Study on the sensitivity of primary tumor cells of patients with cervical cancer to chemotherapeutic combinations

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Summary

Purpose: To study the sensitivity of primary tumor cells from patients with cervical cancer to frequently used chemotherapeutic combinations.

Methods: 54 samples from cervical cancer patients were collected and used to develop cell culture in vitro. Eight frequently used chemotherapeutic combinations (taxol, taxol+cisplatin, taxol+carboplatin, topotecan+cisplatin, gemcitabine+cisplatin, docetaxel, gemcitabine, cisplatin+cyclophosphamide+doxorubicin) were prepared in different concentrations [200,100,50,25, and 12.5% plasma peak concentration (PPC)] and added to tumor cell culture. Adenosine triphosphate (ATP) bioluminescence assay was used to quantify cell cytotoxicity.

Results: Sensitivity of primary tumor cell from patients with cervical cancer to 8 chemotherapeutic combinations were: gemcitabine < docetaxel < topotecan+cisplatin < cisplatin+cyclophosphamide+doxorubicin < gemcitabine+cisplatin < taxol < taxol+carboplatin < taxol+cisplatin.

Conclusion: Using the drug sensitivity test to assess chemotherapeutics in the treatment of cervical cancer patients offers many benefits, being a first step to a clinically individualized chemotherapy.

Key words: cervical cancer, chemotherapeutics, culture in vitro, sensitivity

Introduction

According to latest statistics in cancer incidence, cervical cancer is the sixth most common cancer in Europe and the fourth worldwide in females, and the seventh most common cancer overall [1]. Morbidity of cervical cancer ranks first among gynecologic malignant tumors [2], showing a big difference between different regions of the world, with an increased mortality depending on economy development, culture, medical treatment and public health systems [3,4]. The etiology of cervical cancer is multifactorial, incriminated being viruses such as human papillomaviruses (HPVs), herpes simplex virus type 2, cigarette smoking, vaginal douching, use of oral contraceptives and nutrition that could produce a series of gene mutation that lead to carcinogenesis [5,6].

The sensitivity of tumors with the same pathological pattern to the same chemotherapeutics varies because the biological characteristics of each patient are different, such as immunity, metabolic profile and tumor histology, resulting in different tumor infiltration capacity and sensitivity to chemotherapeutics [7-9]. Also, there are differences in terms of clinical manifestation, pathological pattern and molecular biological characteristics of cervical cancer [10], with varying clinical therapeutic effect.

Researches related to drug sensitivity genetics show that individual reaction to drugs is different and this is generally correlated with enzymatic metabolism [11-13]. A drug may have a good therapeutic effect for one genotype while may

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have poor therapeutic effect for another genotype, even a toxic side effect. Several authors also show that drug resistance of tumor cell to chemotherapeutics such as cisplatin, mitomycin and adriamycin is increased when the lung resistance protein (LRP) gene expression of cervical cancer is increased [14-16]. Upregulated gene expression of Twist causes drug resistance of tumor cell to taxol and vincristin. ATP fluorescence method [17], a drug monitoring technology, has been frequently used in testing chemotherapeutic drug sensitivity, showing high sensitivity and fast detection.

The aim of this study was to investigate the inhibition rate of 8 chemotherapeutic combinations in cervical cancer cells and to correlate with histological type, offering clinical support for personalized therapies and improving the therapeutic effect.

Methods

General material

Fresh tumor tissue samples from 54 patients with cervical cancer were collected from May 2015 to August 2016 in the Department of Obstetrics and Gynecology, West China Second University Hospital, Sichuan University. The patient average age was 42.6±12.5 years. All patients were diagnosed with primary cervical malignant tumor and they hadn't been treated with radiotherapy and chemotherapy before operation. Postoperative pathology indicated that there were 38 cases of squamous cell carcinoma, 14 cases of adenocarcinoma and 2 cases of cervical alveolar rhabdomyosarcoma. Before sample collection, informed consent was signed by patients and their relatives. The Ethics Committee of the hospital discussed and unanimously decided that this research could be conducted in West China Second University Hospital, Sichuan University.

Main equipments and reagents

ATP-chemiluminescence method (ATP-TCA kit, Beijing Jinzijing Biology Medicine Technology Co., Ltd.); ATP extraction solvent (TCE, Beijing Jinzijing Biology Medicine Technology Co., Ltd.); ATP standard liquid (Beijing Jinzijing Biology Medicine Technology Co., Ltd.); Sterility inactivating fetal bovine serum (FBS, Shanghai ShuangRu Biotech Co., Ltd.); RPMI1640 culture medium (Shanghai YuanyeBio-technology Co., Ltd); Complete culture solution (CAM, Shanghai Guangrui Bio-technology Co., Ltd.); Trypan blue (HeifeiBomei Biotechnology Co., Ltd.); 96-well cell culture plate (Shanghai DianRui Instruments Co., Ltd.); Cell counting plate (Shanghai DaPing Instrument Co., Ltd.); Attenuation buffer solution (DB, Shanghai Zhaorui Bio-Tech Co., Ltd.); Luciferin-Luciferase (LU-LU, Nantong Feiyu Biotechnology Co., Ltd.); Benchtop (Guangzhou Saifusi Experimental Facilities Technology Co., Ltd.); Orion II micropore plate chemiluminiscence (Titertek Berthold, Germany); Small high-speed centrifuge (Beijing Bohui Biotechnology Co., Ltd.); -20°C cryogenic refrigerator (Shenzhen Saiyataike Instrument Co., Ltd.); Fluorescence microscope (Shanghai Cewei Photoelectric Technology Co., Ltd.); Micromixer (Changzhou Wanhe Instrument Co., Ltd.); Digital thermostatic water bath (Jinan Laibao Medical Instrument Co., Ltd.).

Drugs

Taxol (TAX), cisplatin (DDP), carboplatin (CBP), topotecan, gemcitabine, docetaxel, cyclophosphamide (CTX), and doxorubicin (ADM) were purchased from Shanghai Puzhen Biotechnology Co., Ltd.

Main solution preparation

Digestive enzyme solution for tumor tissue preparation: 12 ml of complete culture solution was added, fully mixed and dissolved. After filtrate sterilization, it was transferred to a 15 ml sterile centrifuge tube.

Sample soak solution preparation method: the final concentration of penicillin was 100 U/ml and the final concentration of streptomycin was 100 U/ml. Dissolution in RPMI1640 culture medium was performed afterwards.

Luciferin-Luciferase (LU-LU reagent) preparation: 16ml DB buffer solution was used to dissolve LU-LU, and was stored in the dark at room temperature.

Chemotherapeutics experimentation

Chemotherapeutics preparation was performed under aseptic condition. Test concentrations of the chosen chemotherapeutics are shown in Table 1.

Five test drug concentrations (TDC) were used in the experiment, including 200, 100, 50, 25 and 12.5% PPC (plasma peak concentration). Two lines of control-well were set for each TDC and the redundant wells on culture plate were used as no-treatment control (NTC) (cells without chemotherapeutics) and blank well (without cells) for comparison testing. The specific distribution is shown in Table 2.

ATP-TCA detection steps: fresh tumor tissue samples excised from patients with cervical cancer were collected. Blood and necrotic tissue were removed from the tissue samples. Then, tissue samples were immersed in soak solution with antibiotics for 15-20 min and after that were cut into pieces (around 1 mm³) randomly and

Table 1. Test concentrations of 8 chemotherapeutics

Drugs	TAX	DDP	CBP	Topotecan	GEM	Docetaxel	CTX	ADM
100% PPC (μg/ml)	13.8	6.3	25	0.75	25	10	3	3

TAX: Taxol, DDP: Cisplatin, CBP: Carboplatin, GEM: Gemcitabine, CTX: Cyclophosphamide, ADM: Doxorubicin, PPC: plasma peak concentration

Table 2. Micropore design scheme of 8 chemotherapeutic combinations. Gray levels represent a chemotherapeutic combination. 100 shows that, after preparation, 100µl of each drug were added into each well

	1	2	3	4	5	6	7	8	9	10	11	12
	200%	100%	50%	25%	12.5%	200%	100%	50%	25%	12.5%		
A	100	100	100	100	100	100	100	100	100	100	NTC	Blank
B	100	100	100	100	100	100	100	100	100	100	NTC	Blank
C	100	100	100	100	100	100	100	100	100	100	NTC	Blank
D	100	100	100	100	100	100	100	100	100	100	NTC	Blank
E	100	100	100	100	100	100	100	100	100	100	NTC	Blank
F	100	100	100	100	100	100	100	100	100	100	NTC	Blank
G	100	100	100	100	100	100	100	100	100	100	NTC	Blank
H	100	100	100	100	100	100	100	100	100	100	NTC	Blank

NTC: no-treatment control

centrifuged. After centrifuge, tissue digestive enzyme was added and incubated for 2 hrs at 37°C. The samples were fully digested and then centrifuged at 1500 rpm for 5 to 8 min. The supernatant was removed and the undecomposed tumor tissue was filtered using a cell strainer of 200 screen mesh. The collected cell suspension was dissolved in CAM to a final concentration of cells of $(1-3) \times 10^5$/ml. The cell solution was added to a 96-well cell culture plate and cultured in 5% CO_2 incubator for 4 hrs at 37°C. After culture, chemotherapeutics were added to the plate, each chemotherapeutics in 5 concentrations (200, 100, 50.0, 25.0 and 12.5% PPC). Besides, blank well (without cell) and control well (with tumor cell but without chemotherapeutics for blank control group) were set. Then, it was cultured for 5-7 days in 5% CO_2 incubator with 95% humidity and 37°C. After the cultures developed, ATP extracting solution was added, mixed and keep for 15 min at room temperature. Then, 0.05 ml of mixed solution was taken out and detected by micro-plate fluoroanalyzer. The inhibition rate of each chemotherapeutic was calculated according to the equation below:

Inhibition rate = (1−ATP amount of test well/ATP amount of control well) ×100%.

Inhibition rate ≥70% was considered as high sensitivity, inhibition rate of 50-70% was as considered susceptible, inhibition rate of 30-50% was considered as low sensitivity and inhibition rate lower than 30% was considered as drug resistance. When inhibition rate ≥50%, it was recommended for clinical application.

ATP standard curve drawing: 90 µl purified water were added to 1st, 2nd and 3rd lines (except row H) of 96-well detection plate. Ten µl ATP standard liquid were added to three wells in row A, respectively. After mixing it, 10 µl were taken from of row A to add into row B, and so on. Thus, rows A-F were composed of six concentration gradients made by double dilution. Finally, 10 µl were removed from row F. Thus, concentration of ATP standard liquid of row A-G were 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11} and blank. The detection plate with sample was put into detector. Afterwards, 40 µl of LU-LU reagent were added by automatic sample injector into the wells of 1st, 2nd and 3rd lines and detected immediately.

Statistics

SPSS19.0 software was used to analyze data. The measured data was expressed as mean±standard deviation. Samples' average comparison was estimated by t-test and the test criterion was α=0.05. p<0.05 indicated statistically significant difference.

Results

ATP standard curve drawing

Luminescence mean values of different concentrations were identified. LG log Kow for diluting concentrations was set as independent variable, while LG log Kow for determining mean values of results was set as dependent variable. Thus, standard curve was drawn to identify the degree of linearity. When ATP concentration was 10^{-11}-10^{-6}mol/ml, it were in good rectilinear correlation and its coefficient of association was r≥0.975 and p<0.01.

Results of ATP bioluminescence method

Tumor primary cells showed attachment growth, normal cellular morphology, numerous cell numbers, clear cell configuration and clear cell nucleus (Figure 1A). Figure 1B-D shows that there was an obvious difference in cytotoxicity of the experimental group in different micropores. Figure 1 (B) shows that the cell number was highly reduced, cellular morphology was shrank and floating, while cell boundary was fuzzy. Figure 1 (C) shows that tumor cells were hardly seen and their boundary was fuzzy. However, in Figure 1 (D), no tumor cell was seen.

The interpretation of the results of the 54 cases showed that the average inhibition rates of different drugs to squamous and adenocarcinoma were different, being correlated with the histological type (Table 3).

Test results showed that under plasma peak concentration, the inhibition rate of chemotherapeutics to squamous carcinoma of the cervix were TAX+DDP > TAX+CBP > TAX > GEM+DDP > Topotecan+DDP > DDP+CTX+ADM > Docetaxel > GEM, while under plasma peak concentration, the inhibition rates of adenocarcinoma of the cervix were TAX+CBP > GEM+DDP > TAX+DDP >
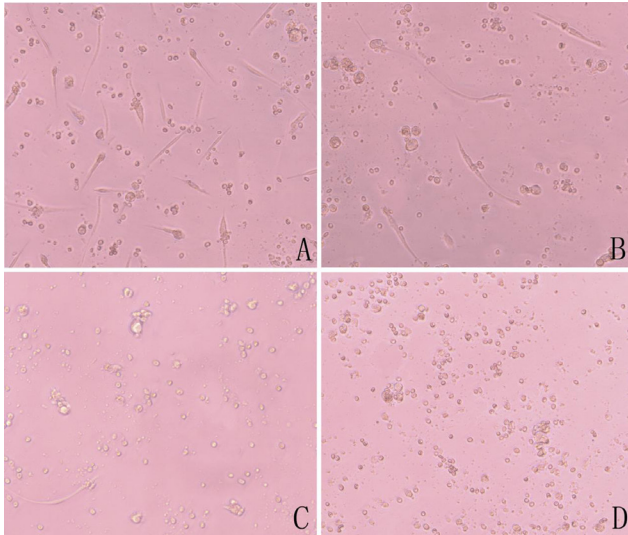

Figure 1. Results of ATP bioluminescence TEST **A:** Blank control group; **B:** GEM, 50% PPC; **C:** TAX, 100% PPC; **D:** TAX+DDP, 200% PPC.

Table 3. Average inhibition rate of drugs to squamous carcinoma and adenocarcinoma of the cervix under plasma peak concentration (%)

Drugs	Squamous carcinoma	Adenocarcinoma
TAX	89.68±9.41	87.97±14.66
TC	93.92±7.83	97.33±1.82
TP	98.11±1.65	92.64±12.66
Docetaxel	65.19±26.92	65.61±28.88
GEM+DDP	75.33±20.86	96.65±19.93
DDP+CTX+ADM	70.59±26.71	89.69±18.35
GEM	46.65±25.76	39.68±31.81
Topotecan+DDP	71.59±24.47	70.16±41.35

p=0.3421

DDP+CTX+ADM > TAX > Topotecan+DDP > Docetaxel > GEM. However, difference of drug sensitivity regarding cervical cancer in different histopathological types had no statistical significance (p>0.05).

Inhibitory effect of drugs to cervical cancer cell in vitro

The mean inhibition rate of drug combinations in different concentrations to tumor cell *in vitro* are shown in Table 4.

It was shown that for the 8 chemotherapeutics, sensitivity and drug concentration were in positive correlation. The inhibition rate of chemotherapeutics to tumor cells was increasing with increasing concentration. Tumor cell growth inhibition effect under different drug combinations in 5 drug concentrations were compared between two groups. The statistical analysis of results by One-Way ANOVA showed a significant p value (p<0.01). Thus, it can be concluded that there was significant difference (p<0.05) in the inhibition effect of chemotherapeutics to individual cervical cancer cells when analyzing the whole tumor inhibition rate. Tumor growth inhibition rate under plasma peak concentration was TAX+DDP > TAX+CBP > TAX > Gemcitabine+DDP > DDP+CTX+ADM > Topotecan+DDP > Docetaxel > Gemcitabine.

IC_{50} (Plasma peak concentration needed for inhibiting the growth of 50% of tumor cell) and IC_{90} (Plasma peak concentration needed for inhibiting the growth of 90% of tumor cells) were assessed according to the percentage of inhibited tumor growth of different drug concentrations. Low IC_{50} and IC_{90} meant tumor cell growth was effectively inhibited by the drug. The results are shown in Figure 2.

The effects of chemotherapeutics to tumor cells were classified into the following 4 grades: when drug concentration was low, tumor cells were susceptible to chemotherapy regimens of platinum combined with TAX. IC_{50} of tumor cell growth was TAX+DDP 18.23% PPC and TAX+CBP

Table 4. Mean tumor growth inhibition ratio under 8 drug combinations in different concentrations

Drug concentrations	200% PPC	100% PPC	50% PPC	25% PPC	12.5% PPC
TAX	96.86±4.67	89.18±10.64	71.40±20.53	54.37±26.54	42.08±26.13
TC	97.83±3.86	94.50±7.28	79.03±16.47	59.12±24.61	46.25±26.25
TP	99.18±0.92	96.68±6.51	86.11±15.72	68.29±23.65	48.87±26.75
Docetaxel	81.89±21.54	65.37±26.49	49.68±27.64	40.22±27.08	34.31±25.42
GEM+DDP	81.49±18.78	77.91±20.57	69.56±22.15	58.11±24.12	44.59±24.22
DDP+CTX+ADM	80.59±20.23	75.18±26.24	66.65±27.65	48.42±27.13	32.13±25.43
GEM	50.61±25.46	44.49±26.37	38.21±26.43	31.43±26.55	26.08±24.51
Topotecan+DDP	76.05±24.33	71.28±25.52	59.65±29.82	45.47±33.73	36.36±35.19

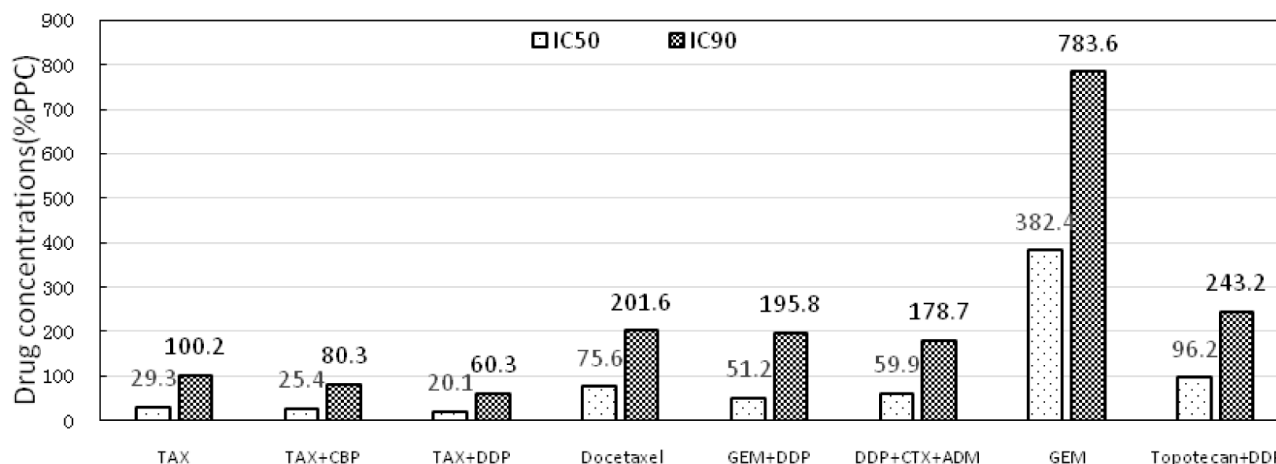For abbreviations see text. One way ANOVA, p<0.01

Figure 2. Drug concentrations under IC_{50} and IC_{90}.

Table 5. Sensitivity of different chemotherapeutic combinations

Sensitivity	TAX+TTP	TAX	TAX+CBP	Docetaxel	GEM+DDP	DDP+CTX+ADM	Topotecan+DDP	GEM
Sensitive	+		+					
Moderately sensitive		+						
Mildly sensitive					+			
Drug resistant				+		+	+	+

For abbreviations see text

21.78% PPC. IC_{90} of tumor cell growth was TAX+DDP 53.88% PPC and TAX+CBP 82.62% PPC. Tumor cells were moderately susceptible to single-drug TAX and the IC_{90} and IC_{50} were 96.87% PPC and 25.69% PPC, respectively. Tumor cells were mildly susceptible to GEM+DDP and its IC_{90} and IC_{50} were 192.82% PPC and 44.54% PPC, respectively. Tumor cells were resistant to Docetaxel, DDP+CTX+ADM, Topotecan+DDP and GEM and IC_{90} was 194.06% PPC, 167.23% PPC, 225.97% PPC and 783.86% PPC respectively, while the IC_{50} was 66.12% PPC, 53.46% PPC, 88.36% PPC and 391.65% PPC. Sensitivity of different chemotherapeutic combination are shown in Table 5.

Discussion

The selection of chemotherapy for cervical cancer should be individualized as there are individual differences between patients because of tumor heterogeneity [18] which is hard to identify. Chemotherapy regimens nowadays are chosen according to experience and are not specific for cervical cancer cell, being similar to colorectal cancer chemotherapy [19]. Thus, the therapeutic effect of chemotherapy varies and sometimes severe adverse reactions may develop, such as immunosuppression with severe infections with germs resistant to antibiotics [20] or fungal infections especially of the genuses Fusarium or Aspergillum [21].

All those 8 chemotherapeutic combinations are primary or secondary regimens and the inhibition shows that tumor cell growth is significantly inhibited by the chemotherapeutic combination. Moreover, the total evaluable rate of the selected fresh tumor tissue was around 100%. Drug sensitivity test showed that when drug concentration increased, the sensitivity increased in a dose-dependent manner. Tumor cells of some patients with cervical cancer are particularly susceptible to some drugs and they are highly susceptible with IC_{50} PPC. However, in some patients, cervical cancer cells show drug resistance and they are only susceptible to drugs when drug concentration reaches 200% PPC or even higher. Thus, the clinical therapeutic effect is improved when drug concentration is increased, providing a good clinical guidance for treating patients needing high-dose chemotherapeutics. Besides, the research results showed that tumor cells are relatively susceptible to the combination of platinum+TAX. TAX is a cycle-specific agent [22,23], which binds specifically to microtubules. Thus, microtubulin assembly is stable and depolymerization function is antagonized, cell cycle being blocked in G_2/M phase and leading to death of cancer cells [24,25]. However, DDP is a cycle nonspecific drug functioning as bispecific alkylating agent, which binds to guanine on the DNA strands [26], forms crosslinks and impair transcription and mitosis [27]. Both CBP and DDP are platinum-derivative

drugs but they have different cytotoxicity and side effect profile [28,29]. DDP dose can't be further increased and the main dose-limiting toxicity is renal [30,31]. Researchers have shown that the inhibition rate of TAX+DDP is higher than that of TAX+CBP in drug sensitive test *in vitro*. In conclusion, the priority selection for patients tolerating high-dose chemotherapeutic concentration is TAX+DDP and the second choice is TAX+CBP.

Drug sensitivity of tumor cells in these 54 cases of patients to different chemotherapeutic combinations is not completely the same, meaning that there are individual differences among patients with the same type of tumor. Topotecan+DDP is the first choice for treating cervical cancer and topotecan can inhibit topoisomerase I and prevent DNA copy of cancer cells [32-34]. The results showed that tumor cytotoxicity of Topotecan+DDP combination elicits drug resistance, while its IC_{90} and IC_{50} are 167.23% PPC and 53.46% PPC, respectively. Statistical analysis of primary data showed that tumor control rate of 14 cases under PPC was no less than 90%. It had even high sensitivity in low concentration and there was no significant dose-dependent effect. Though this chemotherapeutic combination is generally drug resistant, it is of great significance for choosing chemotherapy regimens for susceptible patients. MTS method is adopted to detect endometrial cancer and the sensitivity of patients with cervical cancer to chemotherapeutic drugs [35]. Results of drug sensitivity test and clinical effects proved that they are relevant. Similar techniques could be used to detect sensitivity in other aggressive cancers, such as anaplastic thyroid cancer [36], prostate cancer [37] or renal cancer [38].

In conclusion, the response to treatment could be improved using drug sensitivity test *in vitro* to test chemotherapeutics sensitivity of tumor cells for patients with cervical cancer before choosing a clinical chemotherapy regimen. This test could improve the clinical therapeutic effect, avoid adverse reactions and prevent multidrug resistance. However, this research needs further improvement because the therapeutic effect of anticancer drugs is regulated and restricted by factors *in vivo* and cell growing environment *in vitro* is not the same with that of *in vivo*. Thus, sensitivity testing directly evaluates the interaction between cell and drugs, on which the tumor heterogeneity is not fully expressed. To improve these results, further anticancer drug sensitivity tests should be conducted *in vivo*.

Conflict of interests

The authors declare no confLict of interests.

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