

Molecular mechanisms of paclitaxel and NM-3 on human gastric cancer in a severe combined immune deficiency mice orthotopic implantation model

Jin-Shui Zhu, Ming-Quan Song, Guo-Qiang Chen, Qin Li, Qun Sun, Qiang Zhang

Jin-Shui Zhu, Ming-Quan Song, Guo-Qiang Chen, Qin Li, Qun Sun, Qiang Zhang, Department of Gastroenterology, Affiliated Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China

Guo-Qiang Chen, Chinese academy of science laboratory animals' center, Shanghai Jiaotong University, Shanghai 200233, China

Supported by Natural Science Foundation of Shanghai, No. 02ZB14072

Correspondence to: Jin-Shui Zhu Professor, Department of Gastroenterology, Affiliated Sixth People's Hospital, Shanghai Jiaotong University, Shanghai 200233, China. zhujs1803@hotmail.com

Telephone: +86-21-64369181 Fax: +86-21-64837019

Received: 2007-04-26 Accepted: 2007-05-12

Abstract

AIM: To explore the molecular mechanisms of action of paclitaxel and NM-3 on human gastric cancer in severe combined immune deficiency (SCID) mice.

METHODS: Human gastric cancer cells SGC-7901 were implanted into SCID mice and mice were treated with paclitaxel and NM-3. The effects of paclitaxel and NM-3 on apoptosis of human gastric cancer cells were analyzed using flow cytometry, TUNEL assays, and DNA fragment analyses.

RESULTS: Apoptosis of SGC-7901 cells was successfully induced by paclitaxel, NM-3, and the combination of paclitaxel and NM-3 24 h after injection as shown by the presence of apoptotic hypodiploid peaks on the flow cytometer before G1-S and a characteristic apoptotic band pattern in the DNA electrophoresis. The apoptotic rate detected by TUNEL assay was found to be significantly higher in the paclitaxel/NM-3 compared to the control group ($38.5\% \pm 5.14\%$ vs $13.2\% \pm 1.75\%$, $P < 0.01$).

CONCLUSION: Paclitaxel in combination with NM-3 is able to induce apoptosis of the human gastric cancer cells in SCID mice effectively and synergistically.

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Key words: Gastric cancer; NM-3; Paclitaxel

Zhu JS, Song MQ, Chen GQ, Li Q, Sun Q, Zhang Q.

Molecular mechanisms of paclitaxel combined with NM-3 on the orthotopic implantation model with human gastric cancer in severe combined immune deficiency mice. *World J Gastroenterol* 2007; 13(30): 4131-4135

<http://www.wjgnet.com/1007-9327/13/4131.asp>

INTRODUCTION

Gastric cancer is the common leading cause of cancer death worldwide. Its clinical behavior depends on its capacity to establish metastases of the tumor, and prognosis of advanced gastric cancers is poor. To date, several molecules have been reported to play an important role in gastroenterological tumorigenesis and metastasis^[1-3], but the molecular mechanisms remain to be elucidated^[1-3].

In previous studies using LMD, P²⁷-based RNA amplification, and cDNA microarray, we identified some differentially expressed genes between primary carcinoma cells and lymph node metastatic cells in two patients. Moreover, we further identified four differentially expressed genes in progression of gastric cancer in another group of 15 patients by means of semiquantitative reverse transcribed polymerase chain reaction (RT-PCR), and the expression patterns of these four genes were similar to tumor suppressor genes or oncogenes.

It is now widely accepted that many malignant tumors contain subpopulations of heterogeneous cells. This heterogeneity is exhibited in a wide range of genetic, biochemical and immunologic characteristics. It is likely that specific tumor cells or colonies within the larger heterogeneous tumor specimen are the forerunners of distant metastases^[4]. Thus, many biologic differences might exist between tumor cells in primary and metastatic lesions. Furthermore, the interaction of tumor cells with their living environment may add more differences between these two groups of cells^[2]. As a result, tumor metastasis related genes can be identified by comparing the gene expression profiles between them.

Apoptosis plays a crucial role in the proliferation and turnover of cells in various malignant tumors, and it is enhanced by many anticancer drugs as cytotoxic drugs, hormones, or some recombinant gene, medicine, etc. At present, there is no effective therapy for advanced gastric cancer, as the other malignant tumor, gastric cancer is not only a disease with abnormal cell proliferation and

differentiation, but also a disease with abnormal apoptosis. Thus, the enhanced induction of apoptosis in human gastric cancer cells will be needed to explore. Paclitaxel enhanced the expression of smad3 and smad4 in SCID mice, smad3 and smad4 are kind of multifunction cyclin dependent kinase inhibitors. They play a negative role in cell cycle regulation by inhibiting transition from G0/G1 to S phase. Based on previous studies, we examined the apoptotic indices of human gastric cancer grafted into SCID mice. We investigated its apoptotic effects on human gastric cells, by which we explored its correlated anticancer mechanisms and its synergistic effect combined with NM-3 in order to look for a novel therapy for advanced gastric cancer.

MATERIALS AND METHODS

Materials

RPMI1640 media and TRI201 total RNA isolation kit were purchased from Gibco BRL. The liposome, the trypsin, DMEM culture medium, Hepes and Csc1, 200 bp DNA ladder, dNTP, Taq enzyme and the restriction endonuclease were obtained from Sigma Co.

Drugs and reagents

Paclitaxel was obtained from the Chinese Academy of Science. NM-3 was provided by Doctor Robert, Huston University, USA.

Animal model

Male severe combined immune deficiency (SCID) mice were obtained from Shanghai Experimental Animal Center of Chinese Academy of Sciences. Animals used were 6-7 wk old and weighed 18-22 g. Human gastric cancer SGC-7901 (Shanghai Tumor Institution No: 01842), a poorly differentiated adenocarcinoma cell line, was originally derived from a primary tumor and maintained by passage in the subcutis of nude mice. Animal models were made using orthotopic implantation of histological intact tissue of human gastric carcinoma. Tumors were resected aseptically. Necrotic tissues were cut and the remaining healthy tumor tissues were scissor minced into pieces (about 5 mm × 7 mm in diameter) in Hank's balanced salt solution. Each tumor piece was weighed and adjusted to 50mg. Mice were anesthetized with 4.3% trichloraldehyde hydrate. An incision was made through the left upper abdominal pararectal line. Then the peritoneal cavity was carefully exposed and a part of the serosal membrane in the middle of the greater curvature of the stomach was mechanically injured using scissors. A tumor piece of 150 mg was fixed on each injured site of the serosal surface. The stomach was returned to the peritoneal cavity, and the abdominal wall and skin were closed.

After 12 d, when the tumor reached the size of 0.8-1.0 cm³, mice were randomly separated into four groups, with ten mice per group. Body weight and tumor volume of these orthotopic grafted mice in every group had no obvious difference ($P < 0.05$). *Via* intraperitoneal injection animals received paclitaxel (5 mg/kg), NM-3 (10 mg/kg), paclitaxel (5 mg/kg) combined with NM-3 (10 mg/kg), or

normal saline, respectively. 24 h later mice were sacrificed by cervical dislocation. Samples of the tumor were immediately frozen in liquid nitrogen for later use. Part of the fresh samples were immediately placed into Eppendorf tubes for flow cytometry analysis.

Ten mg of fresh tissue from every animal was minced with blades to millimeter sizes in tissue medium (RPMI 1640). The supernatant was separated and filtered through a 50-mm nylon mesh. The filtered cells were collected by centrifugation and washed twice with PBS.

Detection of cell apoptosis by TUNEL method

Six cell suspensions (1×10^4 cells) of each group (NM-3, paclitaxel, paclitaxel/NM-3, and saline) were placed separately into 60-mm dishes containing cover glass slides (washed and high-pressure sterilized). Then the glass slides were taken out, washed twice with PBS, and fixed in methanol: freezing acetic acid (3:1) for 30 min. The following procedures were carried out according to the kit instruction. The average number of apoptotic cells was determined by counting 1000 cells on each glass slide and the apoptotic index (AI), i.e. the number of apoptotic cells per 100 cancer cells, was calculated.

Chemical staining of X-gal

1×10^6 /L cells of four groups were fixed by 0.5% glutaral pentanediol for 15 min and washed thrice with PBS. X-gal staining solution (20:1) was added and cells were incubated at 37°C for 4-24 h in a humidified atmosphere containing 50 mL/L CO₂. Blue-stained cells, i.e. those with *LacZ* gene expression, were counted under the microscope and the percentage of the positive cells was calculated.

Detection of the expression of the p27mt gene

5 mg fresh tissue specimens of every mouse in four groups were digested by 0.5 g/L trypsin. The cells were collected and washed twice with PBS. After cell lysis in 500 μL SDS-PAGE cell lysis solution and boiling for 5 min, supernatants were collected after centrifugation. Samples were subjected to western blot analysis.

Detection of cells by flow cytometry

Fresh tissue specimens of the four groups were minced with blades to millimeter sizes in tissue medium (RPMI 1640). Then supernatant was separated and filtered through a 50-mm nylon mesh. The filtered cells were collected by centrifugation, stained, washed twice with PBS, and cell suspension was adjusted to a density of 10^6 /L. 100 μL of cell suspension was mixed with 200 μL of DNA-PREP™ LPR, followed by detection using Coulter Epics XL flow cytometer for 15 min. Cell cycle progression and cell apoptosis rate were analyzed.

DNA fragment analysis

SGC-7901 cells, after being collected and washed twice with PBS, were lysed in 500 μL cell lysis solution [1%Np40, 20 mm/L EDTA, 50 mmol/L Tris-HCl (pH 7.5)] in the presence of 10 μL of protease K. Samples were heated in a 56°C water bath for 1-2 h before extraction with phenol/chloroform. After the DNA precipitate had

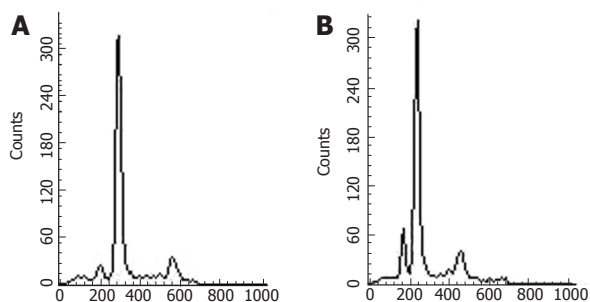


Figure 1 Apoptotic curve line of multidrug-resistant SGC-7901 cells was induced by NM-3 and NM-3 combined with paclitaxel, first apoptotic peak (B) was elevated compared with that of NM-3 group (A).

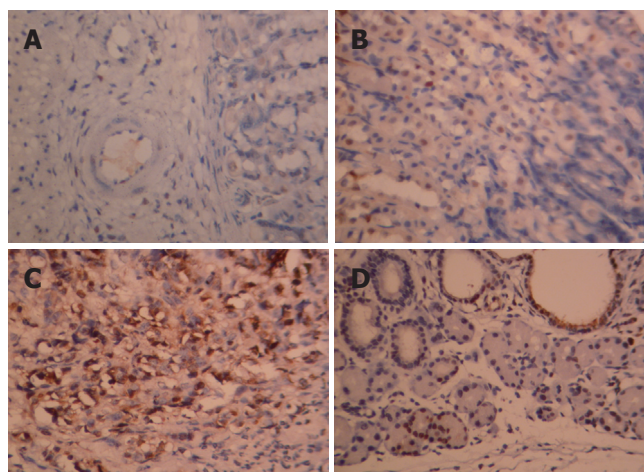


Figure 2 Detection of apoptotic cells by TUNEL method. A: The apoptotic cells appeared yellow in the control group; B: The apoptotic cells appeared yellow in the NM-3 group; C: The apoptotic cells appeared yellow in the paclitaxel group; D: A large number of apoptotic cells appeared in paclitaxel/NM-3 group.

been washed once with 700 mL/L alcohol, 200 μ L of TE was added followed by an overnight incubation with RNase (final concentration 50 μ L/mL) at 37°C. The final DNA was separated by agarose gel electrophoresis (10 g/L) and visualized with the aid of an ultraviolet light lamp.

Statistical analysis

Data were analyzed by *t* test and a *P* value < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS 11.5 for windows.

RESULTS

Detection of cell apoptosis by TUNEL method

The nucleus of apoptotic cells was dark stained, dark brown and the cytoplasm was concentrated and the cell shrank. The AI of samples of the paclitaxel/NM-3 and the control group was 82.6% (\pm 3.12%) and 5.0% (\pm 0.35%), respectively. This difference was statistically significant (*P* < 0.05) showing that paclitaxel/NM-3 could obviously induce apoptosis of gastric cancer cells (Figure 1 and Figure 2).

Cell cycle progression is shown in Table 1. Whereas

Table 1 Effects of paclitaxel/NM-3 on the cell cycle progression (mean \pm SD)

Group	G ₀ /G ₁	S	G ₂ /M
Control	24.13 \pm 1.12	40.12 \pm 1.09	32.17 \pm 1.48
NM-3	34.42 \pm 3.51	22.41 \pm 2.13	18.23 \pm 3.23
Paclitaxel	26.34 \pm 0.35 ^a	39.20 \pm 0.39 ^a	33.10 \pm 0.89 ^a
Paclitaxel/NM-3	87.42 \pm 3.17 ^b	9.12 \pm 0.22 ^b	15.12 \pm 2.40 ^b

^a*P* > 0.05 vs saline group; ^b*P* < 0.01 vs paclitaxel group).

Table 2 Effects of paclitaxel/NM-3 on the cell apoptosis (mean \pm SD)

Group	AI
Control	13.24 \pm 7.75
NM-3	25.62 \pm 6.46 ^a
Paclitaxel	28.90 \pm 5.38 ^a
Paclitaxel/NM-3	38.51 \pm 5.14 ^b

^a*P* < 0.05; ^b*P* < 0.01 vs control group.

the number of cells in G₀/G₁ phase is lower in the paclitaxel and in the control group compared to the others, the percentage of the S phase cells is higher, indicating that transition time of cell cycle was shortened and cell proliferation was active. However, the percentage of G₀/G₁ phase cells increased and the cell cycle was arrested in G₀/G₁ phase in the paclitaxel/NM-3 group, which was significantly different between the control and the paclitaxel group (Table 1 and Table 2).

X-gal chemical staining:

The adenovirus mediated gene transfer rate was evaluated by X-gal staining. The results showed that the infection efficiency could reach 90%, indicating that recombinant adenovirus could effectively transfer genes *in vitro*.

The expression of p27 protein was evaluated after being injected into the cancer cells with paclitaxel *in vitro*: After the cells that could be used in experiment were injected by paclitaxel/NM-3 for 24 h, these cells were collected and lysed with 1 \times SDS PAGE cell lysis solution. After boiling at 100°C for 5 min, the solution was centrifuged. The supernatant was collected and the protein was detected by TMB system western blot kit, KPL USA. Followed by X-gal chemical staining, there was high expression of a 27 kDa protein in the paclitaxel/NM-3 group while only slight traces were seen (endogenous expression) in the paclitaxel and in the control group. Thus, the human mutant p27 recombinant adenovirus constructed in the present study expresses the p27 gene properly in SGC-7901 cells and the protein product could be expressed at a high level in cells.

PCR identification and effect of paclitaxel/NM-3 on p27mt target gene

The pathological change of SGC-7901 cells and their culture fluid were collected and centrifuged. Five milliliters of the supernatant was mixed with 1 mg of protease K, 2 mL of 1% SDS, 10 mmol/L EDTA and 20 mmol/L

Tris-HCl to be digested for 2 h. After being precipitated by dehydrated alcohol, the DNA was collected. PCR reaction was carried out after adding the forward and reverse primers. Finally, a 275-bp target fragment was amplified, which showed that paclitaxel, paclitaxel/NM-3 had already played an inhibitory role on the p27mt target gene.

Detection of cells by flow cytometry

After the animals had been treated with NM-3, paclitaxel, paclitaxel/NM-3, or saline for 24 h, the detection of apoptotic cells was carried out by flow cytometry and repeated six times. The mean value of hypodiploid cells was: 8.23%, 4.67%, 41.0%, and 1.96%, respectively. Statistical analysis revealed a significant difference between the paclitaxel/NM-3 group and the paclitaxel group and the NM-3 group, (paclitaxel/NM-3 *vs* paclitaxel group, paclitaxel/NM-3 group, NM-3 group and paclitaxel group *vs* normal saline group, $P < 0.01$; paclitaxel group *vs* NM-3 group, $P > 0.05$).

Detection of DNA fragment

The result of DNA electrophoresis showed intact genomic DNA in samples obtained from the paclitaxel and the normal control group while there was an obvious 180-200 bp diploid 'trapezia' pattern in samples derived from NM-3 or the paclitaxel/NM-3 injected group, which was in concordance with the characteristic changes of apoptosis.

DISCUSSION

Since paclitaxel was extracted from Taxol which has special anti-tumor effect, paclitaxel had cell toxic side-effects as Taxol either. We found that paclitaxel could inhibit the activation of CyclinE-CDK2 complex and the activation of CyclinD-CDK4 and CyclinA-C DK2 complex as well. In addition, it could also down-regulate the expression of CyclinB, because the arrest of G₀/G₁ was mainly caused by p27 whose accumulation could be induced by exogenous signal. Gastric tumorigenesis was closely correlated with translocation, deletion and mutation of p27 gene and its expression or activity changes^[2,5]. At present, gastric cancer cells treated by paclitaxel/NM-3 in SCID mice were not reported. If the expression level of p27 mt in gastric cancer cell was down-regulated or inhibited by p27, thus the DNA damaged cell could not transit from G₁ phase to S phase directly, that would induced the apoptosis of human gastric cancer cells in SCID mice.

Paclitaxel was first found in 1998, the researchers revealed it effected on cell contact inhibition with TGF- β ^[4]. The degradation of the p27 protein is mainly caused by phosphorylation of the threonine residue at position 187 which is mediated by ubiquitin^[6-8]. Kudo and his colleagues^[9] found that if the 187th threonine of p27 which was mediated by p27 protein, could inhibit cell growth obviously, these inhibitory effects were more obvious on mutant p27 (T187A) than on wild-type p27. The target phosphorylation site of CDK would be protected from phosphorylation. A replication-deficient recombinant adenovirus was constructed which carried p27mt to study apoptosis of the gastric cancer cell line, by which it expected to find a more effective p27 gene

to treat gastric cancer. Koguchi K^[11] reported that they prohibited the viability of the astrocyte when these cells were transfected with exogenous gene p27 carried by adenovirus. Zhang D^[12] reported that the upregulation of the expression of the p27 by retinoic acid significantly inhibited the growth of the oophoroma cell. Koh TY^[13] found high expression of p27 and raised the expression level of cyclinD1 and cyclinE in the cell lines SUN-1066, SUN-1041, SUN-1076 derived from cephalocervical squamous cell carcinoma by transfecting these cells with p27kip1 carried by reconstructed adenovirus, the cancer cells' proliferation were significantly prohibited and the cell cycle analysis showed that the cancer cells were mainly stopped at the G₁-S stage in their study. All this showed that p27 was a very important gene related to the development of carcinoma and had significant impact on the onset, development and prognosis of the tumor.

Nowadays, functional reconstruction of anti-oncogene has been a reasonable strategy of gene therapy for tumor. Sasaki T^[14] found that p27mt had stronger suppressive effects than p27wt on apoptosis and cell proliferation when they applied adenovirus-induced p27mt and p27wt to transfect the cholangiocarcinoma cell lines TFK-1 and HuCCT-1. Park KH^[15,16] got the same results when they used adenovirus mediated p27mt and p27wt to transfect lung cancer cell lines NCI H460, NCI H1264, NCI H358 and NCI H157, and a spongioblast line. In our study, mutant p27 was used to transfect gastric cancer cells and the high expression of this gene was proven by p27 polyclonal antibody. This result supported that adenovirus with reconstructed p27mt could transfect the target gene into a gastric cancer cell which derived from human gastric adenocarcinoma tissue, developed into tumor in SCID mice and expressed endogenous p27. By flow cytometry, rate of apoptosis up to 41.0% in paclitaxel/NM-3 group was proven which had significant difference compared to the control group, DNA analysis showed 180-200bp DNA ladder. By TUNEL technique, a value of apoptotic index up to 82.6% was detected which showed significant difference compared to the control group. The results showed that the gene p27 was an important gene related to the occurrence of the large gastric carcinoma and the down-regulation of p27 may be the main cause of cell differentiation dysfunction and apoptosis dysfunction. Upregulating the expression of p27 by mutant p27, which promotes the apoptosis of the tumor cell, could serve as a new scheme in the treatment of advanced gastric carcinoma. Circle analysis showed that the cleavage of tumor cells was stopped at G₁ stage via suppressing the activity of the cyclin/CDK kinase by p27mt. Winteringham LH^[17] thought that the accumulation of p27 played an important role in apoptotic mechanisms of the gastric cell cycle arrest at the initiation of cell differentiation. Whether other apoptotic factors might affect this process should be explored further.

Recently, although the apoptosis of the human gastric cancer cell was successfully induced by the application of NM-3 and other gene therapy, but the apoptosis of human gastric cancer cells induced by paclitaxel/NM-3 had not been reported in the SCID mice model^[18,19], it was very useful experimental support of tumor-suppressed function

for human gastric cancer treated by paclitaxel/NM-3. The efficacy of this method and the mechanism of apoptosis strongly indicate that paclitaxel/NM-3 is synergistically effective against human gastric carcinomas.

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S- Editor Liu Y L- Editor Mihm S E- Editor Wang HF