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The expression of TP53 pathway-related proteins in ovarian carcinoma transplanted subcutaneously in nude mice

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Abstract. – OBJECTIVE: Given the important functions of TP53 pathway in various biological processes, this study aimed to investigate the expression of TP53 pathway-related proteins in ovarian carcinoma transplanted subcutaneously in nude mice with and without the presence of p53 inhibitor and to explore possible roles of p53 in the development of ovarian cancer.

MATERIALS AND METHODS: Thirty BALB/c-nu female nude mice were randomly divided into model group, control group and p53 inhibitor group (Pftα group). There were 10 rats in each group. The nude mice were subcutaneously inoculated with human ovarian cancer cell line SKOV3, and the tumor growth was observed. Morphological changes of tumor tissue were observed by hematoxylin and eosin (HE) staining. The mRNA and protein levels of TP53 pathway related factors-p53, p21 and mouse double minute 2 homolog (MDM2) were detected by RT-PCR and Western blot.

RESULTS: p53 inhibitor can increase the growth rate of subcutaneously transplanted tumor in nude mice. p53 inhibitor could decrease the expression of p53 and p21 at both mRNA and protein levels and increase the expression of MDM2 at both mRNA and protein levels in ovarian carcinoma transplanted subcutaneously in nude mice.

CONCLUSIONS: TP53 pathway may play pivotal roles in the development of ovarian cancer and TP53 pathway may be a new target for the treatment of ovarian cancer.

Key Words:
TP53 pathway, Ovarian carcinoma, p53, p21, MDM2.

Introduction

Ovarian cancer causes 22,000 new diagnoses and over 140,000 deaths in women worldwide every year[1]. Ovarian cancer is widely metastatic in the abdomen and its manifestation is relatively late. So, most of the ovarian cancers can only be detected in advanced stages, leading to the high death rate[2]. So far, almost all the screening methods for ovarian cancer have been proved to be non-effective[3-5]. Many treatment methods including chemotherapy, radiation therapy, olaparib maintenance therapy and so on have been developed to treat ovarian cancer[6-8]. However, the following observation studies have shown that all the treatment methods failed to improve the survival rate[9]. The development of cancer, including ovarian cancer, is a complex multistep process with a variety of factors including environment factors and cellular factors involved; environmental factors can only induce the development of cancer by affecting the expression of intracellular genes. So, it will be of great clinical values to identify new molecular targets involved in the process of ovarian cancer development to provide evidence for the development of new treatment methods. TP53 (tumor protein p53) also known as p53, is a tumor suppressor gene. The activated p53 protein can inhibit the progression of tumor cells by inducing cell cycle arrest, apoptosis, DNA repair and aging. The expression of p53 protein is a low level in normal cells. MDM2 (murine double minute 2) could bind p53 protein to inhibit p53 mediated transcriptional activity. Therefore, MDM2 could cause tumorigeneisis by preventing apoptosis induced by p53. p53 has been shown to play pivotal roles in the development of various human diseases including Parkinson disease, liver disease, cancer, and so on[10-13]. A previous study has shown

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that mutations in p53 were found in almost all the types of cancer tumors and the mutation rate of p53 in ovarian cancer tumor was proved to be the highest among all the solid cancers[14], indicating that p53 may play important roles in the development of ovarian cancer. However, to the best of our knowledge, no study on the direct effects of p53 and p53 related factor on the development of ovarian cancer has been reported. p21, which is also called CDK-interacting protein 1 or cyclin-dependent kinase inhibitor 1, is a cyclin-dependent kinase inhibitor that usually can be induced by p53. The role of p21 is similar to p53. MDM2 can bind with p21 protein and participate in regulating cell proliferation and apoptosis. The expression of MDM2 was down regulated and the expression of p21 protein increased, reversely. So, p53 and p21 form the cascade to conduct the signal. However, this signal cascade can be blocked by the binding of MDM2 to the N-terminal end of p53[10-14]. MDM2 could cause tumorigenesis. The high expression level of MDM2 can increase the proliferation rate of cancer cells by interacting with p53. So, interactions between p53, p21 and MDM2 may direct or indirect affect the development of ovarian cancer at multiple levels, and it worth to be in-depth investigated. To investigate the role of p53 and p53 related factor in the development of ovarian cancer, we detected the expression level of p53, p21 and MDM2 in ovarian carcinoma transplanted subcutaneously in nude mice with and without the presence of p53 inhibitor at both mRNA and protein levels combined with the observation of tumor growth. Our data indicate that TP53 signaling pathway is important for the development of ovarian cancer and p53 inhibitor can promote the cancer development by decreasing the expression of p53 and p21 and increasing the expression MDM2.

Materials and Methods

Cell Culture

Human ovarian cancer cell SKOV3 (Cell Bank of Chinese Academy of Medical Sciences) was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Carlsbad, CA, USA) containing 10% heat inactivated calf serum (Sijiqing, Hangzhou, Zhejiang, China), 1×10^5 U/L penicillin and 100 μg/L streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C with 5% CO_2.

Establishment of Subcutaneous Transplanted Ovarian Cancer Model in Nude Mice and Grouping

Thirty BALB/c-nu SPF grade female nude mice (Laboratory Animal Center of the Chinese Academy of Sciences) aged from 6 to 8 weeks and weighted from 18 to 22 g, were raised in SPF environment. The protocol was approved by the Ethics Committee of Yantaishan Hospital (Yantai, Shandong Province, China). They were randomly divided into model group, control group and p53 inhibitor group (Pftα group). There were 10 rats in each group. SKOV3 cells were recovered and sub-cultured for 2 to 3 generations. Then, the cells were digested with 0.25% trypsin with the presence of EDTA at 37°C for 1 to 2 min followed by centrifugation. Then RPMI-1640 medium was added to make single cell suspension. After trypan blue staining, the cells were counted under a fluorescence microscope to make sure more than 95% of the cells were alive. The cells were counted with blood counting chamber and the concentration was adjusted to 2×10^6/ml. The P53 inhibitor Pftα was diluted to 10 mg/ml with dimethyl sulfoxide (DMSO) solution. The right armpits of the mice in each group were disinfected and 1 ml SKOV3 cell suspension was subcutaneously injected into the area near the back. When the subcutaneous transplanted tumor was grown to a volume of about 0.12 cm^3, the mice in control group and Pftα group were treated as follows: for the mice in Pftα group, 0.2 ml p53 inhibitor solution was subcutaneously injected into the area 2 to 3 cm from the tumor. DMSO solution was used for the mice in control group. The mice were treated once every other day with a total number of 7 times followed by 15 days' observation.

The Observation of the Dynamic Changes of Tumor Volume in Nude Mice

The general situation and the tumor growth of the nude mice were observed after inoculation. From the day of administration, the longest diameter (a) and the shortest diameter (b) of the subcutaneous tumor were measured with the Vernier caliper. The volume of subcutaneous tumor of the mice in each group was calculated by the formula $V=l/2ab^2$ (V was the tumor volume, a was the longest diameter of the tumor and b was the shortest diameter of tumor).

Tumor Tissue Specimen Collection

On the third day after the 7th treatment (i.e., the 15th day after the start of administration), the

size of the subcutaneous tumor was measured with a Vernier caliper, the tumor growth rate was calculated, and the nude mice were sacrificed by cervical spine. The tumors were collected and weighted. The tumor tissues were washed with phosphate-buffered saline (PBS) and the necrosis and host tissues were removed. Next, half of the tumor tissue was fixed with 10% formalin solution. The other half of tumor tissue was cut into soybeans size and put into the frozen tube and stored in liquid nitrogen.

Histopathological Observation of Subcutaneous Transplanted Tumor

The tumor tissue was sliced, placed in 4% paraformaldehyde for posterior fixation, followed by dehydration with gradient alcohol. Xylene treatment was used to make the tissue transparent. The tissue was embedded with conventional paraffin method and sliced into 5 μm-thick slices. After HE staining (Beyotime, Shanghai, China), the tissue was observed under light microscope.

Detection of p53, MDM2, and p21 mRNA Expression in Tumor Tissue

The expression of p53, MDM2 and p21 mRNA in tumor tissues was detected by RT-PCR. The total RNA was extracted from the tumor tissue by Trizol kit (Invitrogen, Carlsbad, CA, USA) according to the instructions. The reverse transcription reaction was then carried out according to the instructions of the kit (TaKaRa, Otsu, Shiga, Japan). SYBR Green master kit (Applied Biosystems, Foster City, CA, USA) was used for the PCR. The following primers were used for PCR: 5′-TACTCCCCTGCCCTCAACAAGA-3′ (sense) and 5′-ACAACCTCCGTCATGTGCTGTG-3′(anti-sense) for p53; 5'-GTGTGCCGTTGTCTCTTCG -3' (sense) and 5'-GGTCTGCCTC CGTTTTCG -3' (anti-sense) for p21; 5'- ATGAAGCCTGGCTCTGTGT -3' (sense) and 5'- GAAGCCAATTCTCACGAAGG -3' (anti-sense) for MDM2; 5'- CGGGAAATCGTGCGTGACAT -3' (sense) and 5'- GAAGGAAGGCTGGAAGAGTG -3' (anti-sense) for β-actin. All the primers were synthesized by TaKaRa (TaKaRa, Otsu, Shiga, Japan). PCR was performed as follows: 94°C pre-denaturation for 2 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1 min, then 10 min at 72°C. The relative expression level of p53, MDM2, and p21 mRNA was calculated by $2^{-\Delta\Delta Ct}$ method. Three repeats were involved in each experiment.

Detection of p53, MDM2 and p21 Protein Expression in Tumor Tissue

Western blot was used to detect the expression of p53, MDM2 and p21 in tumor tissues. The total protein was extracted according the instructions of the kit. Bicinchoninic acid (BCA) protein quantitation kit (Pierce, Rockford, IL, USA) was used to detect protein concentration. 40 μg protein from each sample was subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis followed by transmembrane to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk for 1 h, after that the primary antibodies of p53, MDM2 and p21 (Zhongshan Golden Bridge, Beijing, China) were diluted with bovine serum albumin (BSA) and incubated with the membrane overnight at 4°C. Then, Tris-buffered saline-tween (TBST) (TBS, 1 ml/L Tween-20) was used to wash the membrane 3 times, 5 min for each time. Horse radish peroxidase (HRP) labeled secondary antibody (1:5000) (TransGen, Beijing, China) was added and incubated at room temperature for 2 h. The color development step was performed in dark room with ECL. The expression of each protein was normalized with the endogenous control β-actin.

Statistical Analysis

Statistical analysis was performed using SPSS19.0 statistical software (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA). The data were expressed as mean ± standard deviation (SD). Single factor analysis of variance was used to compare the data between more than 2 groups. *t*-test was used to compare data between the two groups. $p<0.05$ was considered to be statistically significant.

Results

The Observation of General Condition of The Mice in Each Group

The mice in control group and Pftα group showed irritability and hyperactivity 10 min after treatment, but soon returned to normal state. There was no significant change in the growth status of the nude mice in three groups. No mouse died during the whole experiment, and the rate of tumor formation was 100%. So the treatments used in this study did not affect the general condition of the mice.

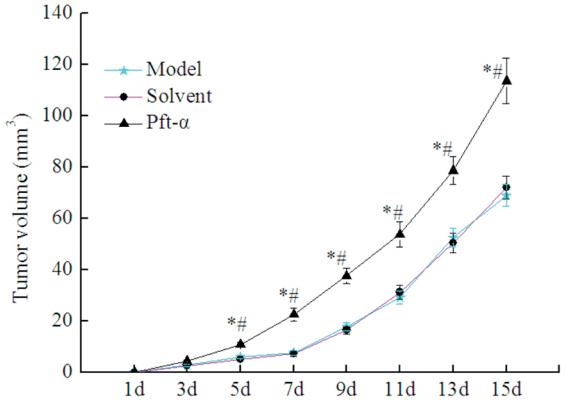

Figure 1. Comparison of the growth rates of subcutaneous transplanted tumor in nude mice of each group. Notes: *Compared with the model group $p<0.05$; #compared with control group $p<0.05$.

Comparison of Tumor Growth in Nude Mice of Each Group

Palpable tumor nodules were formed 3 days after inoculation, and the tumor volume was about 0.12 cm^3. The mice were treated and the tumor volume was measured the day after treatment and was converted into tumor volume growth rate. The averaged data of 10 mice were plotted as growth curves (Figure 1). The tumor growth rate of mice in Pftα group was significantly higher than that of the control group and model group ($p<0.05$), especially after 4 times' treatment, the tumor in mice of Pftα group grew much faster than that of the mice in control group and model group. There is no significant difference in the tumor growth between control group and model group ($p>0.05$). The data suggested that p53 inhibitor can promote the growth of tumor.

Morphological Changes of Tumor Tissue

As shown in Figure 2, HE staining of the subcutaneous tumor tissue sections revealed that the morphological changes of the tumor tissues of each group were consistent with the changes of the differentiated adenocarcinoma. The cancer cells showed a structure as nest or irregular lumps, rich in cytoplasm. There were no significant differences in the microscopic characteristics of tumor tissue sections among the three groups, indicating that p53 inhibitor cannot induce morphological changes of tumor tissue

Expression of p53, MDM2 and p21 mRNA in Tumor Tissue

The expression levels of p53 and p21 mRNA in Pftα group were significantly lower than those in model group and control group ($p<0.05$) (Figure 3), while the expression of MDM2 mRNA in Pftα group was significantly higher than that in model group and control group ($p<0.05$). There was no significant difference in the expression level of p53, MDM2, and p21 mRNA between the model group and the control group ($p>0.05$). Our data suggested that p53 can regulate the expression of p53, MDM2, and p21 at mRNA level.

Expression of p53, MDM2 and p21 Protein in Tumor Tissue

The results of Western blot showed that the expression levels of p53 and p21 protein in Pftα group were significantly lower than those in model group and control group ($p<0.05$) (Figure 4), while the expression of MDM2 protein in Pftα group was significantly higher than that in model group and control group ($p<0.05$) (Figure 4). No significant difference was found in the expression level of p53, MDM2 and p21 mRNA between the model group and the control group ($p>0.05$) (Figure 4). Our data suggested that p53 can regulate the expression of p53, MDM2 and p21 at protein level.

Discussion

As a common transcription factor, the expression level of p53 in healthy cells is relatively low. However, p53 expression can be significantly increased by the stimulations from toxic substances or carcinogenic factors, so p53 is believed to be closely related to the development of cancer[15]. *In vivo* and *in vitro* studies have both shown that p53 has pivotal roles in the regulation of cell cycle, cell differentiation, and apoptosis. Previous studies have shown that p53 can inhibit the development of various cancer tumors by regulating cell proliferation, and the increased p53 protein level can lead to the reduced cancer cell proliferation rate and increased apoptosis rate[16,17]. P53 is encoded by TP53 gene. Previous experimental studies on animal models and clinical studies both have found that mutations happened in TP53 gene can usually promote the development of various cancers[17,18]. The loss of function of TP53 gene in increase the invasion and proliferation ability of cells of a variety of cancer cell lines, and the same time, the apoptosis rate of those cell lines were significantly inhibited after TP53 gene knockdown[17,18]. In our research, the role of p53 in the development of ovarian cancer was tested by the successfully established mice ovarian

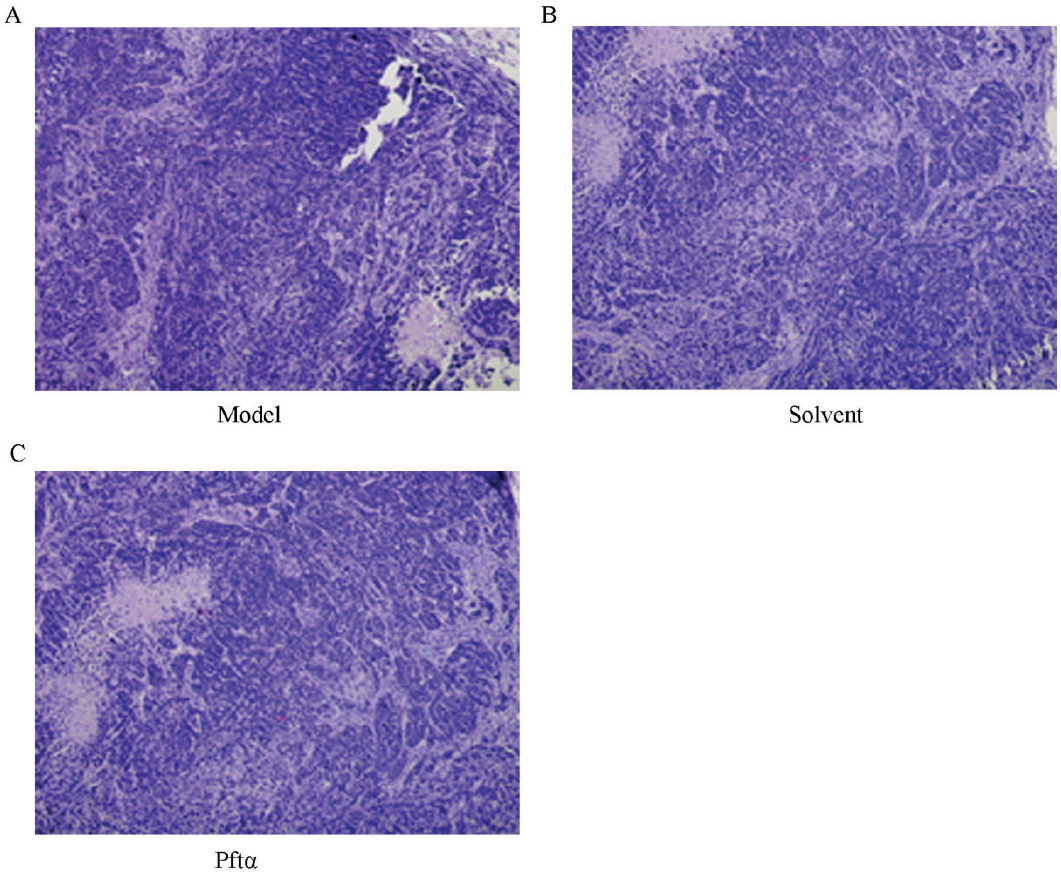

Figure 2. HE staining of the subcutaneous tumor tissue sections (×100). ***A***, model group; ***B,*** solvent group; ***C,*** Pfta group.

cancer model by subcutaneously transplanting ovarian cancer cells. HE staining of the subcutaneous tumor tissue sections revealed that the morphological changes of the tumor tissues of each group were consistent with the changes of the differentiated adenocarcinoma (Figure 2), indicating that the mice ovarian cancer model was successfully established. The effects of p53 in the development of ovarian cancer were explored by the application of p53 inhibitor treatment. After the treatment with p53 inhibitor, the tumor growth rate was significantly increased compared with that of the mice in both control group and model group (Figure 1). Our data suggested that p53 can inhibit the development of ovarian cancer, while p53 inhibitor can inactive p53, thus promoting the growth of the tumor. As a downstream effector of p53, the expression of cyclin-dependent kinase inhibitor p21 can be both induced and significantly promoted by the increased level of p53[19,20]. The increased expression of p21 will lead to the inhibition of cell division by inhibiting the cyclin/cyclin-dependent kinase (CDK) complex, which plays pivotal roles in the process of G1-to-S transition, which in turn inhibits the proliferation cancer cells[20]. So both p53 and p21 play negative roles in the development of cancer. The expression of human homologue of MDM2 can

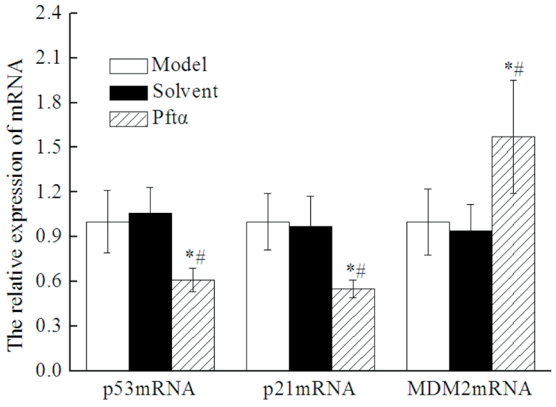

Figure 3. Expression of p53, MDM2 and p21 mRNA in tumor tissue of each group. Notes: *compared with the model group $p<0.05$; #compared with control group $p<0.05$.

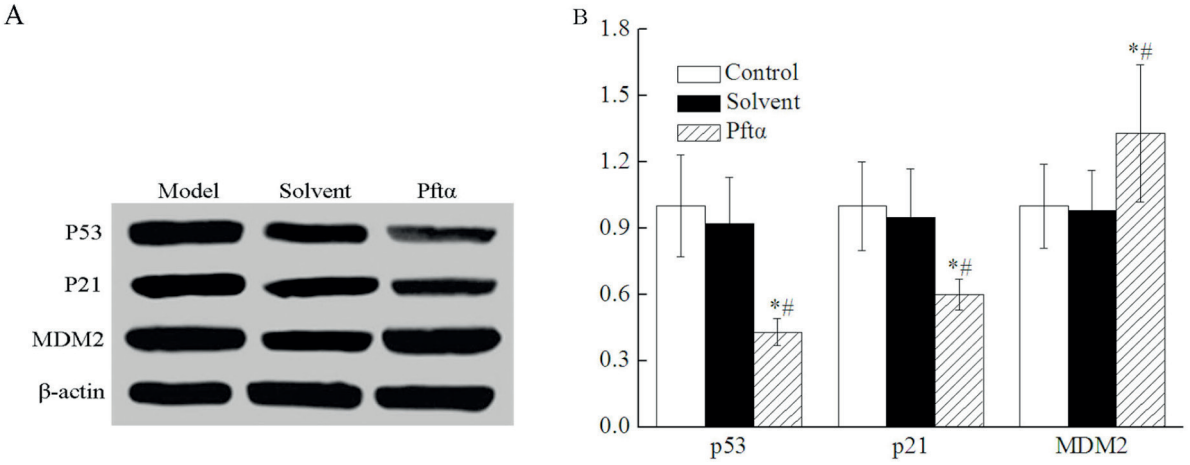

Figure 4. Expression of p53, MDM2 and p21 protein in tumor tissue of each group Notes: *compared with the model group $p<0.05$; #compared with control group $p<0.05$.

also be induced by p53. However, MDM2 can inhibit the function of p53 by forming a complex with it to block the p53 signaling pathway[21]. The blocked p53 signaling pathway will downregulate the expression of the proteins involved in the p53 triggered cancer-inhibition signal conduction cascade, thus promoting the development of cancer[21]. A recent study has shown that the high expression level of MDM2 can increase the proliferation rate of retinoblastoma cells by interacting with p53; in that study, MDM2 knockdown was found to be able to induce p53, which increases the survival rate of retinoblastoma cells through a p53-independent mechanism[22]. Consistent results were found in our study; we found that the expression levels of both p53 and p21 were significantly decreased by p53 inhibitor treatment at both protein and mRNA levels ($p<0.05$) (Figure 4). However, expression levels of MDM2 were significantly increased by p53 inhibitor treatment at both protein and mRNA levels ($p<0.05$) (Figure 4). So, the increased expression level of p53 and p21 came with the decreased expression level of MDM2. Our data suggested that p53 inhibitor can promote the development of ovarian cancer by regulating p53 signaling pathway. So, p53 signaling pathway can be a target for the treatment of ovarian cancer.

Conclusions

p53 inhibitor could increase the growth rate of subcutaneous transplanted ovarian cancer tumor in nude mice. Also, p53 inhibitor could decrease the expression of TP53 pathway related fator-p53 and p21 at both mRNA and protein levels, but could increase the expression of another TP53 pathway related fator-MDM2 at both mRNA and protein levels. So, TP53 pathway may play pivotal roles in the development of ovarian cancer and TP53 pathway may be a new target for the treatment of ovarian cancer.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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