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E2F2 induces MCM4, CCNE2 and WHSC1 upregulation in ovarian cancer and predicts poor overall survival

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Abstract. – OBJECTIVE: To explore the genes co-upregulated with E2F2 in ovarian cancer and their association with survival outcomes in ovarian cancer patients.

MATERIALS AND METHODS: The raw data of GDS3592 was downloaded from GEO datasets for reanalysis. The overlapping subset between the top 150 upregulated genes in ovarian cancer epithelial cells (CEPIs) and the E2F2 positively correlated genes (Pearson's r≥0.5) in ovarian cancer cohort in TCGA was identified. The association between E2F2, MCM4, CCNE2 and WHSC1 and overall survival (OS) and recurrence-free survival (RFS) in ovarian cancer patients were assessed using Kaplan-Meier plotter.

RESULTS: E2F2 is a significantly upregulated transcription factor in CEPIs. MCM4, CCNE2, and WHSC1 are co-upregulated with E2F2 among the 308 ovarian cancer samples (Pearson's r=0.5159, 0.3963 and 0.4941 respectively). Enforced E2F2 expression significantly enhanced MCM4, CCNE2 and WHSC1 transcription in SKOV3 and A2780 cells. High E2F2 and CCNE2 expression are associated with worse OS (high E2F2, HR: 1.48, 95%CI: 1.17-1.85, $p<0.01$; high CCNE2, HR: 1.36, 95%CI: 1.15-1.6, $p<0.01$). High MCM expression might be associated with worse RFS at the margin of significance (HR: 1.18, 95%CI: 1.00-1.39, $p=0.055$).

CONCLUSIONS: MCM4, CCNE2, and WHSC1 are co-upregulated with E2F2 in ovarian cancer. Enforced E2F2 expression significantly increased MCM4, CCNE2, and WHSC1 expression in ovarian cancer cells. High E2F2 and CCNE2 expression are associated with worse OS among ovarian cancer patients.

Key Words:
E2F2, MCM4, CCNE2, WHSC1, Ovarian cancer.

Introduction

Epithelial ovarian cancer is a common female malignancy and is one of the leading causes of cancer-related death among women[1]. The initiation and development of ovarian cancer are associated with complex genetic and epigenetic alterations[2,3]. Therefore, elucidation of the molecular mechanisms underlying the carcinogenesis still remains a challenge.

E2F2 is a member of the E2F family of transcription factors, which plays a crucial role in the control of cell cycle[4]. Expression of E2F2 is elevated in ovarian cancer cell lines compared with normal peritoneal mesothelial cells (HPMCs)[5,6]. Activation of CDK1, CDK7, E2F1 and E2F2 is associated with enhanced cell proliferation in OVCAR-3 ovarian cancer cells[7]. In addition, high E2F1 and E2F2 were associated with higher grade of tumors and also associated with unfavorable disease-free and overall survival (OS)[8]. However, its downstream effectors in ovarian cancer are not clear.

Minichromosome maintenance complex component 4B (MCM4) is a DNA replication licensing factor[9], which has tumorigenic effect in esophageal adenocarcinoma[10], breast cancer[11] and ovarian cancer[12]. CCNE2 is a gene encoding cyclin E2 in human, which regulates the transition from G1 to S phase determining cell division[13]. CCNE2 upregulation is associated with carcinogenesis and aggressive phenotype of breast cancer[14] and gastric cancer[15]. WHSC1 is a gene encodes nuclear receptor binding SET domain protein 2 (NSD2), a histone-lysine N-methyltransferase[16]. This gene is involved in the NF-kappaB signaling for cancer cell proliferation, survival, and tumor growth[17]. Its upregulation contributes to oncogenic RAS-driven transcription in lung cancer cells[18]. High WHSC1 might also be associated with advanced tumor aggressiveness in serous ovarian carcinoma[19].

In this study, we firstly reported that MCM4, CCNE2, and WHSC1 are co-upregulated with

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E2F2 in ovarian cancer. Enforced E2F2 expression significantly increased MCM4, CCNE2 and WHSC1 expression in ovarian cancer cells. In addition, we also observed that high E2F2 and CCNE2 expression are associated with worse OS among ovarian cancer patients.

Materials and Methods

Bioinformatic Data Mining

The microarray that assessed gene expression profiles of ovarian cancer epithelial cells (CEPIs) was searched in GEO datasets. The raw data of one previous Affymetrix Human Genome U133 Plus 2.0 Array (GDS3592)[20] that investigated the dysregulated genes in CEPIs compared to normal ovarian surface epithelia (OSE) was downloaded and reanalyzed. To identify the known protein-protein interactions (PPIs) between E2F2 and the 100 upregulated genes, the gene list was uploaded into the Search Tool for the Retrieval of Interacting Genes (STRING) (http://string-db.org/) for analysis. The minimum required interaction score was set to high confidence (0.7).

The genes positively correlated to E2F2 in the ovarian cohort in TCGA database were identified using cBioportal (http://cbioportal.org)[21]. The heat map of E2F2, MCM4, CCNE2, and WHSC1 expression, measured by RNAseq (ployA+ Illumina HiSeq pancan normalized), as well as regression analysis of their expression were further studied using UCSC Xena (http://xena.ucsc.edu/).

The association between the expression of E2F2, MCM4, CCNE2 and WHSC1 and overall survival (OS) and recurrence free-survival (RFS) in ovarian cancer patients were assessed using Kaplan-Meier Plotter, which is an online tool for genome-wide validation of survival-associated biomarkers in ovarian cancer using microarray data of 1287 patients[22]. The analysis was performed by using the JetSet best probe set, while only patients with serous ovarian cancer were included.

Cell Culture and Transfection

Human ovarian cancer cell lines A2780 and SKOV3 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin. All cells were maintained in a humidified incubator with 5% CO_2 at 37˚C.

Human E2F2 cDNA expression clone (pCMV3-E2F2) and the empty control were obtained from Sino Biological Inc. (Beijing, China). SKOV3 and A2780 cells were transfected with the E2F2 expression vector or empty control using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

QRT-CPR Analysis

Total RNAs in the cell samples were extracted using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Then, the RNA samples were reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's protocol. Then, E2F2, MCM4, WHSC1 and CCNE2 mRNA was measured using qRT-PCR analysis with the following primers (E2F2, F, 5'-AAGTGCATCAGAGTGGATGGCCT-3' and R, 5'-AATGAACTTCTTGGTGAGCAGCCC-3'; MCM4, F, 5'-TTGAAGCCATTGATGTGGAA-3' and R, 5'-GGCACTCATCCCCGTAGTAA-3'; CCNE2, F, 5'-TGATGGTGCTTGCAGTGAAGAGGA-3' and R, 5'- CACAAGGCAGCAGCAGTCAGTATT-3'; and WHSC1, 5'-TTCTGCACCAAGGCCTACCAC-3' and R, 5'-AGGTTTGCCACACACGTCACA-3') and the SYBR® Select Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems). GAPDH was detected as the endogenous control.

Western Blotting

Total protein was extracted from cell samples using a cell lysis buffer. Then, an equal amount of denatured protein samples were loaded and separated in 10% SDS-PAGE gels and transferred to a PVDF membrane. Then, the membranes were incubated with primary antibodies against E2F2 (ab138515, Abcam, Cambridge, UK), MCM4 (ab4461, Abcam), CCNE2 (ab40890, Abcam), WHSC1 (ab28470, Abcam) and β-actin (ab3280, Abcam). After the incubation and washing, the membranes were further incubated with secondary antibodies coupled to HRP. After washing, the protein bands were visualized by using the SuperSignal™ West Femto Chemiluminescent Substrate (Pierce Biotechnology, Rockford, lL, USA).

Statistical Analysis

Data were presented in the form of means ± standard deviation (SD). Data were analyzed for statistical significance by two-tailed Student's *t*-test. *p*-value of <0.05 was considered statistically significant.

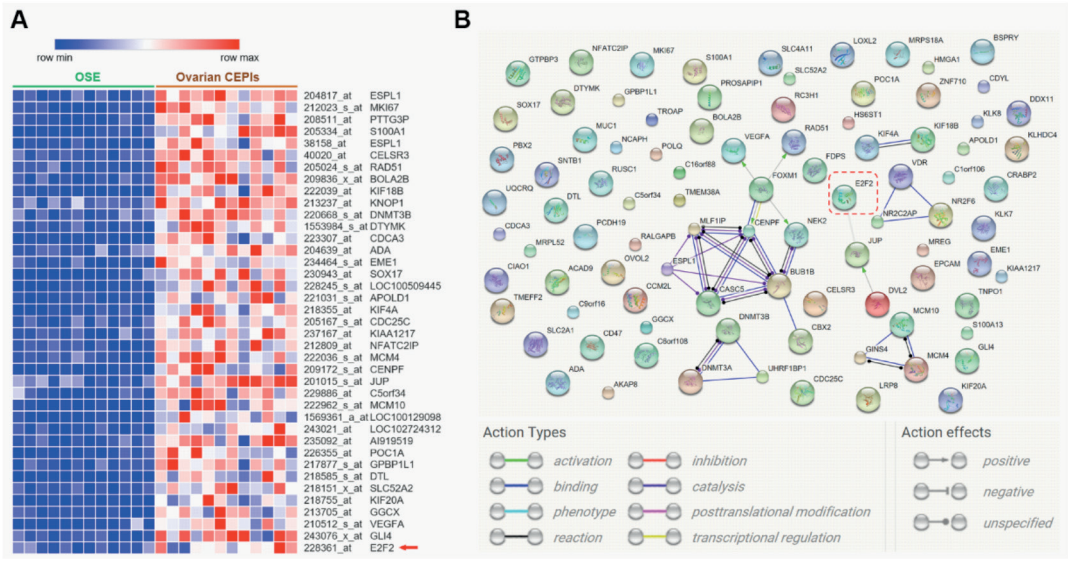

Figure 1. E2F2 is significantly upregulated in CEPIs compared to OSE. ***A.*** Heat map of the most upregulated genes in 12 cases of ovarian CEPIs compared to 12 cases of OSE. Red: up-regulation; Blue: down-regulation. The image was obtained by re-analysis of the raw microarray data of GDS3592. ***B,*** PPI analysis of the known interactions between E2F2 and the 100 most upregulated genes in CEPIs.

Results

E2F2 is Significantly Upregulated in CEPIs Compared to OSE

By reanalysis of the raw data of GDS3592, we identified the most upregulated genes in CEPIs compared to OSE (Figure 1A). Among the upregulated genes, E2F2 is a transcription factor (Figure 1A, red arrow) with oncogenic properties in multiple cancers, including ovarian cancer[5,6]. To identify the known possible interactions between E2F2 and other upregulated genes in ovarian cancer, the gene symbols were uploaded into the STRING tool for analysis of PPI network. The results showed that E2F2 is co-mentioned with JUP homologs in previous studies (Figure 1B). But we did not find any other direct connections between E2F2 and the upregulated genes (Figure 1B).

E2F2 is Co-upregulated with MCM4, CCNE2, and WHSC1 in Ovarian Cancer

To further study whether there is any possible connection between E2F2 and other upregulated genes in ovarian cancer, we identified the overlapping subset of the top 150 upregulated genes in CEPIs and the E2F2 positively correlated genes (Pearson's r≥0.5) in ovarian cancer cohort in TCGA, which includes MCM4, CCNE2 and WHSC1 (Figure 2A-B). Then, we examined the RNAseq data of E2F2, MCM4, CCNE2 and WHSC1 in ovarian cancer cohort in TCGA (Figure 2C). Regression analysis confirmed that MCM4, CCNE2, and WHSC1 are positively correlated with E2F2 among the 308 ovarian cancer samples (Pearson's r=0.5159, 0.3963 and 0.4941 respectively) (Figure 2D-F).

E2F2 Overexpression Results in Increased MCM4, CCNE2, and WHSC1 Expression

To examine whether E2F2 directly increases MCM4, CCNE2, and WHSC1 expression in ovarian cancer, SKOV3, and A2780 cells were transfected with a pCMV3-E2F2 expression vector or the empty control. In these two cell lines, enforced E2F2 expression significantly enhanced MCM4, CCNE2, and WHSC1 transcription (Figure 3A-B) and translation (Figure C).

High E2F2 and CCNE2 Expression Are Associated with Worse OS Among Ovarian Cancer Patients

Since previous studies reported that E2F2, MCM4, CCNE2, and WHSC1 all have oncogenic properties in some cancers, we further investigated whether their expressions are associated with survival outcomes among ovarian cancer patients. Data mining in Kaplan-Meier plotter suggest that high E2F2 and CCNE2 expression are associated with worse OS (high E2F2, HR: 1.48,

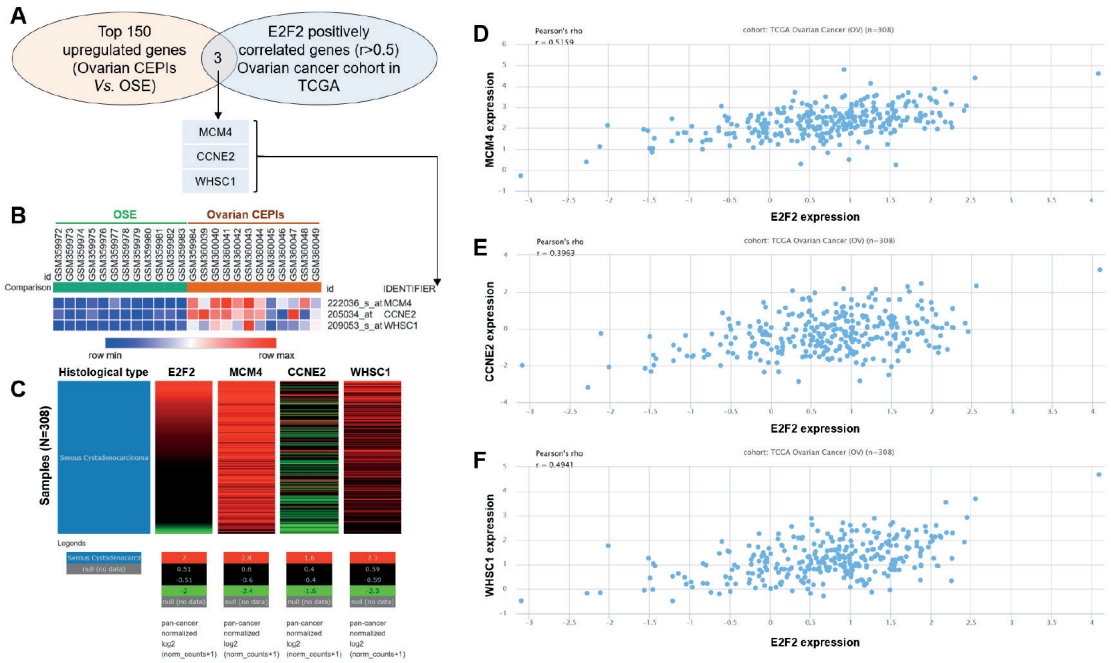

Figure 2. E2F2 is co-upregulated with MCM4, CCNE2, and WHSC1 in ovarian cancer. ***A,*** The overlapping subset between the top 150 upregulated genes in CEPIs and the E2F2 positively correlated genes (Pearson's r≥0.5) in ovarian cancer cohort in TCGA. ***B,*** The heap map of MCM4, CCNE2, and WHSC1 in GDS3592. ***C-F,*** The heat map of RNAseq data (ployA+ Illumina HiSeq pancan normalized) ***(C)*** and Regression analysis ***(D-F)*** between E2F2 and MCM4, CCNE2, and WHSC1 in ovarian cancer cohort in TCGA database (N=308). The analysis was performed using UCSC Xena.

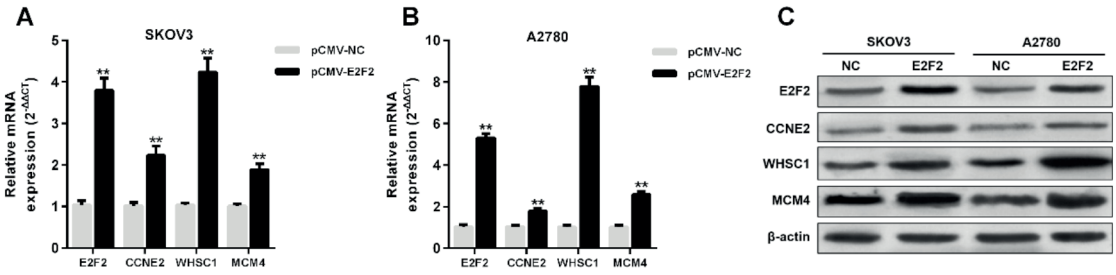

Figure 3. E2F2 overexpression results in increased MCM4, CCNE2, and WHSC1 expression. ***A-B,*** QRT-PCR analysis of E2F2, CCNE2, WHSC1, and MCM4 mRNA expression in SKOV3 ***(A)*** and A2780 ***(B)*** cells 36 h after transfection of a pCMV-E2F2 expression vector or the negative control. ***C,*** Western blot analysis of E2F2, CCNE2, WHSC1, and MCM4 protein expression in SKOV3 and A2780 cells 48 h after transfection of a pCMV-E2F2 expression vector or the negative control.

95%CI: 1.17-1.85, p<0.01; high CCNE2, HR: 1.36, 95%CI: 1.15-1.6, p<0.01) (Figure 4A and C). In addition, high MCM expression might be associated with worse RFS at the margin of significance (HR: 1.18, 95%CI: 1.00-1.39, p=0.055) (Figure 4F). However, no significant association was observed in other groups (Figure 4B, D, E-F and H).

Discussion

E2F2 is a well-characterized regulator of the G1-to-S-phase transition[4]. The oncogenic property of E2F2 is observed in multiple cancers due to its effect on promoting cell-cycle progression. Overexpressed E2F2 is a crucial center of cell cycle regulation in hepatocellular cancer and high expression of E2F2 is significantly associated with poor prognosis[23]. In ovarian cancer, E2F2 also acts as a proliferation-promoting transcription factor, of which the high expression is associated with higher grade tumors and unfavorable survival outcomes[8]. Traditionally, E2F2 has distinct effects on the expression of cdk2, cyclin E and pRB[24]. However, whether other ge-

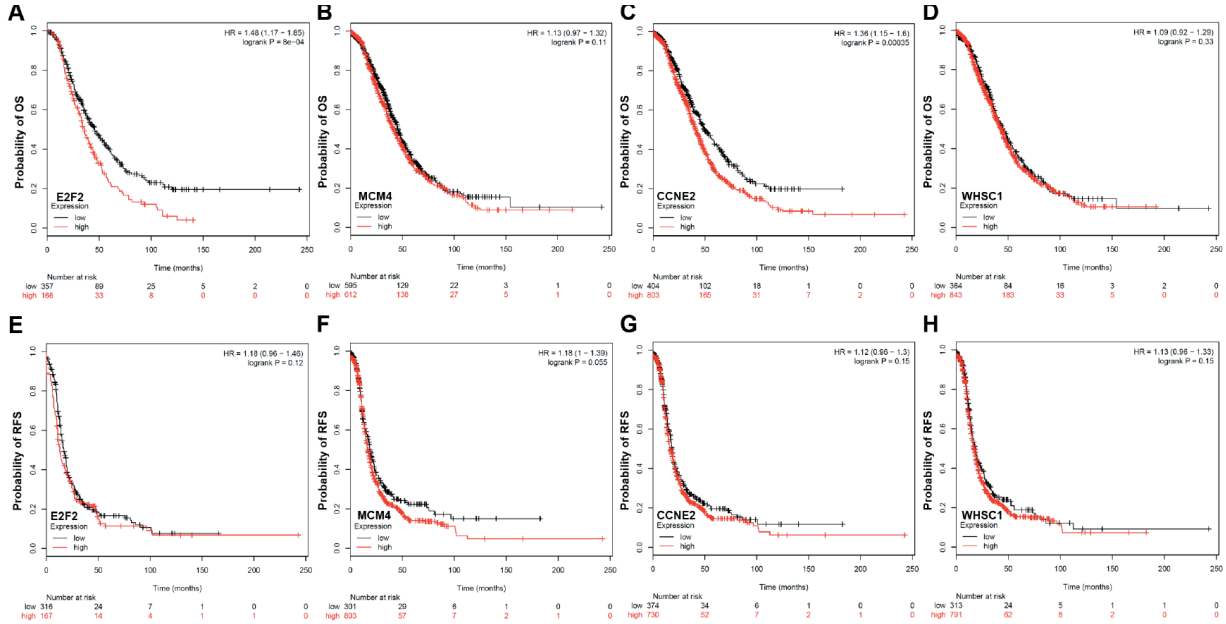

Figure 4. High E2F2 and CCNE2 expression are associated with worse OS among ovarian cancer patients. ***A-H.*** Kaplan-Meier plots of the association between E2F2 (***A*** and ***E***), MCM4 (***B*** and ***F***), CCNE2 (***C*** and ***G***) and WHSC1 (***D*** and ***H***) expression and OS ***(A-D)*** and RFS ***(E-H)*** in ovarian cancer patients. Data was obtained by using Kaplan-Meier Plotter.

nes are involved in its oncogenic effect are not clear.

In the current study, we found that E2F2 is significantly upregulated in CEPIs compared to OSE. However, by performing PPI analysis, we did not find any known and significant association between E2F2 and other significantly upregulated genes. Interestingly, by comparing E2F2 positively correlated genes in ovarian cancer cohort in TCGA database and the upregulated genes in CEPIs, we found that MCM4, CCNE2 and WHSC1 were in the overlapping subset. In addition, our regression analysis also confirmed that MCM4, CCNE2, and WHSC1 are positively correlated with E2F2. In SKOV3 and A2780 cells, enforced E2F2 expression directly increased MCM4, CCNE2, and WHSC1 expression at both mRNA and protein level.

Actually, MCM4, CCNE2, and WHSC1 all have oncogenic properties. High MCM4 expression was correlated with proliferation markers, Ki-67 and cyclin E expression in non-small cell lung cancer cells[25]. MCM4 upregulation also might be a marker of poor response to treatment and prognosis in breast cancer patients[26]. However, the association between MCM4 expression and survivals of ovarian cancer patients was not clear. CCNE2 overexpression is associated with endocrine resistance and aggressiveness in human breast cancer cells[13,14]. Knockdown of endogenous CCNE2 can significantly inhibit cell proliferation in bladder cancer[27] and prostate cancer[28]. Although dysregulated CCNE2 was observed in ovarian cancer in one previous study[29], its association with survivals of ovarian cancer patients was not studied. WHSC1 is involved in the NF-kappaB signaling for cancer cell proliferation, survival, and tumor growth[17]. Its upregulation contributes to oncogenic RAS-driven transcription in lung cancer cells[18]. WHSC1 can also activate TWIST1 to promote epithelial-mesenchymal transition and invasive properties of prostate cancer[30]. High WHSC1 might also be associated with advanced tumor aggressiveness in serous ovarian carcinoma[19]. However, its association with survivals of ovarian cancer patients was also not reported. In this study, data mining in Kaplan-Meier plotter showed that high E2F2 and CCNE2 expression are associated with worse OS, while high MCM expression might be associated with worse RFS at the margin of significance. Therefore, we infer that E2F2 and CCNE2 might be a useful indicator of OS among the patients. However, this hypothesis needs further validation by using multi-variable analysis based on large patient samples.

Conclusions

MCM4, CCNE2, and WHSC1 are co-upregulated with E2F2 in ovarian cancer. Enforced E2F2 expression significantly increased MCM4, CCNE2, and WHSC1 expression in ovarian cancer cells. High E2F2 and CCNE2 expression are associated with worse OS among ovarian cancer patients.

Conflict of Interest

The authors declare no conflicts of interest.

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