Norcantharidin Inhibits the Expression of Id1 mRNA in H446 Cells

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ABSTRACT Objective: To investigate the effects of the expression of Id1mRNA on human small cell lung cancer H446 cells. Methods: The cell viability of H446 was evaluated by MMT assay. The ability of cell migration was determined by scratch wound assay. The cell apoptosis was assayed by Hoechst staining. The quantity of Id1 mRNA of H446 cells was detected by real time quantitative reverse transcription-polymerase chain reaction (RT-PCR). Results: The cell inhibition rate and cell apoptosis rate increased obviously by norcantharidin. The cell migrating distance was shorter in norcantharidin treated cell. Meanwhile, the relative quantity of Id1mRNA reduced significantly by norcantharidin. The quantity of Id1mRNA was inhibited by norcantharidin in a dose dependent manner. Conclusion: Norcantharidin can inhibit Id1mRNA in H446 cells, which may be one important mechanism of decreased cell viability, migration ability and induced cell apoptosis.

Key words: Norcantharidin; Id1; H446 cells

Chinese Library Classification (CLC): R734.2 Dicument code: A

Article ID: 1673-6273 (2012) 07-1225-04

Introduction

Inhibitor of differentiation 1 or inhibitor of DNA binding 1 (Id1) which belongs to the Id family of helix-loop-helix transcription factors plays a role in cellular differentiation, proliferation, apoptosis, invasion and angiogenesis [1-5]. Up regulation of Id1 is frequently found in several types of cancers [6-8], including lung cancer [9]. The increasing of Id1 was correlated with advanced tumor stage and poor prognosis in several other cancer[10-11]. Previous study found that down regulation of Id1 by small interfering RNA in PC3 cells could inhibit the cell growth, induce cell apoptosis and senescence in vivo and in vitro[5]. Norcantharidin (NCTD) is a synthetic demethylated analog of cantharidin. NCTD is now used in China as a routine anticancer drug against different kinds of human cancers[12-13]. NCTD also reduced the growth and migration of human lung cancer A549 cells in vitro and ex vivo [14]. This study was to detect the effect of NCTD on the expression of Id1mRNA in H446 cells.

1 Materials and methods

1.1 Cell culture and reagents

Human small cell lung cancer cell line H446 was obtained from Type Culture Cell Bank of Chinese academy of Sciences (Shanghai, IBCB). H446 cells was maintained in RPMI 1640 medium supplemented with 10% (v/v) FCS (Gebico, MO), 100U/ml penicillin,100 μg/ml streptomycin (Solarbio, USA) at 37℃ in a humidified atmosphere containing 5% CO_2. NCTD (Beijing Shuanghe Pharmaceutical Co., China) was diluted with RPMI1640 into 0.5, 1.0, 2.5 and 5.0 mg/L, respectively for each experiment.

1.2 Cell viability assay

Briefly, H446 cells were seeded in 96-well plates. The cells were treated without (untreated control group) or with various concentrations of NCTD (the four-concentration groups, every concentration × 6) in fresh culture medium. Then the cells were cultured in an incubator with 5% CO_2 at 37℃. Cell viability was examined by 3- (4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as the instruction manual at 48h. The experiment was repeated three times and the average result was calculated.

1.3 Cell migration ability assay

H446 cells were seeded in 24-well plates and grew to 100% confluence. A 120μm broadband of cell erasioning was created in each well. The image was acquired randomly at 6 visual fields in each erasioning band. The cells were treated by various concentrations of NCTD in fresh culture medium at 37℃. 48h later, the image was acquired again. The average distance of cell migrating was measured and calculated by JD801 morphological image analysis software(JEDA, China).

1.4 Cell apoptosis assay

H446 cells were seeded in 24-well plates and treated without (untreated control group) or with various concentrations of NCTD in fresh culture medium, and then were cultured in 5% CO_2 at 37℃. 48h later, the cells were fixed and stained with Hoechst as the manufacturer's protocol, and were then examined under a fluorescent microscope. Cell was considered to be undergoing apoptosis on the basis of the appearance of nuclear fragmentation and chromatin condensation. At least 500 cells were counted in each visual fields and the percentage of apoptotic cell was calculated from over 3000 cells.

1.5 Quantitative Real-time PCR assay

H446 cells were seeded in 24-well plates and treated without (untreated control group) or with various concentrations of NCTD

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(Received: 2011-09-03 Accepted: 2011-09-30)

in fresh culture medium, and were again cultured in 5% CO_2 at 37℃. 48h later, total RNA was isolated from H446 cells using TRIzol reagent (Invitrogen, USA) as the manufacturer's protocol. Total RNA concentration was determined by spectrophotometric analysis at 260 nm. The reverse transcription reaction was performed using the BcaBESTTM RNA PCR Kit (TaKaRa, Japan) with 1 μg of total RNA following the manufacturer's instructions. Real time quantitative PCR was performed using SYBR Green Realtime PCR Master Mix (ToYoBo bio, Japan) on an Applied Biosystems 7500 fast Sequence Detection System. PCR cycling conditions were 95℃ for 30s, 1 cycle; 95℃ for 20s, 60℃ for 30s, 72℃ for 15s, 40 cycles. Primers specificity for Id1, as well as for endogenous β-actin control were designed and provided by TaKaRa biotechnology Co.,Ltd (Dalian,China). Id1: 5'-acgacatgaacggctgttactcac-3'; 5'-ctccaactgaaggtccctgatgtag-3'. β-actin: 5'-attgccgacaggatgcaga-3'; 5'- gagtacttgcgctcaggagga-3'. β-actin was used as a housekeeping gene to normalize the gene expression in each sample. The mRNA expression levels were calculated by relative quantitation using the $2^{-\triangle\triangle CT}$ [15] method. Experiment was performed in triplicate for each data point.

1.6 Statistical analysis

The data were shown as mean ± standard deviation (SD) and analyzed by the SPSS 10.0 software to evaluate the statistical difference. One-way or two-way ANOVA was used to establish whether significant differences existed among groups. A value of $P < 0.05$ was considered to be statistically significant.

2 Results

2.1 NCTD inhibited cell viability of H446

Cell viability was determined by MTT assay at 48h after NCTD treated. The viability of H446 cells reduced obviously by NCTD. The changes of cell inhibition rate were in a dose-dependent fashion in each group as shown in Table 1.

Table 1 Cell inhibition rate of H446(%)

NCTD(mg/L)	N	H446
0	6	3.75± 1.02
0.5	6	14.98± 3.47★★
1.0	6	32.57± 3.69★★
2.5	6	48.49± 4.08★★
5.0	6	69.36± 5.17★★

Note: ★★Significant difference compared in each group, P< 0.01

2.2 NCTD inhibited cell migration of H446

The cell migration ability reduced obviously by NCTD, which was in a dose dependent manner in H446. The distance of cell migrating was significantly decreased in H446 cells treated with different concentration of NCTD as shown in Table 2.

Table 2 Migration distance of H446(m)

NCTD(mg/L)	N	H446
0	6	81.65± 5.11
0.5	6	60.39± 7.15★
1.0	6	49.63± 8.09★
2.5	6	17.82± 6.33★

Note: ★ Difference compared in each group，P< 0.05

2.3 NCTD induced cell apoptosis of H446

The apoptosis of H446 cells induced obviously at 48h by NCTD , and which was in a dose-dependent manner. The apoptosis rate of H446 cells was shown in Table3.

Table 3 Apoptosis rate of H446 (%)

NCTD(mg/L)	N	H446
0	6	5.94± 1.11
0.5	6	11.73± 2.68★
1.0	6	19.84± 2.97★
2.5	6	23.65± 3.42★

Note: ★Significant difference compared in each group，P< 0.05

2.4 NCTD inhibited the expression of Id1mRNA

The expression of Id1mRNA which involved in cell differentiation, proliferation, apoptosis and invasion was detected. The expression of Id1mRNA was significantly inhibited by NCTD in

H446 cells. The relative quantity of Id1mRNA was obviously reduced by NCTD in a dose dependent fashion as shown in table 4.

Table 4 Relative quantity of Id1mRNA in H446

NCTD(mg/L)	N	H446
0	6	1.00± 0.06
0.5	6	0.64± 0.07★★
1.0	6	0.47± 0.07★★
2.5	6	0.35± 0.06★★

Note: ★★Difference compared in each group，P< 0.01

3 Discussion

Id1 is a member of the Id protein family that belongs to the helix-loop-helix family of transcription factors. Id proteins lack a DNA-binding domain but can bind to other helix-loop-helix proteins and inhibit them from DNA binding, hence named as inhibitors of DNA binding [16]. Four Id proteins (Id1-4) have been identified in mammals that share similar structural characteristics. They play different regulatory roles throughout prenatal and postnatal development and tumorogenesis [9]. Id1 has a key role in cellular differentiation, proliferation, apoptosis, invasion and angiogenesis [1-5], hence named as inhibitor of differentiation1. Meanwhile, Id1 plays key role in the development of many human cancers. Study showed that Id1 over expression was related with cell differentiation in human prostate cancers[2] and down regulation of Id1 by small interfering RNA in PC3 cells can inhibit the cell growth, induce cell apoptosis and senescence [5]. Other researchers found that Id1 could inhibit cell proliferation and migration in lung cancer cell lines. The over-expression of Id1 increased cell proliferation while Id1 knockdown decreased cell proliferation in lung cancer cells [17-18]. This study demonstrated that NCTD had inhibition efficacy on Id1mRNA expression of H446 cells and that the cell viability, migration ability and cell apoptosis of H446 were significantly changed by NCTD. The deceased expression of Id1mRNA in our study may be one reason of changed cell viability, migration ability and apoptosis by NCTD. Inactivation of Id1 by NCTD may provide a novel mechanism for treatment of lung cancer. Other researchers found that NCTD could inhibit cell proliferation which might decrease the expression of the proliferation-related gene proteins, PCNA and Ki-67, of GBC-SD cells[19], suggesting that NCTD could inhibit cell proliferation by affecting several genes expression.This study found that NCTD can inhibit the gene expression of Id1 firstly. Down-regulation of Id1 gene by NCTD may be a novel mechanism for NCTD on chemopreventive and chemotherapeutic effects.

References

[1] Schmidt M, Asirvatham AJ, Chaudhary J. Inhibitor of differentiation 1 (Id1) promotes cell survival and proliferation of prostate epithelial cells[J].Cell Mol Biol Lett,2010, 15(2):272-295

[2] Yu XL, Xu XH, Han BJ, et al. Inhibitor of DNA Binding-1 Overexpression in Prostate Cancer: Relevance to Tumor Differentiation[J]. Pathol Oncol Res,2009, 15(1):91-96

[3] Ciarrocchi A, Piana S, Valcavi R, et al. Inhibitor of DNA binding-1 induces mesenchymal features and promotes invasiveness in thyroid tumour cells[J].Eur J Cancer, 2011, 47(6):934-945

[4] Mellick AS, Plummer PN, Nolan DJ, et al. Using the transcription factor inhibitor of DNA binding 1 to selectively target endothelial progenitor cells offers novel strategies to inhibit tumor angiogenesis and growth[J].Cancer Res, 2010, 70(18):7273-7282

[5] Yu XL, Jing T, Shang FF, et al. Down regulation of Id1 by small interfering RNA in prostate cancer PC3 cells in vivo and in vitro[J]. Eur J Cancer Prev, 2011, 20(1):9-17

[6] Healey MA, Deaton SL, Alder JK, et al. Id1 overexpression is independent of repression and epigenetic silencing of tumor suppressor genes in melanoma[J]. Epigenetics, 2010, 5(5):410-421

[7] Dong Z, Liu S, Zhou C, et al. Overexpression of Id-1 is associated with tumor angiogenesis and poor clinical outcome in oral squamous cell carcinoma[J]. Oral Oncol, 2010, 46(3):154-157

[8] Electron K, Miao P, Patrick A. et al. Id1 Gene Expression Is Up-Regulated in Hyperplastic and Neoplastic Thyroid Tissue and Regulates Growth and Differentiation in Thyroid Cancer Cells [J]. J. Clin. Endocrinol. Metab, 2004, 89(12): 6105-6111

[9] Laleh K, John RG, Shiva SF, et al. Increased expression of id family proteins in small cell lung cancer and its prognostic significance[J]. Clinical Cancer Research, 2008, 14(8):2318-2325

[10] Zhang X, Ling MT, Feng H, et al. Id-I stimulates cell proliferation through activation of EGFR in ovarian cancer cells [J]. Br J Cancer, 2004, 91(12):2042-2047

[11] Anido J, Sá ez B A, Gonzà lez J A, et al. TGF-β Receptor Inhibitors Target the CD44 (high)/Id1 (high) Glioma-Initiating Cell Population in Human Glioblastoma [J]. Cancer Cell, 2010, 18(6): 655-668

[12] Huang Y, Liu Q, Liu K, et al. Suppression of growth of highly-metastatic human breast cancer cells by norcantharidin and its mechanisms of action[J].Cytotechnology, 2009, 59(3): 201-208

[13] Hill TA, Stewart SG, Ackland SP, et al. Norcantharimides, synthesis and anticancer activity: Synthesis of new norcantharidin analogues and their anticancer evaluation [J].Bioorg Med Chem, 2007, 15(18): 6126-6134

[14] Luan JL, Duan HY, Liu Q, et al. Inhibitory effects of norcantharidin against human lung cancer cell growth and migration[J]. Cytotechnology,2010, 62:349-355

[15] Arocho A, Chen B, Ladanyi M, et al. Validation of the 2-DeltaDeltaCt calculation as an alternate method of data analysis for quantitative

PCR of BCR-ABL P210 transcripts [J]. Diagn Mol Pathol, 2006, 15 (1):56-61

[16] Zebedee Z, Hara E. Id proteins in cell cycle control and cellular senescence[J]. Oncogene,2001, 20(58):8317-8325

[17] Cheng YJ, Tsai JW, Hsieh KC, et al. Id1 promotes lung cancer cell proliferation and tumor growth through Akt-related pathway [J]. Cancer Lett,2011, 307(2):191-199

[18] Bhattacharya R, Kowalski J, Larson AR, et al. Id1 promotes tumor cell migration in nonsmall cell lung cancers [J]. Journal of Oncology, Published online 2010,2010:1-8

[19] Fan YZ, Fu JY, Zhao ZM, et al. Effect of norcantharidin on proliferation and invasion of human gallbladder carcinoma GBC-SD cells[J]. World J Gastroenterol, 2005, 11(16):2431-2437

去甲斑蝥素对 H446 细胞 Id1mRNA 表达的影响

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摘要 目的:观察去甲斑蝥素对小细胞肺癌 H446 细胞 Id1mRNA 表达的影响。方法:分别利用 MTT 法检测细胞生长活性;用划痕实验分析细胞迁移能力;采用 Hoechst 染色观察细胞凋亡;用实时荧光 RT-PCR 法测定 H446 中 Id1mRNA 的表达。结果:去甲斑蝥素对 H446 细胞的生长有明显的抑制作用,细胞的生长抑制率和凋亡率明显增加,细胞迁移距离明显缩短。去甲斑蝥素可抑制细胞内 Id1mRNA 的表达,其相对定量随去甲斑蝥素的浓度增大而减少。结论:在 H446 细胞中,去甲斑蝥素能抑制 Id1mRNA 的表达,这可能是去甲斑蝥素抑制细胞生长,迁移和诱导细胞凋亡的重要机制之一。

关键词:去甲斑蝥素;Id1;H446 细胞

中图分类号:R734.2 **文献识别码**:A **文章编号**:1673-6273(2012)07-1225-04

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(**收稿日期**:2011-09-03 **接受日期**:2011-09-30)