DHFR silencing effects on biological behaviour of ovarian carcinoma cells in vitro

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Abstract

Background: Dihydrofolate reductase (DHFR) is involved in the DNA synthesis and is expressed highly in platinum resistant ovarian carcinoma tissue . The relationship between DHFR gene and platinum drug resistance in ovarian carcinoma (OC) is still unclear.

Methods: To design targeting hairpin siRNA of DHFR gene, and constructed lentiviral vector carrying DHFR gene, then through flow cytometry-high performance liquid chromatography and electron microscope, DHFR gene expression was down-regulating to investigate the biological behaviour of DHFR in OC.

Results: DHFR mRNA expression in ovarian carcinoma was higher than normal ovarian tissues, in patients with omentum metastasis was lower than that in patients without omentum metastasis, in patients with CR for treatment was lower than that of patients with SD or PD or PR, cisplatin-sensitive patients was lower than that in cisplatin-multidrug resistant cases. The median survival period of patients with DHFR mRNA expression lower than 0.331(Youden index) was 16.4 months, but of that higher than 0.331 was 44.5 months. COX multivariate analysis showed that DHFR mRNA expression was a dependent prognostic factor. The apoptosis rate of DHFR-pGCSIL-SKOV3 was obvious higher than pGCSIL-SKOV3 and SKOV3 cells at 24h and 48h($P \leq 0.05$). G0/G1 stage rate of three groups at 24h-48h and 72h were decreasing but the G2/M and S stage rates were raising. The cells in the cisplatin concentration (2.5-5.0 μ g/mL)at 24h and 48h,the intracellular cisplatin content of DHFR-pGCSIL-SKOV3 cell were significantly higher than pGCSIL-SKOV3 and SKOV3 cells. The microfilament increased and gathered together, mitochondrial structure also change obviously under no drug. But there were rarely microfilament in three group cells induced by IC50 cisplatin concentration(4.4-5.5-4.9 μ g/ml)at 24 and 48 h, while at 72 h, there are obvious increase and inordinate microfilament.

Conclusion: DHFR silencing inhibits cell growth and cisplatin resistance, there were certain contact between resistance increases with microfilament gathered and the change of the mitochondria.The results laid the foundation for us to investigate the molecular mechanisms of multidrug-resistance in tumor.

Background

Ovarian carcinoma(OC) is the highest fatality rate of malignant tumor in the female reproductive system[1], Platinum resistance in the early stages of chemotherapy greatly reduces the chemotherapy sensitivity of ovarian carcinoma patients, leads to recurrence and poor prognosis[2].Drug resistance remains a major challenge in the treatment of ovarian carcinoma[3].Therefore, to research what is the chemotherapy multi-drug resistant mechanism in epithelial ovarian carcinoma and how to improve the sensitivity of chemotherapy has a great significance to treat ovarian carcinoma effectively and improve its survival rate .Our group previously using the method of fluorescence labeling differential display PCR (FDD-PCR) screening and validating the differences of platinum resistance and sensitive ovarian

epithelial carcinoma cells also found that DHFR gene expression exists differences between two group ovarian epithelial carcinoma cells. The DHFR expression was significantly raised after platinum sensitive ovarian epithelial carcinoma cells in vitro was induced into drug-resistant cell [4]. Dihydrofolate reductase (DHFR) is critical gene in the metabolism of synthetic folic acid to tetrahydrofolate following absorption, catalyzing dihydrofolate back into four hydrogen folic acid. Four hydrogen folic acid is one carbon unit transfer, it is necessary for the synthesis of DNA biosynthesis precursor substance thymine DNA nucleoside to provide one carbon unit, participating in the human body important metabolism of life [5]. Recently, pemetrexed is used in clinical whose drug targets is inhibition of DHFR metabolic enzymes to play a role of anti-tumor [6]. Reports that DHFR gene were higher expressed in leukemia cell lines/bone sarcoma with drug-resistant cell line and drug-resistant breast carcinoma cell lines, which plays a certain role in multi-resistant drug [7–9]. During the development of antitumor drugs, DHFR who were related to folic acid metabolism is an important drug targets, may be an important direction in anti-tumor drugs research. However, so far, no studies has reported about the relationship between DHFR gene and ovarian carcinoma ,especially related to platinum resistance.

Methods

Clinical samples and follow-up

Tumor samples were obtained from Guangxi Medical University carcinoma Hospital, and all of them were identified by pathology. The study was endorsed by the Ethics Committee of the Guangxi Medical University. All patients received an explanation for aims of this study and a signed informed consent. They all understood that they could withdraw from the study at any time without influencing their oncological or general medical treatment. 80 cases of ovarian malignant tumor were collected from different ages of people, their ages ranged from 13 to 76 years old, and the average age was 41.1 years old. In accordance with ovarian histology classification made by World Health Organization (WHO, 1973), the 80 samples we collected including 24 cases of mucinous carcinoma, 41 cases of serous carcinoma, 2 cases of endometrial sample carcinoma, 3 cases of embryo sinus tumor within, 1 case of asexual cell tumor, 2 cases of granulosa cell tumor, 5 cases of immature teratoma and 2 cases of other metastases tumor; and according to the clinical stage FIGO (2004) standard, the samples were classified as follows, I ~ II stage: 30 cases, III ~ IV stage: 50 cases. All of patients were accepted tumor destruction reduction operation. 67 cases of epithelial neoplasm and 13 cases of non-breast epithelial neoplasm were accepted treatment of cisplatin plus paclitaxel and treatment pf cisplatin plus bleomycin together with vincristine chemotherapy, respectively. 50 cases of benign ovarian tumor were collected from different ages of people, the average age was 40.1 years old(range: 10–74 years). The 50 samples included 11 cases of mucinous tumor, 36 cases of serous tumor and 3 cases of teratoma. 30 cases of normal ovarian tissues were collected from different ages of people, the average age was 43.1 years old(range: 29–60 years). Among these 30 peoples that the normal ovarian tissues were collected, 28 peoples had uterine fibroids, 1 person had cervical carcinoma and 1 person had breast carcinoma. The normal ovaries were resected while the carcinomas were cut off (agreed by the patients). The resected ovaries were further confirmed

without abnormality by pathology. All of tissue samples were collected during the operation. The primary lesion organization and metastases organization of tumor were stored in liquid nitrogen, respectively, then the samples were ready to RNA extraction and histopathological examination via 10% formalin fixed slice. All of ovarian malignant tumor patients need to followed up for 6 months to 60 months according to WHO level four evaluation standard in 1988. In this study, excepted 5 patients were lost to follow-up, all of the malignant ovarian tumor patients were followed up after treatment until December 2010, and no one was died of carcinoma. The median follow-up time was 29.25 months (range 3–60 months). Short-term curative effect was decided according to WHO standard in 1988. The judgment for tumor patients who resistance or sensitive to platinum drugs were in accordance with previous study.

Real-time fluorescent quantitative polymerase chain reaction(RT-qPCR)was used to quantify DHFR expression in all kinds of ovarian tissues

Organization RNA were extracted using Trizol one-step methods; cDNA were synthesized with RT retrovirus kit (TOYOBO company offers, M-MLV reverse transcriptase Promega company offers). All of experimental steps were strictly followed the specifications. Primers were designed with Primer 5.0 software and synthesized by Invitrogen Company. The DHFR primers were 5'-GTCATGGTTGGTTCGCTAAACTGCA-3'(forward); 5'-ATACATACTTTTTTCAGAGGGAGGG-3' (reverse). The PCR products were separated by 1.5% agarose gel and imaged by gel imaging analysis system, and the mRNA expressions of DHFR was quantified and normalized to that of GAPDH.

Plasmid generation

The hairpin siRNA targeted by DHFR gene was designed to screen out the best silencing fragment of siRNA, and the single-stranded DNA oligonucleotides were synthesized. The primer was annealed to form double-stranded DNA oligonucleotides, named shRNA. The annealed shRNA template was connected to the linear lentiviral vector pGCSIL after enzyme digestion and recovery, after the transformation of DH-5a, positive clones were extracted for sequencing. The sequencing results were compared by BLAST, and the homology was 100%. The recombinant plasmid was named DHFR-pGCSIL. The quality ratio of three plasmid included DHFR-pGCSIL (pGCSIL), pHelper1.0 and pHelper2.0 was 4:3:2, SKOV3 cells were inoculated in 6-well plates with 3×10^5 per well. On the second day, cell adherent growth converged to 70%~80% and then turned transfection, the specific operation was proceeding according to the instruction of LipofectamineTM2000. The experiment was divided into three groups: experimental group (SKOV3 cell group carrying DHFR-pGCSIL gene, negative control group (SKOV3 cell group only carrying pGCSIL virus) and blank control group (parental SKOV3 cell group). SKOV3 cells with DHFR-pGCSIL gene were sorting by flow cytometry .

Western blot assay

The cells were lysed with the radioimmuno precipitation test lysate and centrifuged at $13\,987.5 \times g$ for 5 min ,then extract the total cell protein in the supernatant.The proteins were separated by 10% sodium dodecyl sulfate-polyphenylamide gel electrophoresis, and then electrotransferred to the nitric acid fiber membrane. The buffer solution was washed and sealed by 5% skim milk powder. The antibody was incubated overnight, and the membrane was washed.The photographic film was scanned or photographed, and the relative expression level of the target band was analyzed with the gel image processing system, with GAPDH as the internal reference.

Drug cytotoxicity assay

When the three groups of cells were cultured to achieve a confluence of about 80%, then digested them with 0.25% trypsin to make a single cell suspension.The number of cells was inoculated in 96-well plat accorrding to 8×10^3 per well, and the total volume of each well was 200 uL,which were cultured was cultured at 37 °C for 24 h with 5% CO₂.After 24 h, the three group cells were cultured with cisplatin for 24 h, 48 h and 72 h at the mass concentration gradients of 1.25, 2.5, 5.0, 10.0, 20.0 and 40.0 ug/mL.MTT solution 20 uL was added to each hole, incubated at 37 °C for 4 h, and the supernatant was sucked and discarded. 150uL dimethyl sulfoxide was added to each hole, and then beaten and mixed to make the dirty fully dissolved. All solutions were transferred to another hole plate.The absorbance (D) value of each hole was measured by enzyme-linked immunoassay at 492 nm(ThermoLab Systems, Chantilly, VA, USA), the results were recorded, and the growth curve was plotted.

Apoptosis detection by flow cytometry

The three group cells number were cultured to about 80%~90%, then digested them with 0.25% trypsin to make a single cell suspension.The number of cells was inoculated in 6-well plat accorrding to 5×10^5 per well, and the total volume of each well was 2 mL,which were cultured was cultured at 37 °C for 24 h with 5% CO₂.After 24 h, the three group cells were cultured with cisplatin for 24 h, 48 h and 72 h respectively at the mass concentration gradients of 2.5, 5.0, 10.0, 20.0ug/mL,the total volume of each well was 2 mL. The operation was conducted according to the instructions of the apoptosis kit.

Cell cycle detection by flow cytometry

Three groups Cells (1×10^6) were induced by the IC₅₀ concentration cisplatin drug (4.4~5.5~4.9 µg/ml), after 24, 48 and 72 h, respectively, accordance with the cell cycle kit instructions, 10 000 cells in every group were analyzed using MultiCycle Software for Windows (Phoenix Flow Systems, San Diego, CA, USA), Multicycle software analysis of DNA content, G1, G2, S cell number and proportion of every period.

Cell platinum drugs concentration detection by High Performance Liquid Chromatography

Three groups Cells (1×10^7) were induced by different concentration cisplatin chemotherapy drugs (0, 2.5, 5.0, 7.5 $\mu\text{g/mL}$) after 24, 48 h, respectively. According to the corresponding chromatographic conditions for determination of peak area, making the linear regression with peak area of content, the concentration of DDP in 0.1–1.0 $\mu\text{g/mL}$, the regression equation $Y = 1.24 \times 10^{-5} x + 6.41 \times 10^{-3}$.

Transmission electron microscopy

Three group cells were cultured to a culture flask of 75 cm^2 respectively, when the cell number reached 90%~100%, using IC₅₀ cisplatin mass concentration (4.4 $\mu\text{g/mL}$) stimulated them for 24 h, 48 h and 72 h, then 0.25% trypsin digested the cells, the cell suspension inside the centrifuge tube was centrifuged ($167.7 \times g$, 10 min) into clusters, which were fixed overnight by 3% glutaraldehyde fixation solution, were fixed 1 h again by 1% osmic acid fixation solution. Acetone: isoamyl acetate (1:1) dehydrated them for 10 min and alcohol dehydrated them layer by layer gradient. Epoxy resin 618 embedded and then prepared them into a 1 μm semi-ultrathin section. Transmission electron microscopy (OH7650 TEM, Hitachi, Japan) observed the cell's ultrastructure and took photographs, and then analyzed the ultrastructure changes of cells in every group.

Statistical analysis

The experimental results were analyzed by SPSS 18.0 statistical software and the data were expressed as means $\pm$ SD. The comparison of the three groups data was conducted by ANOVA or Non-parametric Rank Sum Test. $P < 0.05$ was considered statistically significant.

Results

DHFR mRNA expression

DHFR expression was significantly higher in normal ovary and benign tumor tissues than in OC tissues ($P = 0.000-0.027$). Table 1.

Table 1 Real-time PCR detect DHFR expression in ovarian tissues

Groups	cases	DHFR mRNA expression [SD±x]	P
Normal ovary	30	0.584±0.234	P=0.000*
Benign tumor	50	0.895±0.783	P=0.027**
OC	80	0.197±0.412	P=0.000***

Notes: *P<0.05 vs benign tumor, ** P<0.05 vs OC, *** P<0.05 vs normal ovary.

Relationship between DHFR mRNA expression in ovarian carcinoma tissues and its clinical pathologic factors

The DHFR mRNA expression in serous ovarian carcinoma was higher than that in mucinous and endometrioid ovarian carcinoma ($P < 0.05$), in platinum-resistant patients was significantly higher than that of platinum-sensitive patients ($P = 0.011$), the patients with complete remission (CR) was significantly lower than that of patients with partial response (PR)/stable disease (SD)/progressive disease (PD) ($P = 0.000$), while there was no significant difference in DHFR mRNA expression among CR/SD/PD patients. Lower DHFR expression was observed in omentum metastasis ($P = 0.044$). However, there was no correlation between the expression level of DHFR mRNA in ovarian carcinoma and its pathological grade ($P = 0.489$), clinical stage ($P = 0.324$), CA-125 ($P = 0.305$), lymph node metastasis ($P = 0.956$), ascites ($P = 0.254$), organ metastasis ($P = 0.346$). Table 2.

Table 2
Correlation between clinicopathological variables and DHFR expression in OC tissues

Variables		n	DHFR mRNA expression	P
Histopathology	Serous	41	0.401 ± 0.779	P = 0.480
	Mucinous	24	0.222 ± 0.147	
	Endometrioid	2	0.177 ± 0.129	
	germ cells	11	0.199 ± 0.866	
	Granular cells	2	0.269 ± 0.108	
FIGO Stage	I-II	30	0.249 ± 0.167	P = 0.324
	III-IV	50	0.298 ± 0.578	
Grade	G1	16	0.150 ± 0.066	P = 0.489
	G2-G3	64	0.301 ± 0.586	
Lymphnode metastasis	Yes	15	0.199 ± 0.121	P = 0.956
	No	65	0.276 ± 0.508	
Omentum metastasis	Yes	33	0.175 ± 0.129	P = 0.044
	No	47	0.323 ± 0.587	
Organ metastasis*	Yes	7	0.163 ± 0.099	P = 0.346
	No	73	0.278 ± 0.498	
Serum CA-125 (U/mL)	< 35	9	0.187 ± 0.289	P = 0.305
	≥ 35	71	0.296 ± 0.147	
Ascites	< 500 ml	42	0.312 ± 0.662	P = 0.254
	≥ 500 ml	38	0.225 ± 0.143	
Outcome	CR	27	0.106 ± 0.117	P = 0.000 ^a
	PR	25	0.218 ± 0.115	
	SD	12	0.211 ± 0.180	P = 0.373 ^b
	PD	16	0.411 ± 0.651	
Platinum resistance	Resistant	24	0.341 ± 0.701	P = 0.011

^aCR patient DHFR mRNA levels vs PR/SD/PD;^bPR/SD/PD DHFR mRNA. *Metastasis to the liver, lung, brain, bone or spleen

Variables	n	DHFR mRNA expression	P
Sensitive	51	0.130 ± 0.103	
^a CR patient DHFR mRNA levels vs PR/SD/PD; ^b PR/SD/PD DHFR mRNA. *Metastasis to the liver, lung, brain, bone or spleen			

The relationship between DHFR expression and its prognosis

The cumulative survival rates of OC patients with 1, 2, 3, 4, 5 years were 57.0%, 31.0%, 16.0%, 9%, 16.0%, respectively, the median survival time was 15.19 months. ROC curve analysed an Youden index defined DHFR mRNA expression quantity is 0.331, more than 0.331 is DHFR positive, and less than 0.331 is DHFR negative. Using single factor analysis, Kaplan Meier survival curve, Long - rank test shows: The median survival time of DHFR positive was 16.4 months, 44.5 months in DHFR negative (P = 0.018), Fig. 1. Cox proportional hazards model analysis shows that DHFR mRNA expression affect the prognosis of patients with ovarian carcinoma, but not independent prognostic factors. Table 3.

Table 3
Cox proportional hazards model analyse the relationship between DHFR expression and its prognosis

Variables	b	SE(b)	Wald(X2)	P	RR	RR95%CI	
						Lower	Upper
age	.003	.023	.134	.701	1.109	.897	1.028
Histopathology	– .346	.226	0.915	.145	.766	.187	1.251
FIGO Stage	.897	.204	8.870	.032	3.401	1.231	4.342
Grade	– .494	.370	1.781	.182	.610	.354	1.435
Omentum metastasis	– .778	.435	3.132	.044	.385	.134	1.321
Lymphnode metastasis	.445	.421	.599	.439	1.546	.700	3.654
Ascites	.020	.000	1.647	.208	1.000	1.000	1.334
residual tumor	.304	.598	.299	.528	1.752	.356	7.578
CA125	.000	.000	2.467	.111	1.113	.987	1.345
neoadjuvant chemotherapy	– .042	.596	.005	.945	.798	.189	3.097
DHFR expression	.002	.023	.069	.018	1.203	.862	1.013

Silencing of DHFR by lentivirus mediated transfection

The results showed that the difference between the content of DHFR mRNA in the three group transfected cells such as siRNA-DHFR-574/687/768 was not statistically

significant($p = 0.905$),

but the content of siRNA-DHFR-768(5'-GUCUAGAUGCCUUAATT-3') was the lowest among the three groups, means it significantly inhibited DHFR expression. at different time, the difference was statistically significant($P = 0.02$), the lowest DHFR content of three groups was effecting on 72 h. This indicated that siRNA-DHFR-768 fragment had the strongest silencing when effecting on 72 h, and therefore was selected as an optimal shRNA for following experiments. Using PGCSIL /GFP vector as universal primer, the extracted plasmid was used as the template for PCR reaction to amplify the target gene with the full length of 823 bp. The homology was 100% by BLAST comparison. Sequence:

CCGGAAGTCTAGATGATGCCTT AAACCTCGAGTTTAAGGCATCATCTAGACTTTTTTTTG. DHFR plasmid carrying pGCSIL/GFP vector transfected into SKOV3 cells successfully. RT-PCR and Western blot assay were used to detect mRNA and protein expression of DHFR in transfected cells, respectively. When compared with blank control (no pGCSIL) group and NC-pGCSIL group, the mRNA and protein expressions of DHFR were significantly inhibited in cells transfected with DHFR -pGCSIL ($P < 0.05$).

TableS1.

Detection of DHFR mRNA expression in transfection cell group

DHFR gene carry with lentivirus was transfected in SKOV3 cells, after culturing lots of cells, flow cytometry instrument separate from GFP cells and non-GFP cells, separative rate is 65.8% (Fig. 2). Westblot assay test the gray value. SKOV3 grey value/DHFR-pGCSIL-SKOV3 grey value = $0.25/0.11 = 2.4$ times, showed that cell transfected success. TableS2, Fig. 3.

MTT detect cells growth curve in three groups

MTT assay detect the growth curve of DHFR -pGCSIL SKOV3, pGCSIL SKOV3 and SKOV3 cell, the IC50 cisplatin concentrations were 4.4 ug/ml, 5.5 ug/ml and 4.9 ug/ml.

apoptotic changes

Apoptosis rate of DHFR - pGCSIL SKOV3, pGCSIL SKOV3 and SKOV3 cell were raised with the increasing cisplatin concentration (2.5, 5.0, 10.0, 5.0 ug/mL) at 24, 48, 72 h. There was no statistically significant difference between pGCSIL SKOV3 and SKOV3 cell groups($P = 0.977 > 0.05$). After 24 h, < 5.0 ug/mL,

apoptosis rate of 3groups is basically the same; > 5.0 ug/mL, apoptosis rate of DHFR-pGCSIL- SKOV3 cells was significantly higher than the other two groups (P = 0.035, 0.028 < 0.05). After 48 h, three groups have intersection in cisplatin concentration between 4.0 ~ 5.0 ug/mL, before the cisplatin concentrations intersection, apoptosis rate of DHFR-pGCSIL-SKOV3 cells was significantly lower than the other two groups (P = 0.003, 0.036 < 0.05);While after the cisplatin concentration intersection, apoptosis rate of DHFR-pGCSIL SKOV3 cells was significantly higher than pGCSIL -SKOV3 and SKOV3 cell (P = 0.009, 0.047 < 0.05).After 72 h, apoptosis rate of DHFR-pGCSIL-SKOV3 cells was obviously higher than that of other two groups in every drug concentration (P = 0.023, 0.034, 0.012, 0.034 < 0.05).There was no statistically significant difference between pGCSIL SKOV3 and SKOV3 cell groups (P > 0.05). Table 4.

Table 4

Compare apoptosis rate in three groups cells after different cisplatin concentration at different time (%,
 $\bar{x}\pm s$)

group	cisplatin concentration($\mu\text{g/ml}$)							
	2.5	P	5.0	P	10.0	P	20.0	P
24 h		1.023		0.098		0.035		0.028
DHFR-pGCSIL-SKOV3	5.32 $\pm$ 0.24		9.90 $\pm$ 0.49		22.83 $\pm$ 0.44		41.42 $\pm$ 1.05	
SKOV3	5.06 $\pm$ 0.22		10.63 $\pm$ 0.49		12.83 $\pm$ 0.85		27.46 $\pm$ 1.01	
pGCSIL-SKOV3	7.16 $\pm$ 0.49		11.01 $\pm$ 0.87		12.07 $\pm$ 1.22		26.52 $\pm$ 1.13	
48 h		0.003		0.036		0.009		0.047
DHFR-pGCSIL-SKOV3	15.30 $\pm$ 1.32		40.02 $\pm$ 0.79		57.09 $\pm$ 0.89		69.35 $\pm$ 1.35	
SKOV3	20.20 $\pm$ 1.46		37.12 $\pm$ 2.08		47.16 $\pm$ 1.45		64.12 $\pm$ 2.63	
pGCSIL-SKOV3	23.27 $\pm$ 1.35		35.26 $\pm$ 1.38		49.55 $\pm$ 2.02		64.89 $\pm$ 2.81	
72 h		0.023		0.034		0.012		0.000
DHFR-pGCSIL-SKOV3	46.11 $\pm$ 0.68		60.84 $\pm$ 1.48		80.26 $\pm$ 1.32		90.42 $\pm$ 1.47	
SKOV3	29.00 $\pm$ 1.23		46.03 $\pm$ 2.01		66.40 $\pm$ 1.68		83.12 $\pm$ 2.07	
pGCSIL-SKOV3	22.25 $\pm$ 1.16		44.16 $\pm$ 1.87		62.85 $\pm$ 1.89		82.97 $\pm$ 2.28	
note: P values is transfection group compared with negative control group and blank control group								

High performance liquid periods of platinum drugs concentration changes extra and intracellular cells

When there was no platinum drugs effected, cisplatin content of DHFR - pGCSIL SKOV3, pGCSIL SKOV3 and SKOV3 is the same. Different cisplatin concentration (0,2.5, 5.0, 7.5 ug/mL) effected on three groups, at 24 h, intracellular cisplatin concentration of transfection group were significantly higher than that of control group after induced by cisplatin concentration (2.5 ug/mL, 5.0 ug/mL) ($P = 0.006, 0.034 < 0.05$).while in 7.5 ug/mL cisplatin concentration ,which was significantly lower than the control group ($P = 0.034 < 0.05$).At 48 h, the intracellular cisplatin concentrations were all significantly higher than control group ($P = 0.043, 0.032, 0.014, < 0.05$).The intracellular cisplatin drug concentration decreases in transfection group along with the increase of drug concentration, is not a dependency relationship between the control group and drug stimulates concentration. Table 5, Fig. 4.

Table 5

Compare cisplatin concentration in vivo in three groups cells when different cisplatin concentration effect at different time ($\mu\text{g/mL}$, $\bar{X} \pm S$)

Groups	cisplatin concentration($\mu\text{g/ml}$)						
	0	2.5	P	5.0	P	7.5	P
24 h			0.006		0.034		0.034
DHFR-pGCSIL-SKOV3	0.331 ± 0.013	2.458 ± 0.012		1.992 ± 0.031		0.693 ± 0.031	
SKOV3	0.923 ± 0.022	1.721 ± 0.021		1.031 ± 0.032		1.104 ± 0.019	
pGCSIL - SKOV3	0.902 ± 0.019	1.168 ± 0.025		1.560 ± 0.033		1.170 ± 0.027	
48 h			0.043		0.032		0.014
DHFR-pGCSIL-SKOV3	0.331 ± 0.013	1.770 ± 0.023		1.843 ± 0.025		1.741 ± 0.022	
SKOV3	0.923 ± 0.022	1.196 ± 0.023		1.490 ± 0.028		1.265 ± 0.025	
pGCSIL - SKOV3	0.902 ± 0.019	1.135 ± 0.021		1.228 ± 0.025		0.437 ± 0.035	

ultrastructure changes

Electron microscopy detect IC50(Silencing groups,4.4~5.5~4.9 µg/ml) cisplatin concentration in different period in three groups of cell,At 24, 48 h, cell microfilament were rare in 3 groups,DHFR- pGCSIL-SKOV3 group can visit mitochondria swelling disappeared, ridge, cavitation, atrophy, endoplasmic reticulum expansion, gathered the ribosome, did not see the golgi apparatus at 24 h, some mitochondria dissolved disappeared, and a large number of white vesicle appeared at 48 h.However, at 24 and 48 h, endoplasmic reticulum expansion were rare, mitochondria structure change is not obvious, even there are 2–3 golgi apparatus in pGCSIL-SKOV3 and SKOV3 cells;At 72 h ,DHFR-pGCSIL-SKOV3 cells has microfilament gathered together and increase obviously, mitochondria structure also disappeared. Microfilament disordered were arrangement, mitochondrial morphological were diversity in the Other cells.

Table 6, Fig. 5.

Table 6

Use electron microscope to observe ultrastructure changes of three groups cells after IC₅₀ cisplatin induced in different periods

Time		DHFR-pGCSIL - SKOV3	pGCSIL- SKOV3	SKOV3
0 h	mitochondria	Vacuoles, disappear	-	Structure is normal
	Endoplasmic reticulum expansion	-	-	-
	The ribosome aggregation	-	-	-
	microfilament	+++	++	+
24 h after cisplatin induced	mitochondria	The number increased, cavitation	Structural change significantly	Structure is normal
	Endoplasmic reticulum expansion	+++	-	++
	The ribosome aggregation	-	-	-
	microfilament	-	-	+
48 h after cisplatin induced	mitochondria	Many forms are visible and part of solution	The number increased	Number increase, expansion and contraction at the same time
	Endoplasmic reticulum expansion	-	-	-
	The ribosome aggregation	-	-	-
	microfilament	-	+	-
72 h after cisplatin induced	mitochondria	Change significantly	A rod, the C word structure	Structure disappeared
	Endoplasmic reticulum expansion	-	-	-

Note:- means none;+ means less;++ means more;+++ means a little more;++++ means much more

Time		DHFR-pGCSIL - SKOV3	pGCSIL- SKOV3	SKOV3
	The ribosome aggregation	++++	-	++++
	microfilament	Change significantly	disorders	-
Note:- means none;+ means less;++ means more;+++ means a little more;++++ means much more				

Discussion

Extensive research reveal the multi-resistant occurrence mechanism of ovarian carcinoma: (1)ABC (ATP binding cassette) type membrane carrier protein family's drug efflux, such as P - gp, BCRP and MRP1 ;(2) DNA damage repair system disorder; (3) The p53 gene mutation ;(4) Apoptosis pathway obstruction (5) Cell survival pathway activation [10], more and more studies[3] have found multi-resistant may be associated with folate metabolism enzymes.It could inhibit the growth of ovarian carcinoma cells by affecting folate metabolism, and enhance chemotherapy drug toxic effected on the ovarian carcinoma cells, thereby enhancing ovarian carcinoma chemotherapy sensitivity [11].However, tumor cells may reduce the affinity between chemotherapy drug and DHFR via increasing DHFR gene copy number and (or) quantity, then reduce mechanism such as drug uptake and speed up the discharge to induce drug resistance[12].

Dihydrofolate reductase (DHFR) is a monomer enzyme, formed NADPH DHFR - NADPH ternary complexes under the participation of coenzyme, and converting folic acid to the necessary materials of DNA, RNA and protein biosynthesis, therefore, the enzyme plays a very important role for the metabolism of the body.Folic acid prevents the occurrence of tumor via promoting DNA methylation, DNA repair, and prevent the P53 DNA fragmented. Folic acid metabolism is extremely complex, folic acid metabolic pathways in normal cells, precarcinomaous cells and carcinoma cells are not the same. Our research use real-time fluorescent quantitative PCR technology measured DHFR mRNA expression in the OC tissue, benign ovarian tumor and normal ovarian tissue, the result shows that ovarian benign tumor tissue of DHFR mRNA relative expression is significantly higher than OC and normal tissues, indicated that DHFR expression increased may be a compensatory adaptation mechanism for being lack of folic acid to the carcinomaous start or process, mediating the more folic acid into the cell. Due to tumor cells usually have low folic acid content [13], with the development of tumor, it is hard for extracellular carrier folate reduction to be intake of intracellular due because of lacking folate reduction or its activity decline in tumor cells, thus made DHFR expression down regulation. However, normal cells have much more folic acid, through the competitive inhibition of drug effect on DHFR, making DHFR expression level maintained at a higher level, this has the same with Matakidou [7]. The results of the study demonstrated that in malignant tumor tissues, DHFR mRNA expression has no correlation with the clinical stage, pathological classification, lymphatic metastasis, ascitic fluid, the result is consistent with some scholars

researching the other malignant tumors [8, 9]. According to the results of this observation DHFR mRNA expression in serous ovarian carcinoma patients is significantly higher than other ovarian epithelial carcinoma, and DHFR mRNA expression is higher in non-omentum metastasis in OC patients than that of patients with omentum metastasis, the reason maybe that omentum tissue contains phagocytes, when phagocytes can interact with DHFR protein, two fragments in DHFR can close to each other and folds formed active form, restore enzyme activity again.

Noteworthy is the result of this observation found that platinum resistance tissues DHFR expression is significantly higher than platinum sensitive ovarian carcinoma tissue, which not only verify our group experimental results in vitro that DHFR expression have differences between the platinum resistance and sensitive OC, and its DHFR expression was significantly raised after platinum sensitive ovarian epithelial carcinoma cells in vitro was induced into drug-resistant cell [14], also be the same time with Liu. [15] who study leukaemia found DHFR content difference in resistant and sensitive cells up to 17 times in leukemia cell lines. There are also scholars showed that DHFR gene expression of MTX drug resistant in the bone sarcoma cell lines is higher than the original generation cell lines, and with the increase of the cell resistance, DHFR gene expression also is up regulation, which means DHFR gene plays a certain role in tumor multi-drug resistance. Results also found that DHFR mRNA expression in CR patients tissues is lower than the tumor progression, while the median survival time of DHFR mRNA expression positive patients was 16.4 months, DHFR mRNA expression negative was 44.5 months. DHFR mRNA expression may be mediated multi-drug resistance. In order to know the specific molecular mechanism, we research biological behaviour of ovarian carcinoma cells in vitro though silencing and overexpression DHFR gene the pros and cons. The study results show that cell apoptosis rate and drug sensitivity increased after DHFR gene was down regulation. DHFR could suppress the proliferation of SKOV3 cells by inducing apoptosis, then reverse cell multidrug resistance partly. Because of the complexity of the apoptosis regulation, inducing its apoptosis mechanism may be associated with higher apoptosis protein, be up regulation of Fas/FasL, c-myc gene, be down regulation of survivin gene expression, and decreased Mitochondrial membrane potential, which are subject to further study. Knocking out DHFR gene strengthened the retardation of cisplatin on cell cycle, especially the G2/M phase, the normal pathway of the cell cycle was disturb, and its continuity was interrupted, drugs make the cell cycle pathway stagnated in S and G2/M phase, make the monitoring point enter into the next G2/M cell cycle decreased, thus inhibiting the proliferation of the cells. In order to know the mechanism of cisplatin resistance about DHFR gene, high performance liquid detection method study intracellular cisplatin concentration showed that cells of knocking out DHFR gene can make it easier for cisplatin to take into the body, greatly increased the concentration of drug in the target cell, and the time for drugs take into is faster and earlier, which can quickly reach the peak concentration. When drug concentration is larger, knocking out DHFR gene reduce pumping cisplatin in SKOV3 cells, enhance the killing effect on cells. While when high concentrations of cisplatin effect after 48 h, because of its high drug concentration and action time is longer, drug metabolism capacity in the cell increased, results in the decrease of intracellular drug concentration. The cell ultrastructure of knocking out DHFR gene change obviously and earlier time after cisplatin were induced, shows that the sensitivity of chemotherapy drugs was higher than the other two

kinds of cells, and the sensitivity of chemotherapy drugs may be associated with the depolymerization of actin filaments. In the process of cisplatin stimulation, mitochondria and endoplasmic reticulum in cells also change obviously, suggested mechanism of cisplatin treat tumor may be through mitochondria and endoplasmic reticulum kill cells. One research arsenic trioxide drug induced sensitive leukemia K562 cells apoptosis mainly by mitochondria, while induced resistance leukemia K562 / ADM cells apoptosis mainly by a combined way of mitochondria and endoplasmic reticulum. The results also is the same time with Banka [16]. Sun [17] found that cisplatin inducing tumor cell apoptosis and inhibit tumor growth by restoring the function of p53, Klinghoffer [18] at the same time studies results of the mechanism of cisplatin induced apoptosis was consistent with Ma Yanyun' research. Based on the above literature research, the experiment mechanism of apoptosis may be also related to the p53 pathway, but as a result of this experiment did not test the endoplasmic reticulum, mitochondria and protein and gene expression involved in the p53 signal transduction pathways, the specific mechanism that cisplatin induced to increase its drugs sensitivity after silencing DHFR gene is unclear.

Through the research about silencing DHFR gene, we found there was a connection between DHFR expression and platinum drug sensitivity in OC cells, after down regulated DHFR expression, its drug sensitivity increases, when at the certain concentration range, the effect of cisplatin increased along with the increase of drug concentration, after reaching a certain concentration, the incremental effect weakened or disappeared, which illuminated cisplatin concentrations have saturation effect.

The G2 / M phase retardation enhanced along with drugs effecting time increased, effecting 72 h is the most obvious. Among effective cisplatin concentration range, prolonged effect of drug appropriately could be one of the methods to reduce the development of drug resistance or reverse resistance. Silencing DHFR gene in ovarian carcinoma cells could be considered as a multi-resistant reversal mechanism, cell apoptosis, cycle are involved in the study of multi-drug resistance mechanism, how is the concrete mechanism need to be further in depth research.

Conclusions

This study found that DHFR gene has a certain relationship with cisplatin resistance, the higher DHFR gene expression, the more increased resistance, and its drug resistance may be related to gathered microfilament and the change of the mitochondria. Cisplatin kill tumor cells by a complex mechanism, cisplatin and taxol has become a first-line chemotherapy drugs for ovarian carcinoma. Through knowing the resistance mechanism of chemotherapy drug, develop a new generation of chemotherapy drugs to reduce the cell resistance and side effects, which is great significant to the clinical treatment of malignant tumors. This study only test epithelial ovarian carcinoma cell apoptosis and cell cycle along with the DHFR gene expression level changes but not study in-depth for the resistance in the process of related proteins and gene expression, which will be the next research direction.

Abbreviations

DHFR

Dihydrofolate reductase; OC:ovarian carcinoma; CR:complete response;

PR

partial response; SD:stable disease; PD:progressive disease;

RT-PCR

Reverse transcription-polymerase chain reaction; HPLC:High Performance Liquid Chromatography;

FIGO:Federation International of Gynecology and Obstetrics

Declarations

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Authors' contributions

ZL contributed to the conception and design, data collection, analysis and interpretation, manuscript drafting. LL and ZY conceived, designed the study, result discussion and were responsible for the paper's supervision. WZ and QN contributed to the data collection, literature review. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Guangxi Medical University. Written informed consent was obtained from all the subjects before study, and written informed consent was obtained from a parent or guardian for participants under 16 years old.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel R, Naishadham D and Jemal A. carcinoma statistics, 2013. *CA carcinoma J Clin*. 2013; 63: 11-30.
2. Mei L, Chen H, Wei DM, Fang F, Liu GJ, Xie HY, Wang X, Zou J, Han X and Feng D. Maintenance chemotherapy for ovarian carcinoma. *Cochrane Database Syst Rev* .2013; 6: CD007414.
3. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C and Gottesman MM. Targeting multidrug resistance in carcinoma. *Nat Rev Drug Discov*. 2006; 5(3): 219-34.
4. Li L, Luan Y, Li X, Tang B, Zhang W, Li D, Zhao J, Wang G, Ding H, Reed E, Li QQ. Demonstration of differential gene expression between sensitive and resistant ovarian tumor cells by fluorescence differential display-PCR analysis. *Oncol Rep*.2005;13(5): 793-99 .
5. Askari BS, Krajinovic M. Dihydrofolate reductase gene variations in susceptibility to disease and treatment outcomes. *Curr Genomics*.2010; 11(8): 578-83.
6. Matsuo K, Lin YG, Roman LD, Overcoming platinum resistance in ovarian carcinoma. *Expert Opin Investig Drugs*. 2010; 19(11):1339-54.
7. Matakidou A, El GR, Rudd MF, Webb EL, Bridle H, Eisen T, Houlston RS. Prognostic significance of folate metabolism polymorphisms for lung carcinoma. *Br J carcinoma*. 2007;97(2): 247-52.
8. Serra M, Reverter-Branchat G, Maurici D, Benini S, Shen JN, Chano T, Hattinger CM, Manara MC, Pasello M, Scotlandi K, Picci P. Analysis of dihydrofolate reductase and reduced folate carrier gene status in relation to methotrexate resistance in osteosarcoma cells. *Ann Oncol*.2004;15(1): 151-60 .
9. Banerjee D, Mayer-Kuckuk P, Capioux G, Budak-Alpdogan T, Gorlick R, Bertino JR. Novel aspects of resistance to drugs targeted to dihydrofolate reductase and thymidylate synthase. *Biochim Biophys Acta* .2002;1587(2-3): 164-73.
10. Tazi Y, Pautier P, Leary A, Lhomme C. Chemotherapy in epithelial ovarian carcinoma. 2013;*Gynecol Obstet Fertil* 41(10): 611-6.
11. Teodoridis JM, Strathdee G, Plumb JA and Brown R. CpG-island methylation and epigenetic control of resistance to chemotherapy. *Bio-chem Soc Trans*. 2004; 32: 916-7.
12. Staub J, Chien J, Pan Y, Qian X, Narita K, Aletti G, Scheerer M, Roberts LR, Molina J and Shridhar V. Epigenetic silencing of Hsulf-1 in ovarian carcinoma: implications in chemoresistance. *On-cogene* .2007; 26: 4969-78.
13. Webber S, Bartlett CA, Boritzki TJ, Hillard JA, Howland EF, Johnston AL, Kosa M, Margosiak SA, Morse CA, Shetty BV. AG337, a novel lipophilic thymidylate synthase inhibitor: in vitro and in vivo preclinical studies. *carcinoma Chemother Pharmacol*.1996;37(6): 509-17.
14. Wu MF, Hsiao YM, Huang CF, Huang YH, Yang WJ, Chan HW, Chang JT, Ko JL. Genetic determinants of pemetrexed responsiveness and nonresponsiveness in non-small cell lung carcinoma cells. *J Thorac Oncol*.2010;5(8): 1143-51.

15. Liu X, Yu X, Guo Z, Li X, Liu Y, Mao N. Cotransduction of Human *mdr-1* and Dihydrofolate Reductase Genes into Murine Hematopoietic Cells. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* .2000;8(2): 110-3.
16. Banka S, Blom HJ, Walter J, Aziz M, Urquhart J, Clouthier CM, Rice GI, de Brouwer AP, Hilton E, Vassallo G, Will A, Smith DE, Smulders YM, Wevers RA, Steinfeld R, Heales S, Crow YJ, Pelletier JN, Jones S, Newman WG. Identification and characterization of an inborn error of metabolism caused by dihydrofolate reductase deficiency. *Am J Hum Genet*.2011; 88(2): 216-25.
17. Sun S, Gui Y, Jiang Q, Song H. Dihydrofolate reductase is required for the development of heart and outflow tract in zebrafish. *Acta Biochim Biophys Sin (Shanghai)*.2011; 43(12): 957-69.
18. Klinghoffer RA, Frazier J, Annis J, Berndt JD, Roberts BS, Arthur WT, Lacson R, Zhang XD, Ferrer M, Moon RT, Cleary MA. A lentivirus-mediated genetic screen identifies dihydrofolate reductase (DHFR) as a modulator of beta-catenin/GSK3 signaling. *PLoS One* .2009;4(9): e6892.

Figures

Figure 1

Kaplan Meier survival curve between DHFR positive group and DHFR negative group

Figure 2

Sorting needed SKOV3 cells by flow cytometry

Figure 3

Western Blot detect DHFR expression in different groups SKOV3 cells

A

B

C

Figure 4

The establishment of chromatogram standard curve by the high performance liquid chromatography

Figure 5

Use electron microscope to observe ultrastructure changes of three groups cells after IC50 cisplatin induced(4.4 μ g/mL) in different periods

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