Paclitaxel-resistant HeLa cells have up-regulated levels of reactive oxygen species and increased expression of taxol resistance gene 1

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Abstract: This study is to establish a paclitaxel (PTX)-resistant human cervical carcinoma HeLa cell line (HeLa/PTX) and to investigate its redox characteristics and the expression of taxol resistance gene 1 (Txr1). HeLa cells were treated with PTX and effects of PTX on cell proliferation were detected through cell counting and the MTT assay. Levels of cellular reactive oxygen species (ROS), reduced glutathione (GSH), and oxidized glutathione (GSSG) as well as the ratio of GSH to GSSG were measured by the 2,7-difluorescein diacetate (DCFH-DA) method and the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) method. Activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were determined by the nitrite formation method, the molybdate colorimetric method, and the DTNB colorimetric method, respectively. The level of Txr1 mRNA was determined by real-time PCR. Compared with the regular HeLa cells, HeLa/PTX cells were larger in size and had more cytoplasmic granules. The population doubling time for HeLa/PTX cells was 1.32 times of that of HeLa cells ($P < 0.01$). HeLa/PTX cells showed stronger resistance to PTX than HeLa cells with a resistance index of 122.69. HeLa/PTX cells had higher levels of ROS ($P < 0.01$) and Txr1 mRNA ($P < 0.01$), lower level of GSH ($P < 0.05$), and lower activities of SOD ($P < 0.01$) and GPx ($P < 0.05$) than HeLa cells. HeLa/PTX cells, with higher levels of ROS and Txr1 mRNA expression, are more resistant to PTX than HeLa cells

Keywords: cervical carcinoma, paclitaxel, taxol resistance gene 1, reactive oxygen species, antioxidant system.

INTRODUCTION

The occurrence and development of cervical cancers and the tolerance of cervical cancer cells to chemotherapeutic drugs are closely related to levels of intracellular reactive oxygen species (ROS) and the condition of antioxidant systems. For instance, the decreased expression of c-FLIP can promote caspase dependent JNK activation in HeLa cells and increase levels of ROS (Nakajima *et al.,* 2008). The expression of peroxiredoxin II and III in cervical carcinoma is significantly increased (Kim *et al.,* 2009), while thymosin beta-4 (TB4) can induce ROS formation and stability of ROS mediated HIF-1alpha, thereby strengthening the tolerance of HeLa cells to paclitaxel (PTX) (Oh and Moon, 2010). In addition, the mechanism and effect of PTX on cancer cells are closely related to factors of cellular redox. One study shows that the silence of heat shock protein 27 (HSP 27) can lead to increase in ROS levels, thus improving the sensitivity of human ovarian carcinoma H08910 cells to PTX (Song *et al.,* 2009). Meanwhile, PTX can induce apoptosis of chronic myeloid leukemia cells through inducing the intracellular oxidative stress and activation of JNK (Meshkini and Yazdanparast, 2012).

Taxol resistance gene 1 (Txr1) is a new drug resistant gene reported by Lih *et al* (Lih *et al.,* 2006). It can regulate the secretion of thrombospondin, resulting in paclitaxel resistance of human prostate cancer cells. Studies in lung cancer (Papadaki *et al.,* 2009) and breast cancer cells (Bai *et al.,* 2012) revealed that the up-regulation of Txr1 could induce drug resistance of cancer cells. Therefore, the tolerance of cervical cancer cells to PTX might be related to the up-regulation of Txr1. In this study, the HeLa/PTX cell line that were resistant to PTX was established and the ROS levels of HeLa/PTX cells, the state of antioxidant system, and the expression of Txr1 in HeLa/PTX cells were investigated.

METHODS AND MATERIALS

Reagents

Newborn bovine serum, PRMI-1640 culture medium and trypsin were all purchased from Gibco, Invitrogen, Carlsbad, California, USA. PTX, thiazolyl blue tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), 2,7-difluorescein diacetate (DCFH-DA), diethyl pyrocarbonate (DEPC) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Reduced glutathione (GSH) was purchased from Beijing Solarbio Science & Technology Co., Ltd., China. GSH and oxidized glutathione (GSSG) assay kit was purchased from Jiangsu Beyotime Institute of Biotechnology, China. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) assay kits were purchased from Nanjing Jiancheng

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Bioengineering Institute, China. Coomassie brilliant blue G250 was purchased from Shanghai Chemical Reagent Co., Ltd., China. TRIzol was purchased from DBI, Biosciences, USA. Reverse transcription (RT) reagent kit and fluorescence quantitative PCR kit were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd., China.

Cell lines and cell culture

Cervical carcinoma HeLa cells were kindly provided by Institute of Biochemistry and Molecular Biology, School of Medicine, Shandong University. The HeLa cells were cultured by using RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 IU/mL penicillin and 100 mg/L streptomycin. The cells were then cultured in an incubator with 5% CO_2 at 37°C. PTX resistant HeLa cells (HeLa/PTX) were established by exposure to increasing concentrations of PTX, according to previously described method with minor modifications (Zhang *et al*., 2010). Briefly, PTX was added when HeLa cells were in logarithmic growth phase. The cells were cultured for 1 hour. HeLa cells were then washed by drug free medium and were cultured in drug-free medium for passage. Concentrations of PTX were gradually increased from10 μg/L to 20 μg/L, 40 μg/L and finally to 500 μg/L. After 10 months, HeLa/PTX cell lines with good growth state were established in the medium containing 500 μg/L PTX. The cells were recovered after cryopreservation for 2 months, and were cultured for several passages in PTX-free medium. After 3 months, HeLa/PTX cells were still resistant to PTX.

Cell morphology observation

HeLa cells (5×10^4/mL) and HeLa/PTX cells (5×10^4/mL) were seeded in 6-well cell culture plates and were cultured overnight. The growth morphology of the adherent cells was then observed under an inverted microscopy (Nikon, TE 2000, Tokyo, Japan).

Cell growth curve assay

Both HeLa and HeLa/PTX cells with the initial concentration of 1×10^4/mL were seeded in 24-well cell culture plates. The cells were then cultured in the incubator. From the following day, cells from 3 wells were counted each day and this counting was repeated each day within the following 7 days. The average number of cells of 3 wells was then plotted to generate cell growth curve and the doubling time of cells at the logarithmic growth phase was calculated based on Patterson formulation (Wang *et al*., 2006).

Determination of resistant index

Resistant index was determined using the previously described method with minor modifications (Zhang *et al*., 2010). Briefly, 100 μL of HeLa cells (5×10^4/mL) and 100 μL of HeLa/PTX cells (5×10^4/mL) were respectively plated in 96-well plates. After culturing for 24 hours, the culture medium of each well was discarded and 200 μL of culture medium containing PTX was added. The final concentrations of PTX were 1 μg/L, 5 μg/L, 10 μg/L, 50 μg/L, 100 μg/L, 500 μg/L, 1000 μg/L, 5000 μg/L, and 10000 μg/L. After incubation for 72 hours, 20 μL of MTT (5 g/L) was added to each well. After incubation for 4 hours, 150 μL of DMSO was then added to each well. The optical density (OD) at wavelength of 490 nm was measured by a microplate reader (BIO-RAD Model 680, UK). The experiments were repeated five times. The inhibition rate of cell growth was calculated by the formula of drug group OD/control group OD. The median inhibitory concentration (IC50) was then calculated according to improved Koushi method. Therefore, the resistance index (RI) was calculated based on the formula of RI = IC50 of drug resistant cells/ IC50 of parental cells.

DCFH-DA

Intracellular ROS levels were measured with DCFH-DA (Hansen *et al*., 2007). HeLa and HeLa/PTX cells at lorithmic growth phase were collected by trypsin digestion and rinsed by PBS. The cells were resuspended in serum-free cell culture medium at a concentration of 1×10^6/mL. Florescent probe DCFH-DA was added to the cell suspensions to a final concentration of 5 μmol. The mixture was then cultured in an incubator in the dark for 30 min at 37°C. The culture was then rinsed by PBS buffer two times. Flow cytometry (BD FACSCalibur, USA) was applied here to measure the change in fluorescence of 2′, 7 ′- dichlorine fluorine fluorescein (DCF) (excitation wavelength of 488 nm and emission wavelength of 525 nm). Each sample was measured with 1×10^4 living cells by using CellQuest software to analyze the mean fluorescence intensity (MFI). MFI indirectly reflects the cellular levels of ROS.

5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB)

DTNB was used in this study to determine the amount of intracellular GSH and GSSG levels (Tietze, 1969). The specific procedure was performed according to the instructions provided by the GSH and GSSG assay kit. BIO-RAD Model 680 microplate reader was also used.

Measurement of SOD, CAT and GPx activities

SOD, CAT, and GPx activities were determined using the nitrite formation method (Elstner *et al*., 1976), the molybdate colorimetric method (Góth, 1991), and the DTNB colorimetric method (Hafeman *et al*., 1974), respectively, according to the manufacturer's instructions of the assay kits. The activities of the enzymes were detected by 722S spectrophotometer.

Real-time PCR

The total RNA was extracted using Trizol reagent. RNA (1 μg) was used to perform reverse transcription reaction. Primers and fluorescent probes of Txr1 (Papadaki *et al*., 2009) and GAPDH (Sui *et al*., 2011) were synthesized by

Shanghai Shinegene Molecular Biotechnology Co., Ltd., China (table 1). The 5’ end of the probe was labeled with fluorescent reporter group FAM. The 3′ end of the probe was labeled with fluorescent quenching group TAMRA. ABI 7300 (Applied Biosystems, Foster City, Californina, USA) was used for real-time PCR. ABI 7300 SDS Software was used for data analysis. Ct method was used to analyze the quantity of mRNAs.

STATISTICAL ANALYSIS

SPSS software 12.0 was used for data analysis. Measurement data were expressed as $\bar{x}$ ± s. The *t* test was used to perform comparison of two samples. $P < 0.05$ was considered as significant difference

RESULTS

Cell growth curve and doubling time of the obtained HeLa/PTX cell line

HeLa/PTX cell line was established by culture in the presence of PTX. HeLa cells grew rapidly as indicated by the observation results of phase contrast microscopy (fig. 1A). Different mitotic cells were visually recognized in the phase of logarithmic growth. And HeLa cells showed the morphology of polygonal or irregular shape with clear cell border and cell aggregation under microscope. Clear and big nucleus with round or oval shape was also observed in HeLa cells. Conversely, the shape of HeLa/PTX cells was more regular than that of HeLa cells. There were dividing cells, and more cytoplasmic granules were observed in HeLa/PTX cells.

The growth curves of HeLa/PTX and HeLa cells were shown in Figure 1B. The population doubling time of HeLa/PTX cells was (32.50 ± 2.21) h, which was 1.32 folds of that of HeLa cells (24.58 ± 1.56) h ($P < 0.01$). This result suggests that the proliferation rate of HeLa/PTX cells was lower than that of HeLa cells, with cell division peaks shifting backwards and longer population doubling time. These results suggest that HeLa/PTX cells are more tolerant to PTX than HeLa cells.

HeLa/PTX cells and HeLa cells have different resistance to PTX

The sensitivity of HeLa/PTX cells and HeLa cells to PTX

Table 1: Real time quantitative PCR primer and probe sequences

Primers and probes	Sequences
Txr1_F	5'GCAGAAGAAAATGAAGAAAGCTCATAA3'
Txr1_R	5'GGAATGCTTGCCTGCTTGT3'
GAPDH_F	5'CCACTCCTCCACCTTTGAC3'
GAPDH_R	5'ACCCTGTTGCTGTAGCCA3'
Txr1_probe	5'ATGCACAAGCACCAAAAGCACCACAAGTAC3'
GAPDH_probe	5'TTGCCCTCAACGACCACTTTGTC3'

Fig. 1

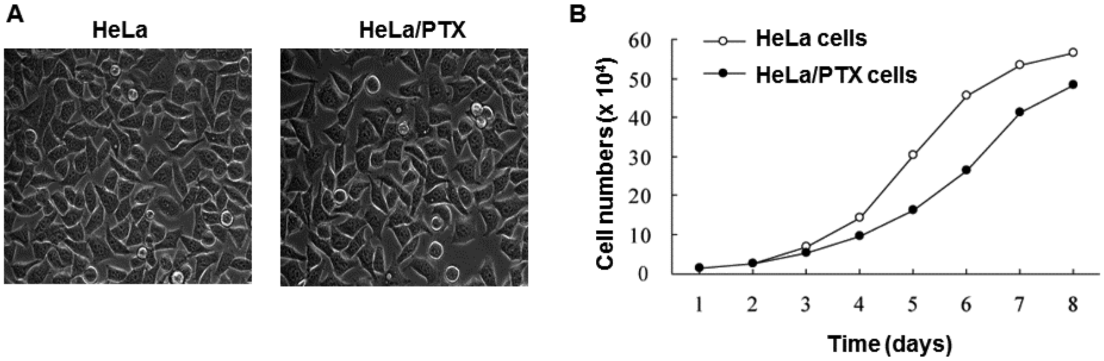

Fig. 1: HeLa and HeLa/PTX cells. Both HeLa and HeLa/PTX cells with the initial concentration of 1× 10^4/mL were seeded in 24-well cell culture plates. The cells were then cultured in the incubator. From the following day, cells from 3 wells were counted each day and this counting was repeated each day within the following 7 days. The average number of cells from 3 wells was then plotted to obtain cell growth curve. Based on the calculation of Patterson formulation, the doubling time of cells at the logarithmic growth phase was calculated. (A) Morphology observation of HeLa and HeLa/PTX cells by invert microscope (× 400). (B) Growth curves of HeLa and HeLa/PTX cells

Fig. 2

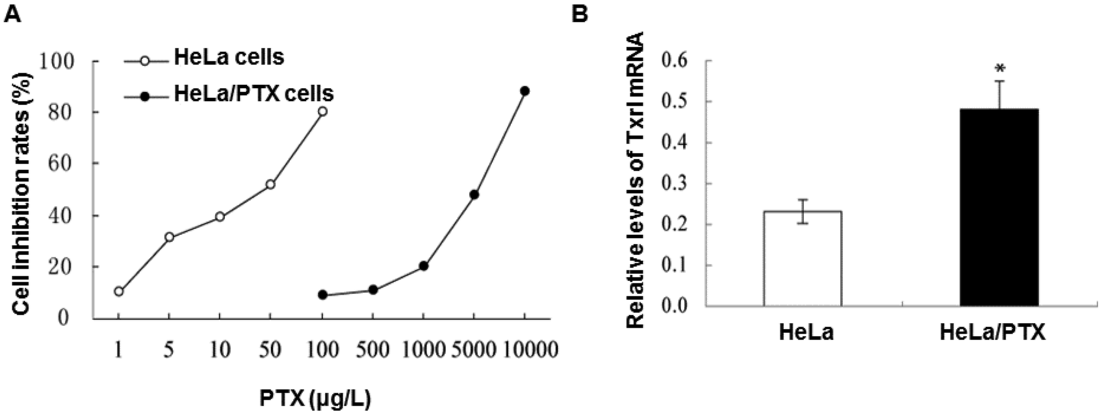

Fig. 2: Effects of PTX on HeLa and HeLa/PTX cells. Both HeLa (5×10^4/mL) and HeLa/PTX cells (5×10^4/mL) were treated with various concentrations of PTX. The experiments were repeated five times. (A) After incubation for 72 hours, 20 μ L of MTT (5 g/L) was added to each well and 150 μ L of DMSO was then added to each mixture after incubation for 4 hours. The optical density (OD) at wavelength of 490 nm was measured. The median inhibitory concentration (IC50) was then calculated according to improved Koushi method. Inhibitory effects of PTX on the growth of HeLa and HeLa/PTX cells were shown. (B) The total RNA was extracted by Trizol reagent and real-time PCR was performed to detect levels of Txrl mRNA in HeLa and HeLa/PTX cells. ABI 7300 SDS Software was used for data analysis. Ct method was used to analyze the quantity of mRNAs. Effects of PTX on levels of Txr1 mRNA in HeLa and HeLa/PTX cells were calculated ($^*P < 0.05$, n = 3).

was determined by the MTT method. As shown in Figure 2A, the IC50 of HeLa/PTX cells was (4159.15 ± 502.37) μg/L, significantly higher than that of HeLa cells, which was (33.90 ± 5.84) μg/L ($P < 0.01$). This result suggests that HeLa/PTX cells obtained the characteristic of significant resistance to PTX, with a resistance index of 122.69.

HeLa/PTX cells have a higher level of Txrl mRNA expression than HeLa cells

Txrl mRNA levels in HeLa/PTX cells and HeLa cells were also determined. As shown in fig. 2B, the expression level of Txr1 mRNA in HeLa/PTX cells was significantly higher than that in HeLa cells ($P < 0.01$). The relative expression level of Txr1 mRNA in HeLa/PTX cells was 2.07 fold of that in HeLa cells. These results suggest that the higher level of Txr1 expression may be related to the higher resistance of HeLa/PTX cells to PTX.

HeLa/PTX cells and HeLa cells have different levels of intracellular ROS, GSH and GSSG

The levels of intracellular ROS, GSH and GSSG in HeLa/PTX cells and HeLa cells were determined. The results were shown in fig. 3. As shown in fig. 3A, the ROS level in HeLa/PTX cells was (508.3 ± 69.1) MFI and in HeLa cells was (155.1 ± 27.1) MFI. Statistically, HeLa/PTX cells had significantly higher levels of ROS (about 3.28 folds) than HeLa cells ($P < 0.01$). The GSH level in HeLa cells was (4.84 ± 0.79) nmol/mg, 1.38 fold higher than that in HeLa/PTX cells ((3.52 ± 0.23) nmol/mg) ($P < 0.05$) (fig. 3B). However, the GSSG level and ratio of GSH/GSSG in HeLa/TPX cells were not significantly different from those in HeLa cells ($P > 0.05$) (figs. 3C and 3D).

HeLa/PTX cells and HeLa cells have different activities of SOD and GPx

Intracellular activities of SOD, CAT and GPx in HeLa/PTX cells and HeLa cells were also determined. As shown in Figure 4, the SOD activity in HeLa/PTX cells was (62.11 ± 3.26) U/mg and in HeLa cells was (126.8 ± 11.39) U/mg. Meanwhile, the GPx activity in HeLa/PTX cells was (31.42 ± 2.22) U/mg and in HeLa cells was (47.09 ± 0.76) U/mg. Thus, the activities of SOD and GPx in HeLa/PTX cells were 49% and 67% of those in HeLa cells, respectively. Statistically, HeLa/PTX cells had significantly lower activities of SOD ($P < 0.01$) and GPx ($P < 0.05$) than HeLa cells (Figure 4A and 4B). However, the activity of CAT in HeLa/PTX cells was not significantly different from that in HeLa cells (fig. 4C).

DISCUSSION

Chemotherapy plays an important role in treating cervical cancer. PTX has become one of the preferred chemotherapeutic drugs for the treatment of cervical cancer (Liu and Mi, 2009; Schwab *et al*., 2013). However, drug resistance is a key factor influencing the efficacy of PTX. It is important to improve efficacy of PTX and to elucidate mechanisms of PTX resistance in cancers (Hasegawa *et al*., 2013; Murakami *et al*., 2013). Studies show that drug resistance of cancer cells is closely related

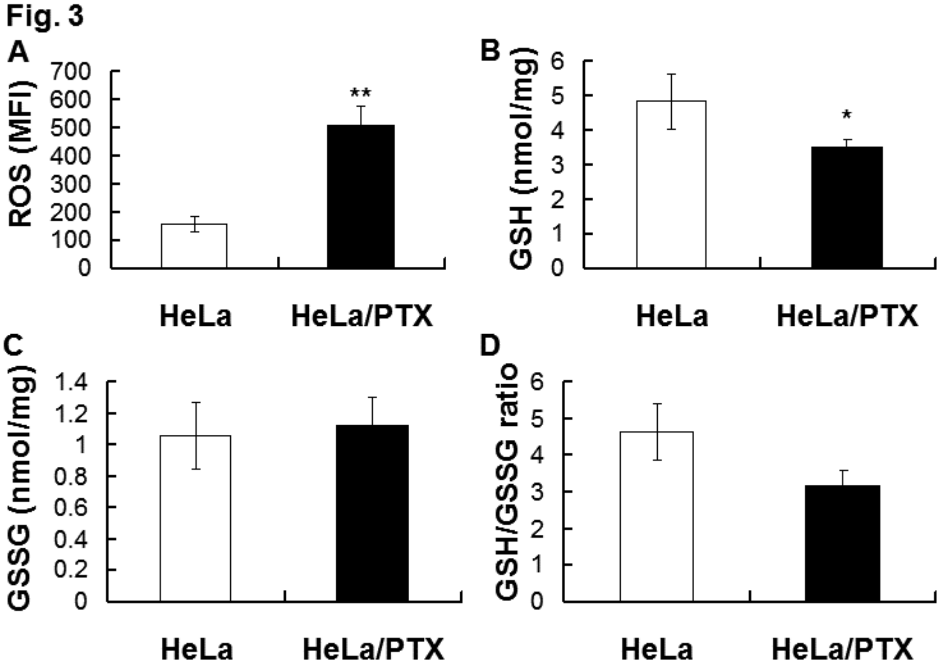

Fig. 3: Intracellular ROS, GSH, and GSSG levels and GSH/GSSG ratio. Levels of ROS, GSH and GSSG in HeLa cells and HeLa/PTX cells were measured. Ratio of GSH/GSSG was analyzed. (A) Intracellular ROS levels measured with DCFH-DA were shown. (B) Intracellular GSH levels measured with DTNB were shown. (C) Intracellular GSSG levels measured with DTNB were shown. (D) Ratios of GSH/GSSG were shown. Data represented $\bar{x}$ ± s of three independent experiments. Compared with HeLa cells, $^{*}P < 0.05$, $^{**}P < 0.01$.

with changes in oxidative stress system and anti-oxidative system of cancer cells. For example, human biliverdin reductase (hBVR) significantly contributes to the modulation of hypoxia-induced chemoresistance of glioblastoma cells by adjusting their cellular redox status (Kim *et al*., 2013). RNH1, which encodes a ribonuclease inhibitor and is highly expressed in HDACi-resistant cell lines, is both necessary and sufficient to induce HDACi resistance. And RNH1 may mediate this resistance through the dampening of HDACi-induced ROS in cancer cells (Zhu *et al*., 2013). Therefore, in this study, the cervical cancer HeLa/PTX cell line resistant to PTX was established and its characteristics of redox were comprehensively analyzed. Our results showed that HeLa/PTX cells and HeLa cells were different in redox status. HeLa/PTX cells had a significantly higher level of ROS compared with HeLa cells, but the level of GSH and activities of SOD and GPx were significantly lower. Pathogenesis of cervical cancer may be associated with changes in oxidative stress which plays an important role in carcinogenesis (Beevi *et al.,* 2007; De Marco *et al.,* 2012). Studies reveal increased lipid peroxidation in patients with cervical carcinoma (Manju *et al*., 2002; Sharma *et al*., 2007). An increase in ROS generation is also observed during cervical cancer development (Warowicka *et al*., 2013). A few studies report the alteration of antioxidant system in cervical cancer tissue (Kolanjiappan *et al*., 2002; Maldonado PA *et al*., 2006). Manoharan *et al*. demonstrated the lowered concentration of GSH and decreased activity of CAT in erythrocytes of cervical cancer patients (Manoharan *et al*., 2002). They also reported decreased activities of antioxidant enzymes (SOD, CAT, and GPx) in the erythrocytes of cervical cancer patients (Manoharan *et al*., 2004). In addition, low levels of GSH and GPx and decreased activity of SOD were observed in the circulation of cervical cancer patients (Manju *et al*., 2002). Thus the changes in levels of serum antioxidants may be responsible for the pathogenesis of cervical cancer (Kim *et al*., 2003). In this study, compared to HeLa cells, the ROS level increased in HeLa/PTX cells whereas the GSH level decreased in HeLa/PTX cells. This result suggests that the redox system, including the oxidative stress system and the anti-oxidative system, was imbalanced in HeLa/PTX cells. Some researches show that the increase of the ROS level is closely related to drug resistance of cervical cancers (Yoon *et al.,* 2004; Cheng *et al.,* 2012). ROS is likely a primary signal in the acquisition of the multi-drug resistance (MDR) phenotype and therefore a potential target when designing drugs for chemoresistance (Tsai *et al*., 2007). The accumulation of ROS during ovarian cancer progression may cause the degradation of MKP3, which in turn leads to aberrant ERK1/2 activation and contributes to tumorigenicity and chemoresistance of

Fig. 4

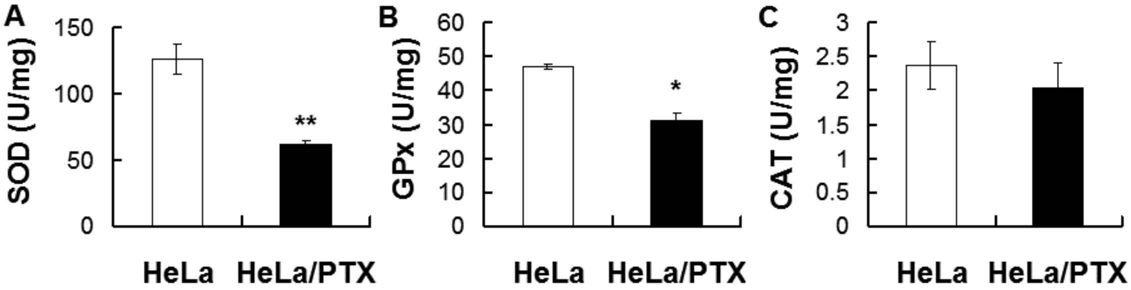

Fig. 4: Intracellular activities of SOD, GPx, and CAT. Activities of SOD, GPx and CAT in HeLa cells and HeLa/PTX cells were detected. (A) Intracellular activities of SOD determined by the nitrite formation method were shown. (B) Intracellular activities of GPx determined by the DTNB colorimetric method were shown. (C) Intracellular activities of CAT determined by the molybdate colorimetric method were shown. Experiments were performed three times and data were expressed as $\bar{x}$ ± s. Compared with HeLa cells, $^{*}P < 0.05$, $^{**}P < 0.01$.

human ovarian cancer cells (Chan *et al*., 2008). Therefore, the resistance of HeLa/PTX cells to PTX may be caused by an increased level of ROS, which causes a more severe imbalance in oxidation/reduction status in cancer cells.

GSH is an antioxidant that chemically detoxifies hydrogen peroxide and protect important cellular components from damages caused by ROS and free radicals (Pompella *et al*., 2003). GPx catalyzes GSH into its oxidized form, GSSG. The decrease in GPx activity causes oxidative stress that induces the depletion of red blood cell GSH in cervical cancer patients (Kim *et al*., 2003; Manoharan *et al*., 2004). The role of SOD is to provide an important antioxidative defense against the potentially damaging activities of the superoxide radical (Khan *et al*., 2010). The decrease of SOD activity leads to the increase of superoxide generation (Naidu *et al*., 2007). A remarkable reduction in the activity of SOD was observed in neoplastic cervical tissue (Balasubramaniyan *et al*., 1994) and venous blood of cervical cancer patients (Srivastava *et al*., 2009). The possible reason for the decrease in SOD activity might be associated with free radical generation, which causes damage to the enzyme by cross linking or damaging the nuclear DNA (Naidu *et al*., 2007). Consequently, the high level of ROS in HeLa/PTX may be caused by the lower level of GSH and the decreased activities of SOD and GPx.

This study also analyzes the expression of Txr1 gene in both HeLa/PTX and HeLa cells. It was found that the level of Txr1 mRNA in HeLa/PTX cells was significantly higher than that in HeLa cells, suggesting that the expression of the drug resistant gene in HeLa/PTX cells was increased. Txr1 is a new drug resistant gene reported by Lih *et al* (Lih *et al.,* 2006). It can regulate the secretion of thrombospondin, resulting in PTX resistance of human prostate cancer cells. Researches in lung cancer cells (Papadaki *et al.,* 2009), breast cancer cells (Bai *et al.,* 2012) and gastric cancer cells (Bi *et al*., 2014) show that the up-regulation of Txr1 could induce drug resistance of cancer cells. Therefore, the tolerance of HeLa/PTX cells to PTX might be related to the up-regulation of Txr1.

This study established the cervical cancer cell line HeLa/PTX that was resistant to PTX. We found that drug resistance of HeLa/PTX cells to PTX may be related with changes in oxidation/reduction system and increased expression levels of drug resistant gene Txr1. Our results provide experimental evidence for the drug resistance mechanism of cervical cancer cells. And our study also indicates that HeLa/PTX cell line may be a useful model for studying the drug resistance mechanism of cervical cancer cells.

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