Cyclin I mRNA Expression Correlates with Kinase Insert Domain Receptor Expression in Human Epithelial Ovarian Cancer

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Abstract. *Background/Aim: Ovarian cancer is the second most common gynecological malignancy after cancer of the uterine corpus, and the fifth leading cause of cancer-related death among women. It has been discovered that cyclin I (CCNI) protein expression correlates with the proliferation of cancer cells and expression of angiogenesis-related proteins, such as vascular endothelial growth factor (VEGF) and VEGF receptor 2/kinase insert domain receptor (VEGFR2/KDR). We examined whether any association exists between mRNA expression of CCNI and KDR genes in epithelial ovarian cancer (EOC) tissues, clinicopathological parameters and patients' response to chemotherapy. Materials and Methods: Expression of CCNI and KDR genes was analyzed by quantitative real-time reverse transcription PCR in 40 human primary EOC tissues and four human ovarian cancer cell lines (TOV-112D, OV-90, OVCAR-3 and Caov-3). Results: CCNI and KDR mRNA expression was detected in all EOC tissues and ovarian cancer cell lines. The mRNA levels of both genes were significantly higher in EOC than in ovarian cancer cell lines ($p<0.001$). Neither CCNI nor KDR mRNA expression in EOC tissues was significantly associated with variables such as age, menopausal status, International Federation of Gynecology and Obstetrics (FIGO) stage, residual disease, patients' response to chemotherapy, tumor histology, grade or sensitivity to chemotherapy. However, we demonstrated a significant positive correlation between the mRNA expression of KDR and CCNI in EOC tissues ($R=0.530$, $p<0.001$). Conclusion: Neither CCNI nor KDR mRNA expression predicts response of patients with EOC to platinum-based first-line chemotherapy. Cyclin I may be involved in angiogenesis in EOC, which needs further investigation.*

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Ovarian cancer is the second most common gynecological malignancy after cancer of the uterine corpus and the fifth leading cause of cancer-related death among women. A total of 21,980 new cases and 14270 deaths are estimated for 2014, which accounts for 2.7% of all new cancer diagnoses and 5.2% of all cancer deaths in women in the United States (1). In Poland, 3,527 new cases of ovarian cancer were reported in 2011, with an age-adjusted incidence rate of 10.9 per 100,000 women. The incidence of ovarian cancer is rising, and despite diagnostic and therapeutic efforts, ovarian cancer-related mortality increased from 6.7 per 100,000 in 1999 (1959 deaths) to 6.9 per 100,000 women in 2011 (2558 deaths) in Poland (2). Due to the lack of early warning symptoms and effective screening methods, the majority of patients with ovarian cancer are diagnosed with the disease at an advanced stage, which is associated with high mortality (1, 3). The standard therapy includes cytoreductive surgery and combination chemotherapy regimens (paclitaxel and carboplatin or cisplatin), however, many patients develop resistance to chemotherapy and die because of recurrence (4, 5). Therefore, there is an urgent need to search for new genes and mechanisms responsible for the development, progression and chemoresistance in ovarian cancer.

Progression of the cell cycle is regulated by complexes of cyclin-dependent kinases (CDKs) and their regulatory partner proteins, the cyclins (6). Cyclin I gene (*CCNI*) expression was detected for the first time in post-mitotic tissues and its mRNA level did not fluctuate during the cell cycle, therefore it was believed that cyclin I had no role in regulating cell proliferation (7-9). However, higher cyclin I protein

expression was discovered in human pancreatic adenocarcinoma than in benign pancreatic diseases and healthy pancreas, which suggested that cyclin I may have a function in carcinogenesis or progression of pancreatic cancer (10). In human breast cancer, immunolabeling for CCNI was correlated with angiogenesis-related proteins, such as vascular endothelial growth factor (VEGF) and VEGF receptor 2/kinase insert domain receptor (VEGFR2/KDR), suggesting a link between CCNI and angiogenesis (11). Furthermore, low CCNI expression was found in normal human breast epithelium, indicating that its presence in breast tumors may play a role in the development or maintenance of breast cancer (12). In our previous study (13), we found a direct correlation between immunostaining for CCNI, and VEGFR2 and proliferation of epithelial ovarian cancer (EOC) cells. VEGFR2, encoded by *KDR* gene, is involved in angiogenesis, which is necessary for growth and spread of tumors. VEGFR2 mediates the angiogenic effects of VEGF (14) and the VEGF/VEGFR2 system enhances neoplastic cell proliferation and survival in numerous types of human cancer, including ovarian tumors (15-18).

The aim of our study was to evaluate the mRNA levels of *CCNI* and *KDR* genes in primary EOC specimens and ovarian cancer cell lines, and to investigate whether an association exists between the expression of analyzed genes in EOC tissues, clinicopathological parameters and patient response to first-line chemotherapy.

Patients and Methods

Cell culture. All human ovarian cancer cell lines (TOV-112D, OV-90, OVCAR-3 and Caov-3) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). TOV-112D and OV-90 cell lines were cultured in 1:1 mixture of MCDB 105 (Sigma-Aldrich, St. Louis, MO, USA) and Medium 199 (PAA, Pasching, Austria) containing 15% fetal bovine serum (FBS) Gold (PAA). Caov-3 cell line was grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) modified to contain 0.584 g/l L-glutamine, 4.5 g/l glucose, 0.11 g/l sodium pyruvate, 1.5 g/l sodium bicarbonate, and 10% FBS Gold (PAA). NIH:OVCAR-3 cell line was cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 0.01 mg/ml bovine insulin (Sigma-Aldrich) and FBS Gold (PAA) to a final concentration of 20%. All cell culture media were supplemented with penicillin and streptomycin (Sigma-Aldrich) to a final concentration of 100 U/ml and 100 μg/ml, respectively. Cells were grown in a humidified atmosphere at 37˚C with 5% CO_2 and maintained as monolayers.

Patients' characteristics. The study group consisted of 40 patients with primary EOC (median age=54.5 years, range=37-85 years), diagnosed and treated between 2006 and 2010 at the First Department of Oncologic Gynecology and Gynecology, Medical University of Lublin, Poland. The optimal debulking status after initial surgery was defined as residual tumor with maximal diameter of 1 cm or less (19). Tumor specimens were collected from women during primary surgery and prior to the initiation of adjuvant therapy. After primary surgery, 32 patients (80.0%) received adjuvant chemotherapy (paclitaxel-platinum, cyclophosphamide-platinum or carboplatin alone). Paclitaxel-platinum chemotherapy was administered to 24 patients (75.00%), six women (18.75%) received cyclophosphamide-platinum chemotherapy, and two patients (6.25%) were treated with carboplatin monotherapy. The response of patients to chemotherapy was assessed according to RECIST criteria ver. 1.1 (20) and platinum sensitivity of tumors was determined as described previously (19). Ethical approval to carry out the study was granted by the Research Ethics Committee of Medical University of Lublin (KE-0254/137/2007).

RNA isolation and quantitative real-time reverse transcription PCR. Total RNA was extracted from samples using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions and digested by DNase I (Fermentas, Vilnius, Lithuania). RNA concentration was determined by spectrophotometry at 260 nm and its purity by A260/280 ratio. The integrity of RNA was verified by ethidium bromide staining on a 2% agarose gels. cDNA synthesis was conducted using Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) and oligo(dT) primer according to the supplier's protocol. Quantitative PCR was carried out using LightCycler® 480 II Instrument and LightCycler® 480 Probes Master (Roche) in the following steps: 10 min at 95˚C and 45 cycles of: 10s at 95˚C, 30s at 60˚C, 10s at 72˚C. Amplification reaction was performed using the following UPL probes (Roche) labelled with fluorescein amidite (FAM) and primers sets for target genes: *CCNI* (UPL#58: 5'-CTCCATCC-3', left: 5'-CGGAAAATGCC TTCAAATCA-3', right: 5'-TTGGCCAGCCATTGAATTAC-3') and *KDR* (UPL#18: 5'-CAGCAGGA-3', left: 5'-GAACATTTGG GAAATCTCTTGC-3', right: 5'-CGGAAGAACAATGTAGTC TTTGC-3'), in duplex with Universal Probe Library (UPL) probe labeled with Yellow 555 and primers set for the reference gene (*GAPD*, Universal Probe Library Human GAPD Gene Assay (Roche): UPL: 5'-CTTTTGCGTCGC-3', left: 5'-CTCTGCTCCT CCTGTTCGAC-3', right: 5'-GCCCAATACGAC CAAATCC-3'. cDNA from TOV-112D cells was used as a calibrator for CCNI and cDNA from EOC tissue with moderate *KDR* gene expression was used as a calibrator for *KDR*. Quantification was carried out by the efficiency method using LightCycler 480 software ver. 1.5 SP3 (Roche) to obtain relative quantity (RQ) values of mRNA expression of *CCNI* and *KDR* genes.

Statistical analysis. The normality of *KDR* and *CCNI* mRNA expression was analyzed using Kolmogorov-Smirnov with Lilliefors significance correction and Shapiro-Wilk tests. Both variables were non-normally distributed in the EOC group, and therefore the group differences were further analyzed with Kruskal–Wallis and Mann–Whitney tests. Correlations between variables were analyzed by Spearman's rank correlation test. *p*-Values lower than 0.05 were considered statistically significant. Statistical analysis was carried out using SPSS ver. 14PL software (SPSS Inc., Chicago, IL, USA).

Results

CCNI and *KDR* mRNA expression was detected in all EOC tissues and ovarian cancer cell lines. Figure 1 shows *CCNI* and *KDR* mRNA RQ values in EOCs and four ovarian cancer cell lines. *CCNI* mRNA level was significantly higher in EOCs than in ovarian cancer cell lines ($p<0.001$,

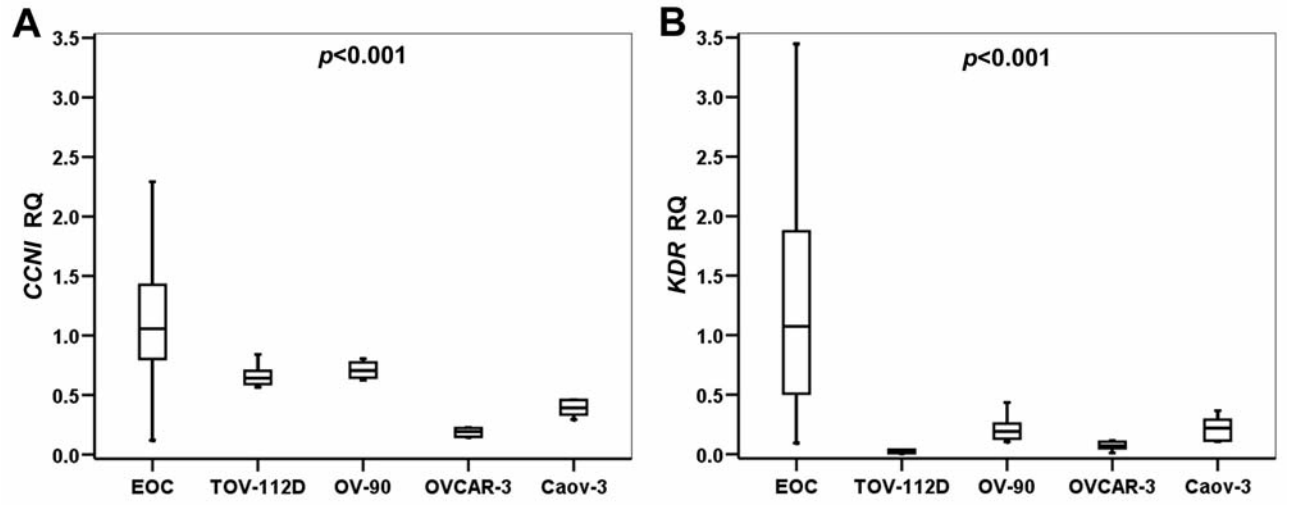

Figure 1. *Cyclin I (CCNI) (panel A) and kinase insert domain receptor (KDR) (panel B) mRNA expression in epithelial ovarian cancer (EOC) tissues and four ovarian cancer cell lines. CCNI and KDR mRNA levels were significantly higher in EOCs than in ovarian cancer cell lines (p<0.001, Kruskal–Wallis test). RQ: Relative quantity; central horizontal line: median; bottom of the box: lower quartile (25 percentile); top of the box: upper quartile (75 percentile); whiskers: values less than 1.5 times the height of the box.*

Kruskal–Wallis test). Among ovarian cancer cell lines, *CCNI* mRNA expression was the highest in OV-90 and TOV-112D cell lines, and the lowest in OVCAR-3 cells ($p<0.001$, Kruskal–Wallis test). We found significantly higher *KDR* mRNA level in EOCs than in ovarian cancer cell lines ($p<0.001$, Kruskal–Wallis test). Among ovarian cancer cell lines, *KDR* mRNA expression was the highest in Caov-3 and OV-90 cell lines, and the lowest in TOV-112D cells ($p=0.001$, Kruskal–Wallis test). Table I shows the association between mRNA expression of *CCNI, KDR* and clinicopathological features of EOCs. mRNA levels of *CCNI* and *KDR* in EOC tissues were not significantly associated with analyzed variables such as age, menopausal status, International Federation of Gynecology and Obstetrics (FIGO) stage, residual disease, patients' response to chemotherapy, tumor histology, grade or sensitivity to chemotherapy. However, we demonstrated a direct and significant correlation between the expression of *KDR* and *CCNI* genes in EOCs (R=0.530, $p<0.001$, Spearman's rank correlation test, Figure 2).

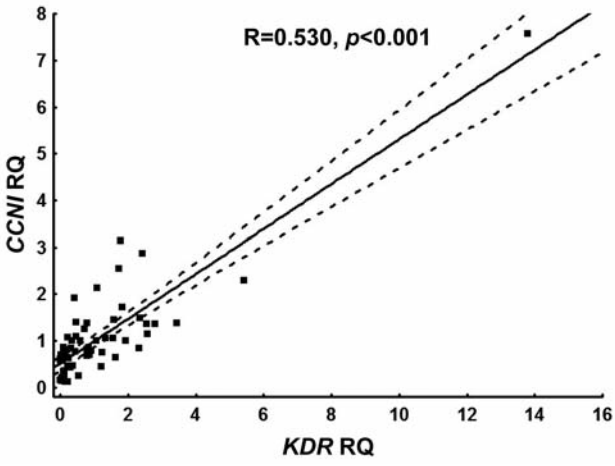

Figure 2. *Correlation between cyclin I (CCNI) and kinase insert domain receptor (KDR) mRNA expression in epithelial ovarian cancer tissues. mRNA levels of CCNI and KDR were positively and significantly correlated (Spearman's rank correlation test). Dotted line: 95% Confidence interval.*

Discussion

Progression of the cell cycle is crucial for cancer cell proliferation, and it is controlled by sequential activation of CDKs, and their regulatory subunits, cyclins, which are often de-regulated in cancer (6). To the best of our knowledge, we are the first to report that *CCNI* mRNA is expressed in EOC tissues and ovarian cancer cell lines. We demonstrated that *CCNI* mRNA expression was significantly higher in EOC tissues than in ovarian cancer cell lines, and it was not associated with tumor grade or disease stage. In our previous study (13), CCNI immunostaining in ovarian cancer cells tended to be higher in poorly differentiated (G3) EOCs and it was directly and significantly correlated with cancer cell proliferative activity. Silencing and reducing CCNI protein expression in cervical cancer HeLa cells inhibited cell proliferation and induced cell-cycle arrest (21), showing the

Table I. *mRNA expression of cyclin I (CCNI) and kinase insert domain receptor (KDR) genes in epithelial ovarian cancer tissues.*

Variable	N	CCNI RQ			KDR RQ		
		Mean±SD	Median (min-max)	*p*-Value	Mean±SD	Median (min-max)	*p*-Value
Age							
<60 years	25	1.14±0.63	1.01 (0.12-2.87)	0.442[a]	1.30±1.16	0.93 (0.09-5.43)	0.434[a]
≥60 years	15	1.69±1.80	1.36 (0.12-7.57)		2.20±3.35	1.25 (0.23-13.80)	
Menopausal status							
Pre	12	1.23±0.73	1.00 (0.45-2.87)	0.813[a]	1.50±0.81	1.38 (0.33-2.57)	0.295[a]
Post	28	1.39±1.38	1.08 (0.12-7.57)		1.70±2.65	0.90 (0.09-13.80)	
FIGO stage							
I and II	13	1.60±1.92	1.00 (0.12-7.57)	0.806[a]	2.17±3.58	1.35 (0.09-13.80)	0.874[a]
III and IV	27	1.22±0.68	1.09 (0.12-2.87)		1.38±1.20	1.07 (0.22-5.43)	
Histology							
Serous	20	1.69±1.59	1.20 (0.12-7.57)	0.254[b]	2.06±2.88	1.61 (0.23-13.80)	0.188[b]
Mucinous	4	1.40±0.63	1.22 (0.86-2.29)		2.26±2.33	1.69 (0.22-5.43)	
Endometrioid	8	0.92±0.44	1.03 (0.12-1.40)		0.78±0.55	0.70 (0.09-1.55)	
Undifferentiated	8	0.89±0.49	0.76 (0.24-1.72)		1.12±1.05	0.79 (0.25-3.45)	
Tumor grade							
G1 and G2	17	1.64±1.68	1.05 (0.12-7.57)	0.359a	2.28±3.26	1.35 (0.09-13.80)	0.389[a]
G3	23	1.13±0.67	1.08 (0.12-2.87)		1.17±0.81	0.84 (0.22-2.81)	
Residual disease							
≤1 cm	15	1.20±0.80	1.00 (0.12-3.13)	0.665[a]	1.24±0.80	1.25 (0.09-2.57)	0.823[a]
>1 cm	25	1.43±1.42	1.09 (0.12-7.57)		1.88±2.77	1.07 (0.22-13.80)	
Patient response to chemotherapy							
CR	15	1.53±1.77	1.05 (0.12-7.57)	0.865[a]	2.23±3.29	1.35 (0.15-13.80)	0.265[a]
PR, SD, and PD	17	1.20±0.69	1.01 (0.24-2.54)		1.30±1.29	1.07 (0.22-5.43)	
Tumor sensitivity to chemotherapy							
Sensitive	23	1.45±1.48	1.05 (0.12-7.57)	0.691[a]	1.94±2.70	1.55 (0.15-13.80)	0.098[a]
Resistant and refractory	9	1.13±0.66	1.01 (0.45-2.29)		1.22±1.62	0.88 (0.22-5.43)	

RQ: Relative quantity; *p*-value by [a]Mann–Whitney test, and [b]Kruskal–Wallis test; FIGO: International Federation of Gynecology and Obstetrics; CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

importance of this protein for maintenance or progression of gynecological cancer. The lack of significant correlation between *CCNI* mRNA and grade or stage of EOCs in our present study may be linked to the observation that *CCNI* mRNA levels did not fluctuate during the cell cycle (7-9). Additionally, it has been demonstrated that CCNI undergoes degradation by ubiquitin-proteasome system (12, 21) and it was proposed that dysregulation of CCNI protein level may be involved in aberrant cell-cycle regulation in breast cancer cells (11). Taken together, these findings suggest that the regulation of CCNI expression necessary for cancer progression could occur on the protein rather than the mRNA level. In the current study, *CCNI* mRNA level in EOC tissues was not significantly correlated with patients' response or tumor sensitivity to platinum-based first-line chemotherapy, which is in accordance with the results of our previous work (13) where CCNI immunostaining was not significantly associated with these variables either.

We found significantly higher *KDR* mRNA level in EOCs than in all analyzed ovarian cancer cell lines. *KDR* mRNA was the highest in OV-90 and Caov-3 cell lines among four analyzed ovarian cancer cell lines. OV-90 is a cell line derived from a metastatic site (ascites) of high grade, advanced serous ovarian adenocarcinoma. Our findings support the current view that autocrine the VEGF/VEGFR2 loop in EOC cells promotes their survival in ascites (15). In EOC tissues, the *KDR* mRNA level was not significantly associated with disease progression, similarly to a previous study (22), where *KDR* mRNA level in EOCs was not significantly associated with FIGO stage. On the other hand, it was discovered that higher VEGFR2 immunostaining in EOC specimens was significantly correlated with the extent of disease, including advanced stage, poor differentiation, residual disease >1 cm, metastases, positive ascitic cytology in early stage, and ascites (13, 23, 24). These findings indicate that VEGFR2 protein expression better reflects the progression of EOCs than the *KDR* mRNA level. In the current study, *KDR* mRNA expression did not predict patients' response or EOC sensitivity to platinum-based chemotherapy. Similarly, VEGFR2 protein expression evaluated by immunohistochemistry (13) and western blot (25) was not associated with the response of patients with EOC to platinum-based first line chemotherapy.

We found the direct and significant correlation between the mRNA expression of *KDR* and *CCNI* genes in EOC tissues. This result is in accordance with the finding of our previous study (13) where we presented the significant positive correlation between immunostaining of VEGFR2 and CCNI in EOC specimens. The same type of correlation was found in human breast cancer (11), which suggests that CCNI may be involved in angiogenesis in human cancer. However, this issue requires further studies.

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