Research on chemotherapy efficacy of twist gene on cervical cancer cells to paclitaxel

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Abstract: The silent Twist gene may increase the sensitivity of cervical cancer cells chemotherapy to paclitaxel, thus was a new idea to improve the efficacy of cancer chemotherapy. The aim was to explore chemotherapy sensitivity of silent Twist gene increased cervical cancer cells to paclitaxel through study the proliferation and apoptosis of cervical cancer Twist gene after paclitaxel treatment. Cervical carcinoma Caski cells and Hela cells was cultured in vitro, mRNA gene expression was detected by using semi-quantitative, fluorescence quantitative PCR, and transferred to Caski cells transiently, and affected with paclitaxel solution of five kinds of different concentrations of 0.001, 0.01, 0.1, 1, 10 umol/L respectively. Then the results of <0.05). Every 12h after 36h, the expression inhibition rate in two groups of Caski cells that has transfected this study was Twist gene expression in Caski cells was higher than in Hela cells, which was of significant difference (p siRNA1 and siRNA2 were 20.3%, 38.2%, 33%, 24%, 68.6%, 50.8% respectively. After 48h in five different concentrations of paclitaxel effect, the cell growth inhibition rate of group siRNA2 with the best transfection efficiency was obviously higher than that of negative control group and blank control group, and the growth inhibition rates showed concentration dependence (p<0.05). It can be concluded that Twist gene in Caski cell was of high expression and the silent Twist gene could inhibit Caski cell proliferation and promote its apoptosis, thus to improve the chemotherapy sensitivity of Caski cells.

Keywords: Paclitaxel; cell growth inhibition rates; Caski cells; silent Twist gene.

INTRODUCTION

In the treatment of cervical cancer, surgery alone, radiotherapy or surgery plus radiotherapy are localized treatment, of which the curative effect on patients in medium and late stage and recurrent stage so far is poor. People use chemotherapy as one of the main treatment means for the patients in medium, late and relapse period that cannot stand the surgery, and also applied it into neoadjuvant chemotherapy and radiotherapy sensitizations to improve its clinical efficacy. But, problems such as insensitivity, rug resistance, etc appeared in chemical treatment process are quite troublesome. The study found that some abnormal genes in cancer occurrence and development process are likely to become the targets of drug candidates for the treatment of malignant tumor and drug resistance reversion (Castanon and Baylies, 2002).

Twist gene belongs to a transcription factor family of highly conserved basic helix-loop-helix (basichelix-loop-helix, bHLH) structure. The combination of human Twist protein with E-box5'-CANNTG-3' sequences on DNA through the basic helix-loop-helix in molecules formed homologous or heterologous dimer and regulated target gene expression (Schrder, 2006), thus to extensively involved in the regulation of cell proliferation and differentiation, organ development, tumori genesis, etc.

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The expression of Twist gene in many malignant tumors is elevated, while the expression in low malignant cancer cells and normal tissues do not exit or is very low. Twist gene can promote tumor formation by inhibiting apoptosis (Shiota *et al.*, 2010), inhibiting tumor suppressor gene p53 (Zhou *et al.*, 2008), promoting tumor angiogenesis (Hoek *et al.*, 2004), etc. It can participate in tumor invasion and metastasis (Kajiyama *et al.*, 2007) by mediating epithelial-Interstitial transformation (EMT), and contribute to the resistance of tumor cells by the anti-apoptotic (Wang *et al.*, 2004), interfere with anti-microtubule drugs (Shen *et al.*, 2011), induce epithelial - mesenchymal transition (Black *et al.*, 2008), induce cancer stem cell-like properties (Li *et al.*, 2009) and impact the tumor microenvironment (McConkey *et al.*, 2009), etc.

Saxena *et al* (2011) found that Twist gene expression in cervical cancer was generally visible. The majority of cancer cells (Twist-positive) showed diffuse distribution. A few Twist-positive cancer cells were in the periphery of cancer nests, and the cancer cell atypia was positively related to invasive. The more obvious atypia, the stronger the invasion; in addition, he concluded that Twist gene expression was related to he degree of tumor differentiation and lymph node metastasis, which prompted that Twist gene was closely related to poor prognosis in patients with cervical cancer. Rho *et al* (2009) found the increased expression of Twist gene was

relevant to the malignant degree of cervical epithelial, and concluded Twist gene can be used as an independent evaluation factor of cervical squamous cell carcinoma lymph node metastasis. Previous studies have proved that Twist gene plays an irreplaceable role in promoting cervical cancer occurrence and development. But current researches related to Twist gene and cervical carcinoma chemo sensitivity are quite few. In order to improve cervical carcinoma by improving the efficacy of chemotherapy and opening up new paths of drug resistance, this paper focused on the use of RNA interference techniques based on the selection of the current clinical cervical carcinoma chemotherapy first-linedrugs paclitaxel, and used specific siRNA designed by Twist gene designed to transfect cervical carcinoma Caski cell, thus to studied the function and value of silent Twist gene for increasing the sensitivity of human cervical carcinoma cells chemotherapy (Crea *et al.*, 2011).

MATERIALS AND METHODS

Main experimental materials

Human cervical carcinoma cell line, Caski cells and Hela cells, small interfering RNA fragments, RPMI1640 medium, Opti-MEM medium, NaCl, KCl, Na2HPO4, KH2PO4, FBS, trypsin digestion, DEPC-treated water, Lip of ectamine TM2000, Trizol Reagent, Dnase, RNase inhibitor, ethidium bromide (EB), chloroform, isopropanol, ethanol, methanol, SYBR Premix Ex TaqTM Kit, PrimeScript RT Master Mix (Perfect Real Time) Kit, ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), thiazolyl tetrazolium (MTT), crystal violet, Annexin V-FITC apoptosis detection kit, stain propidium iodide (PI), paclitaxel powder (bio-exclusive). Configured cell culture, trypsin, cryopreservation solution, 1X phosphate buffered saline (PBS), siRNA, 50xTAE buffer, 1.2% agarose gel, 10x denaturing formaldehyde gel electrophoresis, RNA loading buffer, 10xMOPS buffer solution, 1.2% formaldehyde agarose gel, paclitaxel solution, MTT solution.

Experimental Methods

Firstly, Caski cell lines with high-expression of Twist gene were primarily screened through RT-PCR and quantitative PCR to be the experimental cell line model. Two specific siRNA aimed for Twist gene were compounded through software design, entered into Caski cells by liposome transfection. After that, quantitative PCR were used to test Twist cell gene expression inhibition of transfected siRNA1 siRNA2 groups in 36h, 48h, and 60h respectively.

Secondly, the growth of Caski cells were detected by MTT assay after the silent Twist gene was effect on paclitaxel. The 0.001, 0.01, 0.1,1,10 umol/L taxol were put in si-Twist group, NC-Twist group, Mock group for 48 hours, the OD value was detected by the microplate reader, then converted into the growth inhibition rate. Then, 1umol/L (IC50) paclitaxel was used respectively for 24h, 48h, 72h, 96h, and OD values (cell survival reflects) was statistically analyzed.

To further study the changes of colony forming capacity after paclitaxel was effect on the Caski cells of the silent Twist gene, this experiment adopted flat clone forming experiments to observe 1umol/L paclitaxel treatment to cells in each group, and then made a corresponding study of Caski cell apoptosis situation of silent Twist gene under drugs. That is, 1umol/L paclitaxel was used to deal with cells in each group for 48h, at the same time, the cell cycle was detected.

STATISTICAL ANALYSIS

SPSS 13.0 statistical software was used to conduct statistical analysis and $\overline{x} \pm s$ was used to signify the results. T-test and variance test were adopted to conduct comparison among groups. If $p<0.05$, then it was of statistical significance.

RESULTS

Twist gene has relatively expression differences in Hela Caski cells

Both Caski cell line and Hela cell line express Twist mRNA, but cervical cancer cells Caski cell line Twist mRNA expression was significantly higher than that of Hela cell line, so the relatively high level expression of Caski cell lines of Twist mRNA was chose as the subjects for subsequent experiments.

RNA electrophoresis results, RNA fragment of Small interference and screening of transfection time

With the internal reference of GAPDH, the RQ values of siRNA1 and siRNA2 after 36h of transfection were 0.86 +/- 0.12 and 0.86 +/- 0.13 respectively. There were differences compared with negative control group (1.08 + 0.10), but the difference was not obvious and was of no significant statistical significance ($P>0.05$, table 1); the RQ values of siRNA1 and siRNA2 after 48h of transfection were 0.679±0.14 and 0.679±0.11 respectively, there were significant differences ($p<0.05$, table 1) compared with negative control group (1.10 + 0.8); the RQ values of siRNA1 and siRNA2 after 60h of transfection were 0.75±0.014 and 0.75±0.17 respectively, which was still lower than the negative control group (1.12±0.8) ($p<0.05$, table 1). Compared the RQ values of siRNA1 with that of siRNA2 in 36h, 60h, 48h of transfection, siRNA2 expression decreased obviously, and was significant in 48h of transfection ($p<0.05$, table 1). The results above indicated that both transfection of siRNA1 and siRNA2 have the silent effect on Twist gene, and that of effect on siRNA2 was much better, and such effect was significant in 48h.

Table 1: Twist mRNA expression of transient transfection siRNA1 and siRNA2 in different times (x±s)

Group	36h		48h		72h	
	RQ	Inhibition rate	RQ	Inhibition rate	RQ	Inhibition rate
Mock	1.03±0.04		1.08±0.03		1.09±0.13	
NC	1.08±0.10		1.10±0.08		1.12±0.18	
siRNA1	0.86±0.12	20.30%	0.679±0.14*	38.20%	0.75±0.04*	33%
siRNA2	0.82±0.13	24%	0.345±0.11*#^	68.60%	0.55±0.17*#	50.80%

Note: Real-timePCR, n = 4, siRNA1, siRNA2 vs NC, *p < 0.05; siRNA1 vs siRNA2, #P < 0.05; siRNA2 48h vs 36h/72h, ^p < 0.05

Table 2: The habitation rate of different concentrations of paclitaxel on three groups of Caski cell for 48h (x+/-s %)

Paclitaxel concentration	Inhibition ratio (%)		
	Mock Group	NC–Twist group	si-Twist group
0.001	11.42±1.43	13.06±1.70	18.05±1.03*
0.01	12.08±1.26	14.13±2.07	22.44±1.65**
0.1	19.27±2.12	21.35±1.88	31.21±2.21**
1	31.11±3.01	32.56±2.28	47.34±2.03**
10	44.36±2.15	46.28±2.08	68.86±2.23**

Note: MTT, n = 5, si-Twist vs NC-Twist, *p < 0.05, **p < 0.01

Table 3: Median Inhibitory Concentration (IC50)

Group	si-Twist	NC-Twist	Mock
IC_{50} (umol/L)	1.00±0.13**	32.82±3.25	48.33±3.54

Note: n = 5, si-Twist vs NC-Twist, **p < 0.01

Table 4: Twist gene silence, after the effect of paclitaxel, Caski cell clone formation rate (x ± s,%)

Group	Clone rate %
NC+DMSO	73.00±3.77
si-Twist+DMSO	45.60±2.09*
NC+Taxol(luM)	21.00±2.12
si-Twist+Taxol(luM)	8.20±0.71*

Note: Flat clone forming experiment, n = 5. si-Twist + DMSO vs NC+DMSO, *P < 0.05;si-Twist + Taxol vs NC+Taxol, #p < 0.05.

Table 5: Caski cells apoptosis rate (x ± s,%)

Group	Apoptosis rate%
NC+DMSO	3.21±1.63
si-Twist+DMSO	20.61±3.44*
NC+Taxol(luM)	26.16±4.38
si-Twist+Taxol(luM)	43.21±4.19#

Note: FCM, n = 4 si-Twist + DMSO vs NC + DMSO, *P < 0.05; si-Twist + Taxol vs NC+Taxol, #p < 0.05.

Table 6: The situation of Caski cell cycle distribution of each group

Group	Cell cycle distribution		
	G0/G1 phase	S phase	G2/M phase
A: NC-Twist+DMSO	68.62±2.87	18.35±2.46	13.03±1.44
B: si-Twist+DMSO	77.71±5.38*	21.29±3.16	1.00±0.54*
C: NC-Twist+Taxol	22.70±3.48	29.12±1.55	48.18±3.97
D: si-Twist+Taxol	56.50±6.33#	21.59±2.54#	21.90±3.71#

Note: FCM, n = 4, si-Twist + DMSO vs NC + DMSO, *P < 0.05; si-Twist + Taxol vs NC + Taxol, #p < 0.05

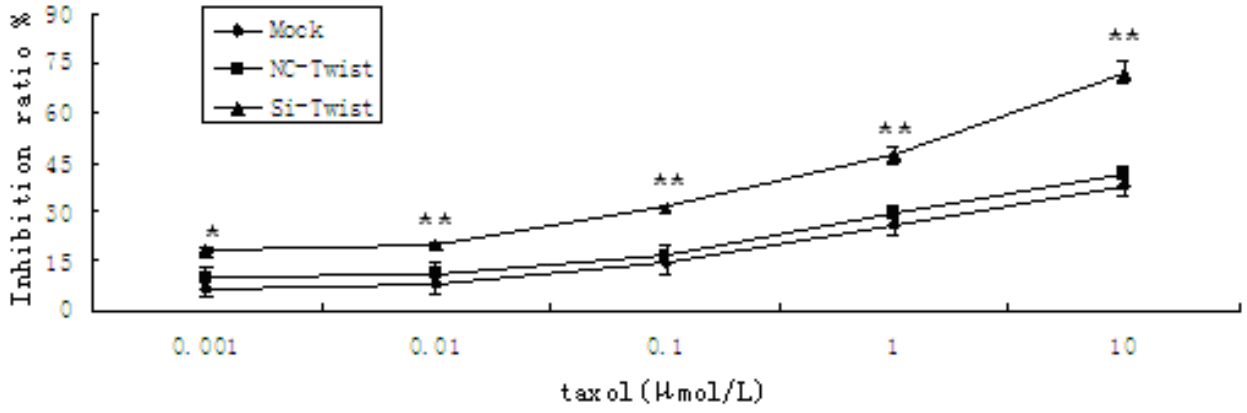

Fig. 1: The relational diagram of the growth of Caski cells in each group after the effect of paclitaxel of different concentrations
(MTT, n=5, si-Twist vs NC-Twist, *p<0.05, **p<0.01)

The growth situation of Caski cell after giving paclitaxel to silent twist gene

With the increase of paclitaxel concentrations, cell growth inhibition rate of each group also increased. The Caski cell growth inhibition rate of si-Twist group was significantly increased then that of NC-Twist group and Mock group, and such difference was statistically significant (p<0.05, table 2, fig. 1). There was no obvious difference of cell inhibition rate between NC-Twist group and Mock group (p<0.05, table 2, fig. 1). The IC50 value of each Si-Twist group, NC-Twist group, Mock group was shown in table 3. The IC50 value of Si-Twist group was significantly lower than the other two groups, the difference was statistically significant (p<0.05). MTT results showed that under the concentration of 1umol/Taxol, over time, si-Twist group compared with NC-Twist group and Mock respectively, Caski cell survival rate was significantly decreased. The difference was statistically significant (p<0.01). Within 0-48h, cell survival rate of si-Twist group reduced gradually along with the time progress, however after 48h, cell survival rate was of upward trend, but still significantly lower than NC-Twist group and Mock group (p<0.01).

The effect to cervical cancer Caski cell clone formation ability after paclitaxel on silent twist gene

Flat clone forming experiment was adopted to understand the ability of forming monoclonal by cell division, thus to further study the influence of Twist gene on cell proliferation. The results are as shown in table 4. The results indicated that clone formation ability of Caski cells from transfection si-Twist RNA was significantly weaker than the negative control group.

The effect of Caski cells apoptosis after giving paclitaxel to silent twist gene

Flow cytometry was adopted to determine cell apoptosis percentage, thus got to know the effect of chemotherapy drugs on Caski cell apoptosis after the adjustment under Twist gene expression. The result showed that without paclitaxel processing, cell apoptosis rate of si-Twist group was significantly increased than NC-Twist group, as shown in table 5. Thus we can see cell apoptosis rate increased significantly after the function of paclitaxel with cervical cancer Caski cells of low Twist gene expression.

The Caski cell cycle changes after giving paclitaxel to silent twist gene

It was observed that by flow cytometry academic without paclitaxel processing, as shown in table 6, cell proportion in si-Twist group increased than in NC-Twist group G0/G1, cell proportion in S phase was of no significant difference, however cell proportion in G2/M1 period decreased significantly. After 1umol/L paclitaxel effect, Caski cell cycle of transfection si-Twist group was still mainly of proliferation block in G0/G1 phase, as shown in table 6. The results indicated that after siRNA - Twist transfection Caski cell, cell cycle arrested in G0/G1 phase, thus improved the sensitivity of paclitaxel.

DISCUSSION

This experiment was aimed to study whether chemotherapy sensitivity of Caski cells to paclitaxel enhanced after Twist gene silencing. Firstly, the fluorescent quantitative PCR in transfection 36h, 48h, 60h respectively tested the Twist gene expression inhibition

rate intransfection siRNA1 and siRNA2 two groups were 20.3%, 38.2%, 33%; 24%, 68.6%, 50.8% respectively. As we can see, the effect of siRNA1 to twist gene silence was better than that of siRNA2, and the transfection efficiency in 48h was better than that of other time period, of which the differences were all significant. The optimal transfection time, the optimal experimental cell lines and the smallest snippet were screened out. After the detection to Caski cells growth situation by MTT colorimetry on silent Twist gene to paclitaxel, we found with the increase of paclitaxel concentrations, each cell inhibition rate also increased, which presented the concentration-response relationship. And the inhibition rate of silent Twist gene increased significantly than that of the other two groups, the differences were statistically significant; the IC50 value of silent Twist gene group (that is si-Twist group) was lower than the negative control group and blank control group two group, the differences were statistically significant; we can see the sensitivity of Caski cells to paclitaxel enhanced after Twist gene silencing. Under the effect of 1umol/L paclitaxel, cell survival rate of si-Twist group in 0-48h decreased over time, which presented the concentration-response relationship. But the cell survival rate presented the upward trend after 48h, which was assumed to be associated with the decrease of transfection efficiency in the process of RNA interference, but did not rule out other factors. RNAi can be used for screening of drug targets. As a simple and effective genetic tool instead of the gene knockout, with the application of RNAi technology, people found out the bottleneck problems in siRNA clinical application were laid in its easy degradation, poor stability, low transfer efficiency, etc (Donnenberg and Donnenberg, 2005). As embodied in the test results, with the passage of time, the efficiency of siRNA silence was no longer in the upward trend, or even in the downward trend, which also led to cervical cancer cells appeared signs of rebounding after the drop of survival rate under the effect of chemotherapy drugs, though cancer gene Twist was silent. So, in the future design of experiment, we can improve the efficiency of transfection by using a suitable method to modificate siRNA so as to achieve the expected better experimental results.

In order to further confirm the clone forming ability of Caski cells of silent Twist gene been affected by paclitaxel was dropped; this experiment selected the flat clone formation experiment to detect each group of cells after drug treatment. Caski cell clone formation rate in transfection siRNA group was significantly lower than the negative control group, the difference was statistically significant. Thus we can see silent Twist gene can inhibit the clone forming ability of Caski cells and enhance the sensitivity of chemotherapy drugs. After the corresponding research of Caski cells apoptosis under the effect of drug after Twist gene silence, Caski cells apoptosis rate of silent Twist gene was found increased significantly than that of not silent group by using flow cytometry, and the susceptibility to paclitaxel also increased. When not treated with drugs, compared silent Twist gene group with negative control group, cell number of the cell cycle in G0/G1 phase increased, and cells number change is not obvious in S phase, cells number decreased in S phase. The cell cycle was mainly in G0/G1 phase proliferation block, compared with negative control group, the sensitivity to taxol increased in G0/G1 phase.

To sum up, the high Twist gene expression was one of the important reasons that lead to the sensitivity decrease of Caski cells to paclitaxel. But the mechanism of Twist genes reducing the sensitivity of cervical cancer to paclitaxel and generate resistance is unclear. Through experimental studies, Wang, *et al.* (2004) assumed that it maybe associated with destruction of Twist gene to paclitaxel antimicrotubule formation. But Zhang, *et al.* (2007) through the nasopharyngeal carcinoma cell line study and Hiscox S, etc. (Hiscox *et al.*, 2006) through breast cancer cell line MCF-7 studies, found that Twist plays the role of inhibiting apoptosis, mediate cancer cells to produce paclitaxel resistance. So, the mechanism of causing cervical cancer chemotherapy sensitivity reduction and paclitaxel resistance production needs further research.

This paper reduced the Twist expression, and increased the chemotherapy sensitivity of Caski cells to paclitaxel by using RNAi technology. Cervical cancer nude mice model was constructed to prepare monoclonal antibody for silent Twist gene, thus to effect on cervical cancer nude mice, and the same to the chemotherapy drug pretreatment. This paper studied whether silent Twist gene could improve the chemotherapy sensitivity of cervical cancer cells so as to improve the chemotherapy effect. Thus we can see this experiment results provide the experimental basis for the cervical cancer treatment, lay a solid foundation for further research on the relationship between Twist gene and cervical cancer chemotherapy sensitivity and also provide the new ideas and research direction for cervical cancer clinical treatment.

CONCLUSION

For the expression of Twist gene in Caski cell was higher than that in Hela cell, Caski cell with high expression level was selected as the experimental subject to conduct subsequent experiment. The silence effect experiment of siRNA1 and siRNA2 transfection was established. The silence effect of siRNA2 segment was better, and the effect was more apparent at 48h of transfection. Through the effect of paclitaxel on Caski cell after Twist gene silence, the growth and proliferation ability of Caski cell were dramatically decreased. The IC50 was obviously

lowered, and the clonality of Caski cell was suppressed. The apoptosis of Caski cell was apparently increased, and cell cycle was mainly retardant in G0/1 phase. Therefore, silent Twist gene can increase the chemo sensitivity of cervical cancer cell to paclitaxel and enhance the chemotherapeutic effect, which provide new ideas for the clinical treatment of cervical cancer.

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