Norcantharidin downregulates programmed cell death 4 expression in human gastric cancer cells

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Abstract: **OBJECTIVE** To investigate the mechanisms by which programmed cell death 4 (PDCD4) is down-regulated by norcatharidinon(NCTD) in human gastric cancer cells. **METHODS** Cell viability was detected by MTT assay in human gastric cancer BGC-823 cells treated with NCTD 5, 10, 20, 40, 80, 160, 320 and 640 μmol·L^{-1} for 24, 48 and 72 h. The effect of NCTD 0, 6, 30 and 60 μmol·L^{-1} on PDCD4 protein expression was detected by Western blotting. BGC-823 cells were treated with NCTD 60 μmol·L^{-1} for 20 h and proteasome inhibitor MG132 10 μmol·L^{-1} for 4 h, then the PDCD4 protein was detected using Western blotting. The PDCD4 mRNA level were detected by RT-PCR after BGC-823 cells were treated with NCTD 60 μmol·L^{-1} for 24 h. The level of microRNA-21 (miR-21) in BGC-823 cells was analyzed with RT-qPCR after treatment by NCTD 60 μmol·L^{-1} for 6, 12 and 24 h. The PDCD4 protein level was detected by Western blotting after BGC-823 cells were transfected with miR-21 inhibitor and the cells treated with NCTD 60 μmol·L^{-1} for 24 h. **RESULTS** Cell viability obviously decreased in NCTD groups. The IC_{50} of NCTD in BGC-823 cells was 74.5, 35.0, and 10.3 μmol·L^{-1} at 24, 48 and 72 h, respectively. NCTD 6, 30 and 60 μmol·L^{-1} down-regulated PDCD4 protein by 9%, 47%, and 62%, respectively. The level of PDCD4 mRNA did not change in NCTD-treated BGC-823 cells. Compared with the cells treated with NCTD alone, the level of PDCD4 protein did not change in cells treated with both MG132 and NCTD. The miR-21 expression in NCTD-treated cells increased dramatically compared to that in control cells. The expression of PDCD4 protein was up-regulated dramatically by miR-21 inhibitor in NCTD-treated cells. **CONCLUSION** NCTD downregulates PDCD4 expression in BGC-823 cells through activation of miR-21.

Key words: norcantharidin; programmed cell death 4; miR-21; human gastric cancer cell line, BGC-823 cell
CLC number: R966 **Document code**: A **Article ID**: 1000-3002(2013)04-0622-07
DOI: 10.3867/j.issn.1000-3002.2013.04.003

Programmed cell death 4 (PDCD4) gene, first cloned from mice in 1995[1], was found to play an essential role in tumor suppression, implicating this protein as a promising target for antineoplastic therapy[2-3]. Recent data showed that PDCD4 expression can be up-regulated[4-5] or down-regulated[6] by antitumor drugs, but the mechanism remains unclear.

MicroRNAs (miR), the short 20-25 nucleotide RNA molecules, are a class of endogenous small non-coding RNAs that regulate target genes expression by targeting mRNA for translational repression[7]. The tumor suppressor PDCD4 is an important functional target of miR-21[8]. It was reported that PDCD4 was down-regulated by expression of miR-21 in breast cancer MCF-7 cells[9-13], colorectal cancer cells[10], glioblastoma T98G cells[11], and cardiac myocytes[12]. PDCD4 protein was also reported to correlate inversely with the level of miR-21 in gastric cancer tissues[13].

Norcantharidin (NCTD), a synthetic analogue of cantharidin, inhibits the proliferation of several tumor cell lines as well as transplanted tumors. NCTD is now in use as a routine anticancer drug against different type of carcinomas, including primary hepatoma[14], gastric cancer[15-16], esophagus carcinomas[16], and lung cancer[17] in China, without any depressive effect on bone marrow cells[18]. In our previous study, we found NCTD could induce cell apoptosis and arrest the cell cycle at G_2/M phase in BEL-7402 human hepatoma cells[19] and human myeloid leukemia K562 cells[20], which might be related to NCTD interference in tubulin polymerization in tumor cells[21].

Foundation item: The project supported by Foundation of Beijing University of Chinese Medicine (2009JYBZZ-2JS038); Foundation of Beijing University of Chinese Medicine (2011JYBZZ-XS037); and Foundation of Beijing University of Chinese Medicine (2011JYBZZ-XS045)

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In this study, we investigated the mechanism of PDCD4 suppression induced by NCTD in human gastric cancer cells, which might help discover new formulas to improve the clinical chemical drug sensitivity in gastric cancer therapy.

1 MATERIALS AND METHODS

1.1 Cell lines and cell culture

Human gastric cancer cell line BGC-823 (Cell Resource Center of Institute of Basic Medical Sciences of Chinese Academy of Medical Sciences, Beijing) was maintained in RPMI 1640 medium (Gibco, USA) with 10% fetal bovine serum, penicillin 100 kU·L^{-1}, and streptomycin 100 g·L^{-1} (Invitrogen, USA), and incubated at 37℃ in 5% CO_2. The response of tumor cells to NCTD was studied during the logarithmic growth phase. BGC-823 cells were serum starved in RPMI 1640 medium within NCTD treatment for 24 h prior to next-step experiments.

1.2 Antibodies, reagents and equipments

The rabbit anti-PDCD4 antibody (1∶2000 dilution) was kindly provided by Dr. Hsin-sheng YANG (University of Kentucky, USA). The rabbit anti-β-actin and goat anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology Inc (USA). The enhanced chemiluminescence (ECL) reagent and bicinchoninic acid (BCA) protein assay kit were purchased from Thermo Scientific Inc (USA). NCTD and the proteasome inhibitor MG132 (or Z-Leu-Leu-Leu-al) were purchased from Sigma (USA). Protease Inhibitor Cocktail Set Ⅲ was from Calbiochem Inc (USA). TRIzol, Lipofectamine 2000, and SuperScript Ⅲ one-step RT-PCR system with Platinum ® *Taq* DNA polymerase were from Invitrogen (USA). M-MLV reverse transcriptase was purchased from Promega Corporation (USA). MiR-21 inhibitor was purchased from GenePharma Inc (Shanghai, China). Microplate reader Spectra Max190 was purchased from Molecular Devices Corporation (USA). The software Quantity One was purchased from Bio-Rad Laboratories Inc (USA). ABI PRISM ® 7500 Sequence Detection System was from Applied Biosystems Corporation (USA).

1.3 MTT assay for cell viability

Human gastric cancer cell line BGC-823 was seeded onto the 96-well plates with 1×10^3 cells per well. After 24 h, the medium was replaced by fresh medium containing NCTD 5, 10, 20, 40, 80, 160, 320 or 640 μmol·L^{-1}, respectively and incubated for 24, 48 and 72 h. Then MTT solution was added to each well under sterile conditions (with a final concentration of 500 g·L^{-1}), and the plates were further incubated for 4 h at 37℃. The supernatant was replaced by DMSO (150 μl per well). The cell plate was vibrated for 10 min and the optical densities were measured by the microplate reader at 550 nm. Each experiment was performed in triplicates.

1.4 Western blotting analysis for PDCD4 protein expression

BGC-823 cells treated with NCTD 0, 6, 30 or 60 μmol·L^{-1} for 24 h were processed and analyzed for PDCD4 protein level by Western blotting[19]. The cells were lysed in the lysis buffer [Tris-HCl (pH 8.0) 50 μmol·L^{-1}, NP-40 1%, NaCl 150 μmol·L^{-1}, SDS 0.1%, and protease inhibitor cocktail set Ⅲ 1%]. Protein concentration was determined using BCA protein assay kit. Proteins were separated on a 10% SDS-PAGE and then transferred to the nitrocellulose membrane, which was blocked in 5% non-fat milk in PBS/Tween-20 and blotted with the rabbit anti-PDCD4 antibody followed by horseradish peroxidase-linked secondary antibody. β-Actin on the same membrane was used as a loading control. The PDCD4 protein was visualized by ECL detection reagents followed by exposure to film. The band intensity of the target protein was quantified using Quantity One.

1.5 Western blotting analysis for PDCD4 protein expression in cells treated with proteasome inhibitor MG132 for the ubiquitin degradation pathway

Two sets of BGC-823 cells were either untreated or treated with NCTD 60 μmol·L^{-1} for 20 h. After that, one set of untreated and treated cells was incubated with dimethyl sulfoxide (DMSO) for 4 h and the other set was incubated with proteasome inhibitor MG132 10 μmol·L^{-1} for 4 h before harvest[22]. The PDCD4 protein level was detected by Western blotting.

1.6 Reverse-transcriptase PCR for detecting PDCD4 mRNA expression

BGC-823 cells were cultured in the absence of fetal bovine serum without or with NCTD 60 μmol·L^{-1} for 24 h. Then total RNAs were extracted from BGC-823 cells using TRIzol reagent. PDCD4 mRNA was quantified by SuperScript Ⅲ

One-Step RT-PCR System with Platinum® *Taq* DNA polymerase against the internal control GAPDH. PCR primers were as follows: PDCD4, 5′-GCCAAGGCAAAAAGGCGACTA-3′ (forward) and 5′-TTCCCCTCCAATGCTAAGGAT-3′ (reverse), and GAPDH, 5′-GGTCGGAGTCAACGGATTTG-3′ (forward) and 5′-ATGAGCCCCAGCCTTCTCCAT-3′ (reverse). The PCR products were examined using gel-electrophoresis analysis on agarose gels and visualized by ethidium bromide staining. To avoid the plateau phase of PCR reaction, PCR cycles in different numbers were tested initially. Then the optimal number of reaction cycles was chosen to detect the level of PDCD4 mRNA. The band intensity of the target mRNA was quantified using Quantity One.

1.7 Real-time RT-PCR for detecting miR-21 mRNA expression

SYBR miRNA assays were used to detect the expression level of mature miR-21 relative to U6-small nuclear RNA. BGC-823 cells were either untreated for 24 h or treated with NCTD 60 μmol·L^{-1} for 6, 12 and 24 h. Total RNA was obtained from cells using TRIzol reagent. Then the level of miR-21 was detected with real-time RT-PCR using the ABI PRISM® 7500 Sequence Detection System according to the manufacturer's instructions. For RT reactions, the first strand cDNA of mR-21 was synthesized using M-MLV reverse transcriptase. The PCR reaction was conducted at 94℃ for 10 min followed by 34 cycles of 94℃ 5 s, 60℃ 34 s. The threshold cycle (Ct) was calculated by the instrument software (7500 Fast System). The miR-21 RT primers: 5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACA-3′. The PCR primers for PCR were as following: miR-21, 5′-CCGCGCTAGCTTATCAGAC-3′ (forward) and 5′-AGTGCAGGGTCCGAGGT-3′ (reverse), and U6 snRNA, 5′-GGACTAGCTTATAGACTG-3′ (forward) and 5′-GGAACGCTTCACGAATTT-3′ (reverse). The level of mRNA was calculated by $2^{-\triangle\triangle Ct}$ method.

1.8 Western blotting for PDCD4 protein expression in BGC-823 cells transfected with miR-21 inhibitor

BGC-823 cells were seeded in a 6-well plates with 2×10^5 per well and cultured in antibiotic-free medium for 24 h to achieve >50% confluence on the day of transfection. The cells were divided into four groups: vector control, vector + NCTD 60 μmol·L^{-1} for 24 h group, scrambled siRNA control and miR-21 inhibitor 40 nmol·L^{-1} + NCTD 60 μmol·L^{-1} for 24 h group. The cells were treated with Lipofectamine 2000 Reagent, scrambled siRNA or miR-21 inhibitor under serum-free conditions for 6 h, then the cells were treated without or with NCTD. The cells were processed and analyzed for PDCD4 protein level by Western blotting analysis.

1.9 Statistic analysis

Data were expressed as $\bar{x}\pm s$. The data were statistically analyzed by one-way analysis of variance (ANOVA) and *t*-test using SPSS 17.0 software.

2 RESULTS

2.1 Effect of NCTD on BGC-823 cells viability

As shown in Fig. 1, IC_{50} of NCTD for BGC-823 cells at 24, 48 and 72 h was 74.5, 35.0 and 10.3 μmol·L^{-1}, respectively. This result indicated that NCTD inhibited the growth of BGC-823 cells in a time- and concentration-dependent manner.

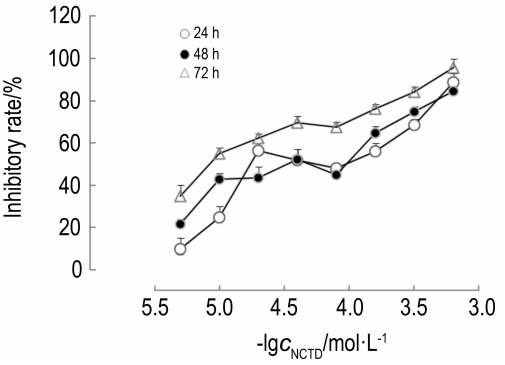

Fig. 1 Effect of norcantharidin (NCTD) on BGC-823 cells survival. BGC-823 cells were treated with NCTD 5, 10, 20, 40, 80, 160, 320 and 640 μmol·L^{-1}, respectively. $\bar{x}\pm s$, $n=3$.

2.2 Effect of NCTD on PDCD4 protein expression

As shown in Fig. 2, the protein level of PDCD4 in BGC-823 cells was down-regulated by NCTD in a concentration-dependent manner compared to untreated cells ($r=-0.459$, $P<0.01$). The PDCD4 level decreased by more than 50% in cells treated with NCTD 60 μmol·L^{-1}.

2.3 Effect of MG132 on down-regulation of PDCD4 in NCTD-treated BGC-823 cells

Western blotting analysis revealed that the amount of PDCD4 protein did not change obviously in the presence of NCTD and MG132 group compared with the group treated with NCTD alone

(Fig. 3), suggesting that PDCD4 protein was not restored after MG132 was added to BGC-823 cells. This result indicated the possibility that NCTD reduced PDCD4 protein level not by promoting the ubiquitin degradation pathway.

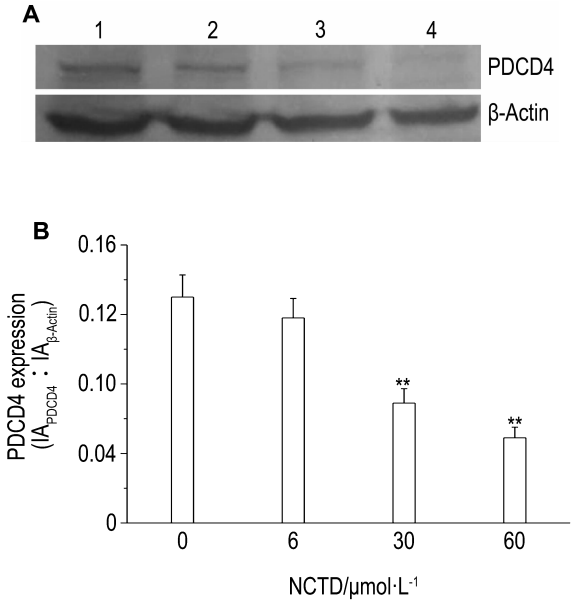

Fig. 2 Effect of NCTD on programmed cell death 4 (PDCD4) protein expression by Western blotting. B was the semiquantitative result of A. Lane 1: normal control group; lanes 2,3 and 4: NCTD 6, 30 and 60 μmol·L^{-1} for 24 h, respectively. $\bar{x} \pm s$, $n = 3$. ** $P < 0.01$, compared with NCTD 0 μmol·L^{-1} group.

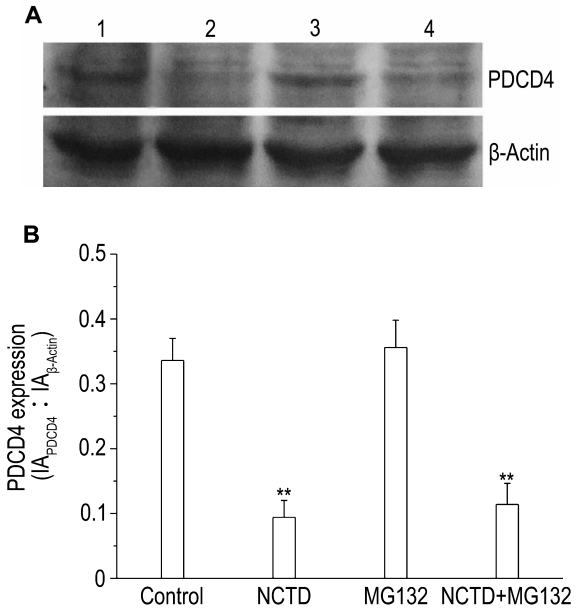

Fig. 3 Effect of MG132 on down-regulation of PDCD4 induced by NCTD. B was the semiquantitative result of A. Lane 1: normal control group; lane 2: NCTD 60 μmol·L^{-1} for 20 h; lane 3: MG132 10 μmol·L^{-1} for 4 h; lane 4: NCTD 60 μmol·L^{-1} for 20 h, then MG132 10 μmol·L^{-1} added for 4 h. $\bar{x} \pm s$, $n = 3$. ** $P < 0.01$, compared with normal control group.

2.4 Effect of NCTD on PDCD4 mRNA expression

As shown in Fig. 4, the mRNA level of PDCD4 in NCTD-treated BGC-823 cells was similar to that of normal control cells (0.69 ± 0.08 *vs* 0.64 ± 0.07), suggesting that the decrease of PDCD4 protein in NCTD treated BGC-823 cells was not due to lowered transcription.

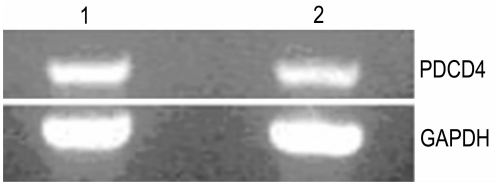

Fig. 4 Effect of NCTD on PDCD4 mRNA expression by reverse-transcriptase PCR. Lane 1: normal control group; lane 2: NCTD 60 μmol·L^{-1} for 24 h.

2.5 Effect of NCTD on miR-21 expression

As shown in Fig. 5, the miR-21 expression increased significantly in cells treated with NCTD for 12 and 24 h compared to untreated cells ($P < 0.01$). This result implied that NCTD might have down-regulated PDCD4 expression through up-regulation of miR-21.

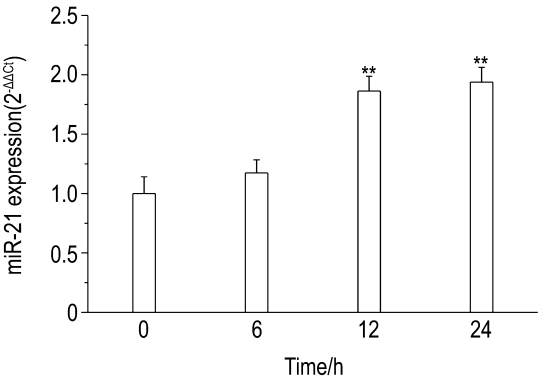

Fig. 5 Effect of NCTD 60 μmol·L^{-1} on miR-21 expression by real-time RT-PCR. $\bar{x} \pm s$, $n = 3$. ** $P < 0.01$, compared with normal control (0 h) group.

2.6 Effect of NCTD on PDCD4 protein expression in BGC-823 cells transfected by miR-21 inhibitor

As shown in Fig. 6, the PDCD4 protein expression was up-regulated significantly in the cells transfected with miR-21 inhibitor and treated with NCTD ($P < 0.01$), compared with the cells transfected with scrambled siRNA and treated with NCTD, indicating again that NCTD reduced PDCD4 protein via up-regulation of miR-21.

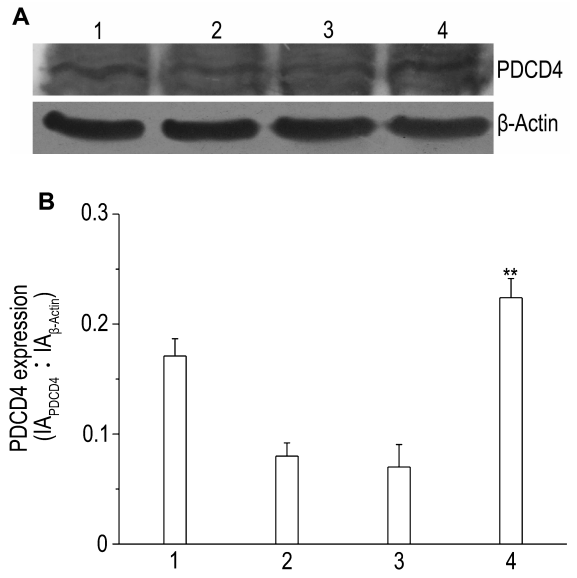

Fig. 6 Effect of NCTD on PDCD4 protein level in BGC-823 cells transfected with miR-21 inhibitor by Western blotting. B was the semiquantitative result of A. 1: vector control group; 2: vector + NCTD 60 μmol·L^{-1} for 24 h; 3: cells transfected with scrambled siRNA then treated with NCTD 60 μmol·L^{-1} for 24 h; 4: cells transfected with miR-21 inhibitor then treated with NCTD 60 μmol·L^{-1} for 24 h. $\bar{x} \pm s$, $n=3$. $^{**}P<0.01$, compared with group 3.

3 DISCUSSION

NCTD has been used as an anticancer drug against different kinds of carcinomas, including gastric cancer in China. In this study, NCTD was shown to inhibit the growth of BGC-823 cells while inducing the miR-21 expression and down-regulating the PDCD4 expression in BGC-823 cells. Moreover, the level of PDCD4 protein was up-regulated dramatically by miR-21 inhibitor in NCTD-treated cells, which suggested that NCTD decreased PDCD4 protein by up-regulating miR-21.

PDCD4 is one of the most intensively studied target genes of miR-21. It was reported that PDCD4 is directly regulated by miR-21 in breast cancer cells. The PDCD4 protein level is 3.5-fold up-regulated by miR-21 inhibition[9]. A similar result was observed in HeLa cervical carcinoma cells. PDCD4 level increased when miR-21 inhibitor was transfected into HeLa cells. In addition, Yao *et al*[23] provided direct evidence that the 3′untranslated region (3′UTR) of PDCD4 is the target region for miR-21 to function. It has been reported that miR-21/PDCD4 pathway plays an important role in carcinogenesis and inflammation[24], suggesting that the use of such agents as anti-miR-21 nucleotides along with NCTD improve the efficacy of anticancer drugs in cancer therapy.

The expression of PDCD4 can be up-regulated or down-regulated by anticancer drugs. It was reported that PDCD4 gene expression was induced by retinoic acid receptor agonists, antiestrogen and HER-2/neu antagonist in breast cancer T-47D cells[25]. The expression of PDCD4 protein was also found to be up-regulated in human gastric cancer BGC-823 cells upon hydroxycamptothecine treatment[26]. Conversely, etoposide and other DNA damage agents were reported to down-regulate PDCD4 in HepG2 and other tumor cells[27]. Further investigation is needed to determine the relationship between PDCD4 expression and chemosensitivity of NCTD in cancer cells.

MiR-21/PDCD4 pathway was reported to play an important role in carcinogenesis and inflammation[24], suggesting that the use of such agents as anti-miR-21 nucleotides along with NCTD improve the efficacy of anticancer drugs, but more attention should be paid to possible inflammation and second carcinogenesis when a big dose of NCTD is applied clinically.

It is known that in response to mitogens, PDCD4 was rapidly phosphorylated on Ser67 by the protein kinase S6K1 and subsequently degraded via the ubiquitin ligase SCF TRCP. Ubiquitin degradation pathway was also analyzed in NCTD treated BGC-823 cells. The protein level of PDCD4 did not increase significantly when proteasome inhibitor MG132 was added to cells. In addition, both PI3K and mTOR play important roles in mitogens induced PDCD4 degradation through ubiquitin proteasome[22]. However, PI3K inhibitor LY294002 and mTOR inhibitor sirolimus (Rapamycin) had no effect on PDCD4 protein level in NCTD treated cells (data not shown). These results collectively suggest that NCTD does not down-regulate PDCD4 level in BGC-823 cells through ubiquitin/proteasome pathway.

The mechanism of how NCTD activates miR-21 and then down-regulates the expression of PDCD4 remains unclear. It was reported that the miR-21 transcription could be induced by numerous factors including reactive oxygen species[28]. NCTD induces oxidative stress in cultured cells[29], which might activate the miR-21 expression and then suppress PDCD4 translation.

Recent data showed that regulation of PDCD4 level and function depends on numerous factors and is cell type specific[2]. Although PDCD4 pro-

tein was also found to be down-regulated by NCTD in human ovarian cancer SK-OV-3 cells and myeloid leukemia K562 cells (unpublished results), the down-regulation of PDCD4 by NCTD needs to be studied in more tumor cells. The molecular mechanism of NCTD induced down-regulation of PDCD4 and the relationship between NCTD induced suppression of cell viability and down-regulation of PDCD4 level in human gastric cancer BGC-823 cells require further investigations for better application of NCTD in gastric cancer therapy.

ACKNOWLEDGMENTS: We would like to thank Dr. WANG Qing (University of Kentucky, USA) for critical reading the manuscript.

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去甲斑蝥素降低人胃癌细胞程序性细胞死亡因子 4 的表达

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摘要: 目的 研究去甲斑蝥素(NCTD)降低程序性细胞死亡因子 4(PDCD4)表达的机制。**方法** MTT 法测定NCTD 5 ~ 640 μmol·L^{-1}与人胃癌 BGC-823 细胞作用 24,48 和 72 h 细胞存活率;Western 蛋白质印迹法测定 NCTD 0, 6, 30 和 60 μmol·L^{-1}作用 BGC-823 细胞 24 h PDCD4 蛋白表达水平;NCTD 60 μmol·L^{-1}作用 20 h 后加入 MG132 10 μmol·L^{-1}作用 4 h 对 PDCD4 蛋白表达的影响;逆转录 PCR 法测定 NCTD 60 μmol·L^{-1}作用 BGC-823 细胞 24 h 后 PDCD4 mRNA 表达的变化;实时荧光定量 PCR(qRT-PCR)测定 NCTD 60 μmol·L^{-1}作用 BGC-823 细胞 6, 12 和 24 h 后 microRNA-21(miR-21)的表达。Western 蛋白质印迹法测定细胞转染 miR-21 抑制剂对 PDCD4 蛋白表达的影响。**结果** NCTD 作用后 BGC-823 细胞存活率明显下降,NCTD 作用 BGC-823 细胞 24, 48 和 72 h IC_{50} 分别为 74.5, 35.0 和 10.3 μmol·L^{-1}。NCTD 6, 30 和 60 μmol·L^{-1}作用于 BGC-823 细胞 24 h,PDCD4 蛋白分别降低 9%, 47%和 62%。NCTD 对 PDCD4 mRNA 表达无影响。与 NCTD 处理组相比,MG132 和 NCTD 共处理对 PDCD4 蛋白表达无明显影响。NCTD 60 μmol·L^{-1}作用 BGC-823 细胞 12 和 24 h 后,细胞中 miR-21 的表达显著升高($P<0.01$)。细胞转染miR-21抑制剂后,可抑制 NCTD 降低 PDCD4 蛋白表达的作用。**结论** NCTD 通过调控 miR-21 降低 PDCD4 蛋白的表达。

关键词: 去甲斑蝥素; 程序性细胞死亡因子 4; microRNA-21; 人胃癌细胞, BGC-823

基金项目: 北京中医药大学基金项目(2009JYBZZ-JS038); 北京中医药大学基金项目(2011JYBZZ-XS037); 北京中医药大学基金项目(2011JYBZZ-XS045)

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(收稿日期: 2013-02-06 接受日期: 2013-07-20)
(本文编辑: 乔 虹)