  | 598  | 48.00| 38.57 | 9.43       |
| ORC1            | 533  | 490  | 45.73| 37.03 | 8.70       |
| PLK1            | 677  | 346  | 40.00| 43.93 | -3.93      |
| **Platinum-based** |      |      |      |       |            |
| AURKA           | 610  | 326  | 45.77| 38.77 | 7.00       |
| CCNA2           | 306  | 630  | 40.54| 44.13 | -3.59      |
| MYBL2           | 390  | 546  | 48.27| 39.77 | 8.50       |
| ORC1            | 485  | 451  | 46.13| 37.93 | 8.20       |
| **Platinum+taxol** |      |      |      |       |            |
| CCNA2           | 352  | 220  | 45.63| 40.00 | 5.63       |
| CDK1            | 157  | 415  | 50.00| 41.60 | 8.40       |
| ORC1            | 364  | 208  | 45.47| 38.57 | 6.90       |
| TOP2A           | 236  | 336  | 48.37| 41.00 | 7.37       |
| TTK             | 425  | 147  | 45.63| 38.47 | 7.16       |

**Table 2** The significant impact of the key genes on the progression-free survival time of advanced-stage, SOC patients treated with different strategy of chemotherapy.
| Gene expression | Groups (patients) | Median PFS (months) |
|-----------------|-------------------|---------------------|
|                 | Low | High | Low | High | Difference |
| **Platinum-based** |     |      |     |      |            |
| AURKA           | 226 | 681  | 14.00 | 16.63 | -2.63    |
| BIRC5           | 639 | 268  | 15.00 | 19.00 | -4.00    |
| CCNA2           | 294 | 613  | 15.00 | 16.53 | -1.53    |
| CCNB2           | 290 | 617  | 14.83 | 16.93 | -2.10    |
| CDC20           | 348 | 559  | 14.37 | 16.85 | -2.48    |
| CDK1            | 651 | 256  | 15.00 | 18.30 | -3.30    |
| ORC1            | 678 | 229  | 17.00 | 14.00 | 3.00     |
| PLK1            | 475 | 432  | 15.00 | 17.00 | -2.00    |
| RRM2            | 649 | 258  | 15.00 | 18.23 | -3.23    |
| TOP2A           | 560 | 347  | 15.00 | 18.30 | -3.30    |
| TTK             | 269 | 638  | 14.37 | 17.00 | -2.63    |
| **Platinum+taxol** |     |      |     |      |            |
| BIRC5           | 358 | 204  | 15.00 | 17.50 | -2.50    |
| CCNB2           | 191 | 371  | 14.37 | 16.13 | -1.76    |
| CDC20           | 249 | 313  | 14.00 | 16.37 | -2.37    |
| MYBL2           | 140 | 422  | 14.27 | 16.00 | -1.73    |
| PLK1            | 292 | 270  | 15.00 | 16.23 | -1.23    |
| TOP2A           | 378 | 184  | 15.00 | 16.83 | -1.83    |
| TTK             | 341 | 221  | 14.03 | 18.00 | -3.97    |