Abstract. Polo-like protein kinase 1 (PLK1), P53 and P21WAF1 are relevant to cell cycle checkpoints and cancer biology. Misregulation of PLK1, P53 and P21WAF1 has been detected in several types of malignant tumors. The present study aimed to clarify the role of PLK1, P53 and P21WAF1 in the prognosis of ovarian cancer. PLK1 and P53 shRNA lentiviral plasmids were transfected into SK-OV-3 cells, respectively. Cell proliferation, apoptosis and invasion were examined by MTT assay, flow cytometry and Matrigel assay, respectively. Survival time of the animals was observed in a xenograft model. Expression levels of PLK1, P53 and P21WAF1 were detected in different ovarian tissues by immunohistochemistry and western blot analysis. Their correlations to the clinicopathologic characteristics of the epithelial ovarian cancer (EOC) cases and their interrelationships were analyzed. Risk factors of prognosis for EOC were determined by logistic regression analysis. The survival time of EOC patients was measured by Kaplan-Meier analysis. After PLK1 or P53 knockdown, proliferation of the SK-OV-3 cells was inhibited, the apoptosis rate was increased, and cell invasion was suppressed in vitro, and the survival time was prolonged in the animals. Expression levels of P53, p-P53 (Ser15), P21WAF1, growth arrest and DNA damage-inducible gene 45 (GADD45) and 14-3-3σ were upregulated in the SK-OV-3 cells after PLK1 knockdown, but downregulated after P53 knockdown. Higher expression levels of PLK1 and P53 were observed in patients with a higher FIGO stage and worse histological differentiation, but lower P21WAF1 was noted at a higher FIGO stage. Negative correlations were observed between expression of PLK1 and P53 and P53 and P21WAF1 in the EOC cases. PLK1, P53 and P21WAF1 could be used to assess the prognosis of EOC, respectively, but only PLK1 was found to be an independent prognostic factor. The overall survival time of subjects exhibiting PLK1-positive/P53-positive expression and PLK1-positive/P21WAF1-negative expression was obviously shorter than the other patient groups at the end of the follow-up. These results indicate that PLK1 is implicated in ovarian carcinogenesis and may owe its ability to inhibition of the activity of P53. In addition, misregulation of PLK1 coincident with P53 and P21WAF1 in EOC suggests poor prognosis.

Introduction
In spite of the recent advances in cytoreductive surgery and chemotherapeutic agents, epithelial ovarian cancer (EOC) remains the leading cause of cancer-related death in women diagnosed with gynecologic malignancies (1). Cell cycle dysregulation is closely related to the development of many malignant tumors, including ovarian cancer.

Polo-like protein kinase 1 (PLK1) is a highly conserved cyclin-dependent serine/threonine kinase, which is closely related to several events in eukaryotic cells during mitosis, such as centrosome replication, spindle formation, chromosome segregation, cytokinesis, and DNA damage repair (2,3). The activities of PLK1 are elevated in a variety of human cancer cells and is implicated in the poor prognosis of lung cancer, colorectal cancer, breast cancer, prostate cancer, malignant melanoma, lymphoma and bladder cancer (4-10). Tumor-suppressor gene P53 is implicated in the regulation of cell cycle and in tumorigenesis, and also plays an important role in the cell cycle G2/M DNA damage checkpoint regulation and replication arrest (11). More than 50% of human cancers contain P53 gene mutations and mutant P53 protein expression (12). P21WAF1 promotes cell cycle arrest in response to many stimuli, such as DNA damage, and acts as an important transcriptional target of P53, and regulates cyclin-dependent kinase (CDK)1 and cyclinB1 complexes in the G2/M DNA damage checkpoint and cell cycle transition (13). P21WAF1 is a tumor suppressor, but it also can behave as an oncogene in certain cellular contexts in human tumors (14).

In the present study, we inhibited the expression of PLK1 and P53 in ovarian cancer SK-OV-3 cells by RNA interference and detected the expression levels of PLK1, P53 and P21WAF1 in different ovarian tissues. We evaluated the associations between their expression levels and the clinical pathological factors of the EOC cases. We also determined the survival time of EOC patients by Kaplan-Meier analysis, and aimed to...
explore the role of concomitant PLK1, P53 and P21WAF1 expression in the prognosis of ovarian cancer.

Materials and methods

Cell transfection. Human ovarian carcinoma SK-OV-3 cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China), and cultured in completed RPMI-1640 medium (HyClone, Logan, Utah, USA), at 37°C with 5% CO2. Cells were harvested in a logarithmic phase of growth for all experiments as described below. PLK1 and P53 shRNA lentiviral plasmids (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for cell transfection, respectively, which was performed following the protocol of the shRNA Plasmid Transfection reagent (Santa Cruz Biotechnology). Stably transfected SK-OV-3 cells were isolated by puromycin (Clontech, Mountain View, CA, USA) selection after transfection for 48 h. Three cell groups were used for the next step study: SK-OV-3, PLK1 shRNA SK-OV-3 and P53 shRNA SK-OV-3 cells.

qRT-PCR. Cell total RNA was isolated using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and first-strand cDNA was synthesized from 1 µg total RNA according to the protocol of the RevertAid First Strand cDNA Synthesis kit (Fermentas, EU). Primers used in qRT-PCR were PLK1, P53 and P21WAF1 (Santa Cruz Biotechnology), and β-actin sense, 5'-ACGCACCCCAACTACAACCTC-3' and anti-sense, 5'-TCTTATGTCACGCACGCA-3'. PCR cycling parameters included: denaturation (94°C, 30 sec), annealing (56°C, 30 sec) and extension (72°C, 30 sec). Equal amounts of PCR products were electrophoresed on 1.2% agarose gels and visualized by ethidium bromide staining. The specific bands of the PCR products were analyzed by Image-Pro Plus 6.0 system, β-actin was used as a control for normalization. RT-PCR was performed 3 times independently.

Western blot analysis. The antibodies used in the western blot analysis, following the manufacturer's protocols, were mouse anti-human monoclonal PLK1, rabbit anti-human polyclonal P53, mouse anti-human monoclonal P53, rabbit anti-human monoclonal phospho-P53 (Ser15), mouse anti-human monoclonal P21 (Santa Cruz Biotechnology), and mouse anti-human monoclonal β-actin (Santa Cruz Biotechnology). Total protein was extracted using RIPA lysis buffer (Beyotime Biotechnology, Haimen, Jiangsu, China). Total protein was separated by 10% SDS-PAGE and transferred onto PVDF membranes. The detection of hybridized protein was performed using an enhanced chemiluminescence kit (Zhongshan Golden Bridge Biotechnology, Peking, China), β-actin was used as a control for normalization. The relative values of specific bands were analyzed by Image-Pro Plus 6.0 system.

MTT assay. Cells (1x10⁴ cells/well) were planted into 96-well plates, and 100 µl medium containing 10% FBS was added into each well. Five duplicate wells were set up for each group. Cells were cultured continuously for 7 days, and 20 µl MTT reagent (5 µg/ml; Sigma-Aldrich, St. Louis, MO, USA) was added into each well, and incubated for another 4 h. The initial medium was aspirated and 150 µl DMSO was added. The absorbance of the samples was measured by a microplate spectrophotometer (Thermo Spectronic, Madison, WI, USA) at 492 nm. All experiments were conducted in triplicate. A cell growth curve was plotted vs. time by Origin 8.0 software.

Flow cytometric analysis. Approximately 1x10⁶ cells were put into a single-cell suspension and treated with PBS solution, and were prepared following the manufacturer's protocol of the Annexin V-FITC Apoptosis Detection kit (Beyotime Biotechnology). Then, the rates of apoptosis were analyzed with the FACScan system (BD Biosciences, San Jose, CA, USA).

Matrigel invasion assay. A Transwell chamber (8-µm pore size; Millipore, Bedford, MA, USA) covered with 100 µl of 1 µg/ml Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was used to measure cell invasive ability. Cells (1x10⁵) were seeded into each upper chamber with 200 µl fresh medium without FBS, and 500 µl medium with 20% FBS was added into each lower chamber. Three duplicate wells were set up for each group. After 12 h, the cells were fixed with methanol for 5 min, and the cells were stained by hematoxylin for 30 min. The upper chamber was cleaned and inverted, and cell numbers were counted on the lower membrane under a high power lens (x400) in 5 random visual fields.

Animal model. The experimental protocol was approved by the Zhengzhou University Ethics Committee for Animal Experimentation. Female BALB/c nude mice (4-5 weeks old, 13-17 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Peking, China), and were randomly assigned into 3 groups with 10 mice per group. Approximately 1x10⁵ cells were suspended in 2 µl PBS and intraperitoneally injected into each mouse, respectively. The survival time was recorded for each mouse.

Patients and tissue samples. Ninety-one ovarian specimens were obtained from patients during surgery at the Department of Gynaecology, The First Affiliated Hospital of Zhengzhou University (from May 2008 to August 2010). The samples consisted of 20 specimens of normal ovarian tissues (obtained from patients who underwent hysterectomy and oophorectomy for multiple uterine myoma other than ovarian tumors), 19 specimens of benign ovarian tumor tissues (10 serous and 9 mucinous cystadenomas), and 52 specimens of EOC tissues (30 serous and 22 mucinous cystadenocarcinomas). The median age of the ovarian cancer patients was 53 years (range from 21 to 75). All of the ovarian cancer patients did not receive preoperative radiochemotherapy. The enrolled ovarian cancer patients all received comprehensive surgical staging and standardized postoperative chemotherapy (paclitaxel combined with platinum), and relapsed patients received second-line chemotherapy drugs for combination chemotherapy. The tissue samples were collected after surgical resection immediately and promptly saved in liquid nitrogen. The consent of all enrolled patients was obtained for sample
collection before surgery, and the present study was approved by the Local Ethics Committee of Zhengzhou University. All tissue samples were verified independently by 2 pathologists before IHC by H&E staining.

**Immunohistochemistry.** Immunohistochemical staining was performed following the protocol of the Universal SP kit (Zhongshan Golden Bridge Biotechnology). For PLK1, P53 and P21WAF1 protein, staining localized in the nucleus was considered positive. Immunoreactive scoring was performed by 2 pathologists independently using Image J; semi-quantitative counting method was used to determine positive staining.

**Statistical analysis.** Values are expressed as mean ± standard deviation (SD). Count data were analyzed by χ² test and Fisher's exact test. Measurement data were analyzed by one-way ANOVA and Bonferroni's test using SPSS 17.0 software package. A difference was considered statistically significant when P-value was <0.05.

**Results**

**Suppression of cell proliferation after RNA interference.** After knockdown of PLK1 or P53, cell proliferation was obviously suppressed from day 2 when compared with the control cells, and a difference in proliferation was also detected between the PLK1-null and P53-null SK-OV-3 cells (P<0.05) (Fig. 1).

**Cell apoptosis is induced by RNA interference.** After inhibition of PLK1 or P53, the cell apoptosis rates were markedly increased (F=236.833, P<0.05), and a difference in apoptosis was also detected between the PLK1-null and P53-null SK-OV-3 cells (P<0.05) (Fig. 2).

**Cell invasion is inhibited by RNA interference.** In the Matrigel invasion assay, the number of invaded cells on the lower membrane was markedly decreased in the PLK1-null and P53-null SK-OV-3 cells compared to the control (F=437.469, P<0.05), and a difference was also detected between the PLK1-null and P53-null SK-OV-3 cells (P<0.05) (Fig. 3).

**Survival time of animals is prolonged after RNA interference.** The survival time of the animals injected with PLK1-null and P53-null SK-OV-3 cells was obviously prolonged when compared to the survival of mice injected with the SK-OV-3 cells (F=95.703, P<0.05), and a difference was also detected between the PLK1-null and P53-null SK-OV-3 cell groups (P<0.05) (Fig. 4).

**Protein and mRNA expression changes in the SK-OV-3 cells after RNA interference.** As a result of PLK1 knockdown, a significant upregulation in the levels of P53, p-P53 (Ser15), P21WAF1, GADD45 and 14-3-3σ was observed in the PLK1 shRNA SK-OV-3 cells. After P53 knockdown, levels of P53, p-P53 (Ser15), P21WAF1, GADD45 and 14-3-3σ were markedly decreased in the P53 shRNA SK-OV-3 cells, without obvious changes in PLK1. However, differences in the fold-change were noted in the PLK1-null and P53-null SK-OV-3 groups (Figs. 5 and 6).

**Expression of PLK1, P53 and P21 WAF1 in the different ovarian tissues.** In the EOC tissues, positive staining for PLK1 and P53 was obviously higher than that in the benign ovarian tumor and normal ovarian tissues (χ²=17.266, P<0.05; χ²=42.656, P<0.05). P21WAF1 was markedly lower in the ovarian cancer tissues (χ²=20.270, P<0.05) (Table I and Fig. 7). Consistent results were observed in the western blot analysis (Fig. 8).

**Association of PLK1, P53 and P21 WAF1 expression with the clinicopathological characteristics of the ovarian cancer case.** As shown in Table II, the expression levels of PLK1 and...
P53 were significantly associated with FIGO stage and histological differentiation, and P21^{WAF1} was obviously associated with FIGO stage in the ovarian carcinoma cases (P<0.05).

Correlations between PLK1, P53 and P21^{WAF1} in 52 EOC cases. Firstly, 14 subjects were PLK1-positive and P53-negative, and 29 were PLK1-negative and P53-positive, which suggested a negative correlation between the expression of PLK1 and P53 (r=-0.629, P<0.05). Secondly, 29 subjects were P53-positive and P21^{WAF1}-negative, and 11 patients were P53-negative and P21^{WAF1}-positive, which indicated a negative correlation between P53 and P21^{WAF1} (r=-0.476, P<0.05). Thirdly, no correlation was observed between expression of PLK1 and P21^{WAF1} (P>0.05).

Univariate and multivariate logistic regression analysis. Based on the present data, PLK1 (OR=3.333, P=0.040, 95% CI: 0.102-1.092), P53 (OR=15.080, P<0.05, 95% CI: 3.713-65.252), P21^{WAF1} (OR=21.459, P=0.017, 95% CI: 3.192-68.521), FIGO stage (OR=26.867, P<0.05, 95% CI: 5.584-129.274), histological differentiation (OR=18.238, P=0.035, 95% CI:
1.267-48.121) and lymph metastasis (OR=5.000, P=0.025, 95% CI: 1.221-20.483) were the prognosis factors of ovarian cancer shown by univariate logistic regression analysis. However, only PLK1 (OR=2.288, P=0.025, 95% CI: 0.105-50.050) was shown by univariate logistic regression analysis. However, only PLK1 (OR=2.288, P=0.025, 95% CI: 0.105-50.050) was

Table I. Expression of PLK1, P53 and P21^WAF1^ in the different ovarian tissues as detected by immunohistochemistry.

| Tissue types                  | PLK1 |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |          |
|-------------------------------|------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
|                               | N    | -        | +        | ++       | Positive | rate (%) | -        | +        | ++       | Positive | rate (%) | -        | +        | ++       | Positive | rate (%) |
| Normal ovarian tissues        | 20   | 20       | 0        | 0        | 0.0      |          | 20       | 0        | 0        | 0.0      |          | 2        | 18       | 0        | 90.0     |
| Benign ovarian tumors         | 19   | 18       | 1        | 0        | 5.6      |          | 19       | 0        | 0        | 0.0      |          | 7        | 12       | 0        | 63.2     |
| Ovarian carcinoma tissues     | 52   | 32       | 11       | 9        | 38.5^ab  |          | 17       | 13       | 22       | 67.3^a   |          | 35       | 11       | 6        | 32.7^a   |

^Compared with benign ovarian tumor and normal ovarian tissues, P<0.05; ^Fisher exact test; PLK1, polo-like protein kinase 1.

Figure 6. Protein changes in the SK-OV-3 cells after RNA interference as measured by western blot analysis. Histogram shows the relative values of the different proteins vs. β-actin. Each bar represents the mean ± SD.

Figure 7. Expression of PLK1, P53 and P21^WAF1^ in the different ovarian tissues as detected by immunohistochemistry (DAB staining, magnification x400). PLK1, polo-like protein kinase 1.
an independent prognostic factor for ovarian cancer, which was analyzed by multivariate logistic regression analysis.

*Kaplan-Meier analysis.* Up to December 2012, the median follow-up was 29 months for 52 ovarian cancer patients (range from 11 to 55 months); 45 patients were alive at the end of the follow-up, 34 subjects had relapsed or succumbed to the disease (Table III). When PLK1 and P53 were combined to evaluate the overall survival time, we found that the overall survival of PLK1-positive/P53-positive subjects was obviously shorter than the survival of the other expression groups in the 52 ovarian cancer patients ($\chi^2=17.246, P=0.037$). When assessing PLK1 combined with P21WAF1, the overall survival time of the subjects with PLK1-positive/P21WAF1-negative expression was markedly shorter than patients in the other expression groups ($\chi^2=48.428, P<0.05$) (Fig. 9).

**Discussion**

PLK1 exhibits high activity in tissues and cells with a high mitotic index, and plays an important role in cell cycle progression. Inhibition of the activity of PLK1 enables cell cycle arrest in the G2/M phase (15,16). Wild-type P53 is a tumor-suppressor gene, which plays an important role in the regulation of the cell cycle and the induction of apoptosis in response to cellular stress. The combination of PLK1 and P53 may provide a new target for ovarian cancer treatment.
cycle and tumor outcome. P53 upregulates cell growth arrest and apoptosis-related genes in response to stress signals, thereby influencing programmed cell death, cell differentiation, and cell cycle control mechanisms. P53 is required for DNA damage checkpoint and functions to upregulate expression of its 3 transcriptional targets, GADD45, 14-3-3σ and P21WAF1 (17,18). In response to DNA damage and other stimuli, P21WAF1 can bind to and inhibit the kinase activity of CDKs leading to growth arrest at specific stages in the cell cycle (14). GADD45, a protein involved in DNA replication and repair, binds to both CDKs and PCNA, and serves as a link between P53-dependent cell cycle checkpoint and DNA repair (18). In response to tumor growth, 14-3-3σ increases the rate of P53-regulated inhibition of G2/M cell cycle progression (19).

It has been proven that P53 is a critical target of PLK1, and its function is abrogated through the physical interaction with PLK1. PLK1 binds to P53 at the P53-binding domain, amino acid sequence comprising residues from 99 to 329 of PLK1, and inhibits its transactivation activity as well as its pro-apoptotic function (20). The present in vitro results showed that the cell proliferation was inhibited, the apoptosis rate was increased, and cell invasion was suppressed after PLK1 or P53 knockdown in SK-OV-3 cells. The survival time was prolonged after PLK1 or P53 knockdown in the animals. Furthermore, a notable phenomenon shown in the present study, was that the inhibitory effects of PLK1 knockdown were more obvious than P53 knockdown in the SKOV-3 cells. The survival time was prolonged after PLK1 or P53 knockdown in SK-OV-3 cells. Inhibition of PLK1 in SKOV-3 cells increased the expression of P53, p-P53 (Ser15), P21WAF1, GADD45 and 14-3-3σ. In contrast, knockdown of P53 decreased the expression of P53, p-P53 (Ser15), P21WAF1, GADD45 and 14-3-3σ, without obvious changes in PLK1. These data suggest that abnormal expression of PLK1 implicated in ovarian carcinogenesis may owe its ability to inhibition of the activity of P53.

Increasing evidence suggests that the level of PLK1 expression has prognostic value for predicting outcomes in patients with various types of cancers such as lung cancer, squamous cell carcinomas of the head and neck, melanomas, and ovarian carcinomas (21,22). Mutation and overexpression of P53 are common in EOC; positive staining for P53 was detected in almost all different subtypes of ovarian cancer, and the group of patients with P53-positive tumors showed worse survival (23,24). Overexpression of P53 is not a feature of benign epithelial ovarian tumors or early-stage borderline ovarian tumors (25). P21WAF1 is often misregulated in human cancers including ovarian cancer, and patients with downregulation of P21WAF1 were found to show worse survival time in ovarian serous carcinoma (26,27). Our previous study also showed that P21WAF1 was downregulated in EOC tissues, and was related with a later FIGO stage (28).

In the present study, abnormal expression levels of PLK1, P53 and P21WAF1 protein in EOC tissues were detected by immunohistochemistry and western blot analysis. The expression levels of PLK1 and P53 were significantly higher in the EOC tissues, while P21WAF1 was obviously lower. Higher expression levels of PLK1 and P53 were detected in patients with later FIGO stage and worse histological differentiation, but lower P21WAF1 in later FIGO stage. These results suggest that misregulation of PLK1, P53 and P21WAF1 are implicated in the progression of ovarian cancer.

P53 plays key roles in cell cycle signal cross-talk, can receive various upstream cell stimuli, and can be directly or indirectly mediated by different signal transduction proteins, such as PLK1, ATM and ATR (20,29,30). The present study showed that PLK1, P53 and P21WAF1 could be used to assess the prognosis respectively, but multivariate logistic regression analysis verified that only PLK1 was an independent prognostic factor for ovarian cancer. The overall survival time assessed by Kaplan-Meier curves analysis showed that the overall survival time of the patients with PLK1-positive/P53-positive or PLK1-positive/P21WAF1-negative was obviously shorter than that of other patients at the end of the follow-up. These results indicate that misregulation of P53 and P21WAF1 may be a common event in ovarian cancer, and may be used to assess the prognosis, but only PLK1 could serve as an independent prognostic factor for ovarian cancer. These results indicate that misregulation of PLK1 coinciding with P53 and P21WAF1 in EOC highly suggests poor prognosis.
In conclusion, according to the above in vitro data and due to the limited number of enrolled patients with follow-up, the present study showed that abnormal expression of PLK1 is implicated in ovarian carcinogenesis and may owe its ability to inhibition of the activity of P53, and misregulation of PLK1 coincident with P53 and P21WAF1 in EOC suggests poor prognosis.

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