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Effect of Verapamil on the Expression of EGFR and NM23 in A549 Human Lung Cancer Cells

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Abstract. *Despite the advances in the detection and treatment of lung cancer, the overall 5-year survival is only 10-20%. Accumulating evidence suggests that verapamil, a calcium channel antagonist, is a potential anticancer agent. Epidermal growth factor receptor (EGFR) is a key therapeutic target in many types of cancer, whereas nm23 is a putative metastasis suppressor gene. In this study, the effect of verapamil on the expression of nm23 and EGFR in A549 human lung cancer cells was investigated by quantitative real-time reverse transcription-polymerase reaction and immunohistochemical assays. The expression of EGFR and nm23 was also determined in lung cancer patients. Verapamil significantly reduced EGFR expression at both the mRNA and protein levels in A549 cells ($p<0.01$). Verapamil also significantly increased the protein levels of nm23 in these cells ($p<0.01$), although the mRNA levels of nm23 were not changed after verapamil treatment. Furthermore, the expression of EGFR in human lung cancer tissues was significantly higher than in normal lung tissues ($p<0.001$). However, the expression of nm23 was not different between lung cancer and normal tissues. Our data suggest that verapamil may regulate the expression of EGFR and nw23 in lung cancer cells by transcriptional and post-transcriptional levels, respectively. EGFR may be a promising therapeutic molecular target for lung cancer treatment using verapamil and/or chemotherapeutic agents.*

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Despite the advances in the detection and treatment of lung cancer, the overall 5-year survival still remains grim. Lung cancer is also a leading cause of malignancy-related deaths in China, and the 5-year patient survival rate remains at 14%, despite diagnostic imaging and therapeutic improvements over the past decades. The frequency of different types of lung cancer is also changing. Adenocarcinoma has become the most frequent histological type (approximately 50%), while squamous cell carcinoma was previously the most common, and accounts for approximately one third of all lung cancer cases, while small cell cancer comprises 15% (1).

Calcium (Ca^{2+}) channel antagonists, such as verapamil, are known to augment the effects of chemotherapy drugs in many different types of cancer (1). Although verapamil was initially thought to have anticancer effects by interference with Ca^{2+}-dependent secondary messenger systems, it appears that other mechanisms account for this effect. It was also reported that verapamil-treated meningiomas were less vascular and smaller, with lower cell proliferation and greater apoptosis (2). In addition, adriamycin resistance of human breast cancer cells was reversed by hyperthermia combined with verapamil and interferon alpha (3). These results indicate that verapamil is a potential anticancer agent or adjuvant in cancer treatment. However, the mechanisms of the anticancer action of verapamil are not fully understood.

Growth factors secreted by either host or tumor cells play a major role in tumor cell progression. Besides stimulating cell division, growth factors may also stimulate cell migration and modulate matrix metalloprotease production. In particular, epidermal growth factor receptor (EGFR) is highly expressed in a variety of tumors, including lung cancer, and plays an important role in tumor growth, infiltration and metastasis.

The nm23 gene family is highly conserved among a wide variety of eukaryotic species (4). The identification of nm23, the first metastasis suppressor gene, indicated the existence of genes that specifically regulate metastasis (5, 6).

In this study, we investigated the effect of verapamil on the expression of EGFR and nm23 in A549 human lung cancer cells by quantitative real-time transcription-polymerase reaction (qRT-PCR) and immunohistochemical staining. We also validated the expression of EGFR and nm23 in lung cancer patients.

Materials and Methods

Reagents. Muriatic acid verapamil was obtained from Shanghai Harvest Pharmaceutical Co., Ltd. (Shanghai, P.R. China). Mouse anti-EGFR and anti-nm23 monoclonal antibodies (mAb) were purchased from Maixin Bio Co. (Fuzhou, P.R. China)

Cells and treatments. A549 human lung cancer cells were purchased from the American Type Culture Collection (Rockville, MD, USA). A549 cells were maintained in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% calf serum, penicillin (10 U/100 μl) and streptomycin (10 mg/100 μl) in a humidified atmosphere containing 5% CO_2 at 37˚C. A549 cells were seeded in 10-cm dishes at (1.5×10^6) cells per dish and cultured for 24 h. Cells were then treated with verapamil at 10-40 μg/ml for 72 h. Verapamil was resolved in PBS and added to medium by concentration accordingly. Following treatment, cells were harvested from the substrate using 0.05% trypsin and 0.02% EDTA, and washed twice with phosphate-buffered saline (PBS). The treated cells were used for RNA extraction.

Patients. Twenty lung cancer and normal lung tissue samples were obtained from patients with lung adenocarcinoma by curative surgical treatment at the Second Affiliated Hospital of Qiqihaer Medical College between 2006 and 2007. Patients who received preoperative (neoadjuvant chemotherapy and radiation therapy) therapy were excluded. Clinical information was obtained from a review of hospital and physician charts. This study was performed after approval by a local Human Investigations Committee. Informed consent was obtained from each patient.

Real-time RT-PCR. Total RNA was extracted from A549 cells using RNAiso Reagent (Takara Biotechnology, Dalian, P.R. China) and cDNA was synthesized with the First-Strand cDNA Synthesis kit (Takara Biotechnology, Dalian, P.R. China), as described previously (7). The primer sets for EGFR and nm23 were as follows: EGFR forward 5'-ACTCCAAGCCTGGGACCATC-3' and reverse 5'-TCCACAGAATCACTGCCATGTATAA-3'; nm23 forward GGTGC GAATGACAGTAGCATTATGA-3' and reverse 5'-AAAGGT GGGCTCCTAACTAGCTGAA-3'. The qRT-PCR was carried out using LightCycler Fast-Start DNA Master SYBR Green 1 (Roche Applied Science, Indianapolis, IN, USA). The protocol was 1 cycle of 95˚C for 10 s, 40 cycles of 95˚C for 5 s and 60˚C for 31 s. Quantitative analysis of the data was performed using ABI PRISM 7300 software (Applied Biosystems, Foster City, CA, USA). Standard curves for templates of EGFR, nm23 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were generated by serial dilution of the PCR products.

Immunohistochemical staining. The protein expression of nm23 and EGFR in A549 cells was examined by immunohistochemical assay. Briefly, the cells were seeded in cover slides prepared in 12-well plates for 24 h. The sterile glass cover slides were placed in cell culture dishes and the cells grew on the top of the cover slides. The cells were then treated with verapamil at 10-40 μg/ml for 72 h. Following treatment, the cells were stained using the Ultra Sensitive™ Immunohistochemical Staining Kit (Maixin Bio, Fuzhou, P.R. China) according to the manufacturer's instructions. The intensity of the immunoreactivity was analyzed by integral optical intensity (IOD) with Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). Five fields were selected from each slide to measure the optical intensity.

EGFR and nm23 expressions in lung cancer samples and paired normal tissue were also examined using immunohistochemistry, as described elsewhere (8). Briefly, antigen retrieval was performed by microwave heating sections in 10 mM sodium citrate buffer (pH 6) for 10 min. After endogenous peroxidase activity was quenched and nonspecific binding was blocked, the slides were incubated at 4˚C overnight with monoclonal antibody anti-EGFR and anti-nm23 mAbs. The secondary antibody was biotinylated rabbit anti-mouse antibody (Dako, Carpinteria, CA, USA) used for 30 min at 37˚C. After further washing with Tris-buffered saline, sections were incubated with complex/horseradish peroxidase (1:100 dilution) for 30 min at 37˚C. Immunolocalization was performed by immersion in 0.05% 3,3'-diaminobenzidine tetrahydrochloride as chromagen. Slides were counterstained with hematoxylin before dehydration and mounting. The intensity of the immunoreactivity was analyzed by IOD with Image-Pro Plus (Media Cybernetics).

Statistical analysis. All determinations were repeated three times and results are expressed as the mean±SD. Quantitative experiments were analyzed by the Student's *t*-test. Statistical software SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used and $p<0.05$ was considered statistically significant.

Results

Effect of verapamil on nm23 and EGFR protein levels in lung cancer cells. The protein expression of EGFR was slightly down-regulated when A549 cells were treated for 72 h with low and medium concentrations of verapamil (10 and 20 μg/ml). When the concentration of verapamil was increased to 40 μg/ml, however, the expression of EGFR was significantly down-regulated compared with that of controls ($p<0.001$). In contrast, the up-regulation of nm23 expression was observed with medium and high concentrations of verapamil (20 and 40 μg/ml) ($p<0.001$), although a low concentration of verapamil (10 μg/ml) did not affect expression (Table I and Figure 1).

Effect of verapamil on EGFR and nm23 mRNA levels in lung cancer cells. Because the treatment with verapamil down-regulated the expression of EGFR and up-regulated the expression of nm23, we further examined by qRT-PCR whether treatment of lung cancer cells with verapamil regulates mRNA levels of EGFR and nm23. The mRNA levels of EGFR significantly decreased when A549 cells were treated for 72 h with verapamil (10-40 μg/ml) ($p<0.01$) (Table II and Figure 2). In contrast, the mRNA levels of nm23 in A549 cells were not changed after verapamil treatment (10-40 μg/ml).

Table I. *Effect of verapamil on the protein levels of EGFR and nm23 in A549 cells.*

Group	Concentration of verapamil (μg/ml)	IOD (Mean±SD)	
		EGFR	nm23
Control	0	69.23±12.40	66.56.1±9.42
Low dose	10	58.16±13.90	69.52±6.16
Medium dose	20	57.51±15.10	105.54±11.21*
High dose	40	44.52±10.08*	106.14±14.49*

A549 cells were treated with or without verapamil at 10-40 μg/ml for 72 h, washed, and tested by immunohistochemical assay for the protein expression of EGFR and nm23; *$p<0.001$ *versus* control.

Table II. *Effect of verapamil on the mRNA levels of EGFR and nm23 in A549 cells.*

Group	Concentration of verapamil (μg/ml)	Relative value (Mean±SD)	
		EGFR/GAPDH	nm23/GAPDH
Control	0	1.01±0.14	1.02±0.20
Low dose	10	0.77±0.08*	1.03±0.10
Medium dose	20	0.34±0.04*	1.08±023
High dose	40	0.25±0.06*	1.17±0.17

A549 cells were treated with or without verapamil at 10-40 μg/ml for 72 h, washed, and tested by real time RT-PCR for the mRNA expression of EGFR and nm23; *$p<0.001$ *versus* control.

EGFR and nm23 expressions in lung cancer and normal lung tissues. We further examined by immunochemistry the expression of EGFR and nm23 in lung cancer and normal lung tissues derived from 20 patients. EGFR was expressed in the cytoplasm and membrane, and nm23 only in the cytoplasm of both normal lung tissue and lung cancer cells. EGFR expression was detected in 20 (100%) lung cancer samples (Figure 2). In contrast, EGFR expression was detected in 14 out of 20 (70%) normal lung tissue specimens. Nm23 expression was detected in 19 (95%) lung cancer samples and in 18 (90%) normal lung tissue samples, respectively. Furthermore, the IOD of EGFR in cancer tissues was significantly higher than that in normal tissues ($p<0.001$) (Table III). In contrast, the IOD of nm23 was not different between cancer and normal tissues.

Discussion

Accumulating evidence suggests that verapamil, a Ca^{2+} channel antagonist, is a potential anticancer agent (9). In this report, we provide, for the first time, evidence demonstrating that verapamil has a regulatory effect on EGFR and nm23,

Table III. *The expression of EGFR and nm23 in lung cancer and normal lung tissues.*

Group	IOD (Mean±SD)	
	EGFR	nm23
Normal lung tissue	27.02±4.03	40.88±6.21
Lung cancer tissue	55.45±11.38*	46.86±12.72

IOD of EGFP and nm23 expression in lung cancer and normal lung tissues was determined by immunohistochemical assay as described in Material and Methods section. *$p<0.001$, lung cancer *versus* normal lung tissues.

which play critical roles in tumor proliferation, invasion and metastasis. We found that verapamil down-regulated expression of EGFR, while up-regulating expression of nm23 in A549 human lung cancer cells. We also found that the expression of EGFR in lung cancer tissues was significantly higher than in normal lung tissues, although the expression of nm23 was not different between lung cancer and normal tissues. These findings suggest that verapamil might modulate the expression of EGFR and nm23, and that EGFR and nm23 may be involved in verapamil-mediated cytotoxicity.

Several reports have shown that Ca^{2+} channel antagonists, including verapamil, have anticancer effect by inducing cytotoxicitty and apoptosis in cancer cells, such as human colon cancer and breast cancer cells (10). It has been shown that verapamil reversed the resistance of cancer cells to some chemotherapeutic agents (11). It was also observed that multidrug-resistant cells have a greater intracellular concentration of Ca^{2+} than nonresistant cells, which contributes to their increased sensitivity to calmodulin antagonism compared with that of nonresistant cells (12). However, the mechanisms of verapamil anticancer activity is not fully understood.

EGFR is an important member of the tyrosine kinase receptor family and plays critical roles in cell growth, differentiation, motility and survival (13). It has been reported that EGFR was a promising therapeutic molecular target for cancer including non-small cell lung cancer (NSCLC). The tyrosine kinase inhibitor gefitinib, a synthetic anilinoquinazoline which targets EGFR, had a beneficial effect in some patients with refractory, advanced NSCLC (14). It was also reported that the sensitivity to growth inhibition by gefitinib in NSCLC cell lines under basal growth conditions was associated with dependence on protein kinase B/AKT and extracellular signal-regulated kinase (ERK) activation in response to EGFR signaling for survival and proliferation (15). It is therefore possible that verapamil might be capable of modulating the expression of EGFR in lung cancer cells. The present study showed that verapamil down-regulated both the

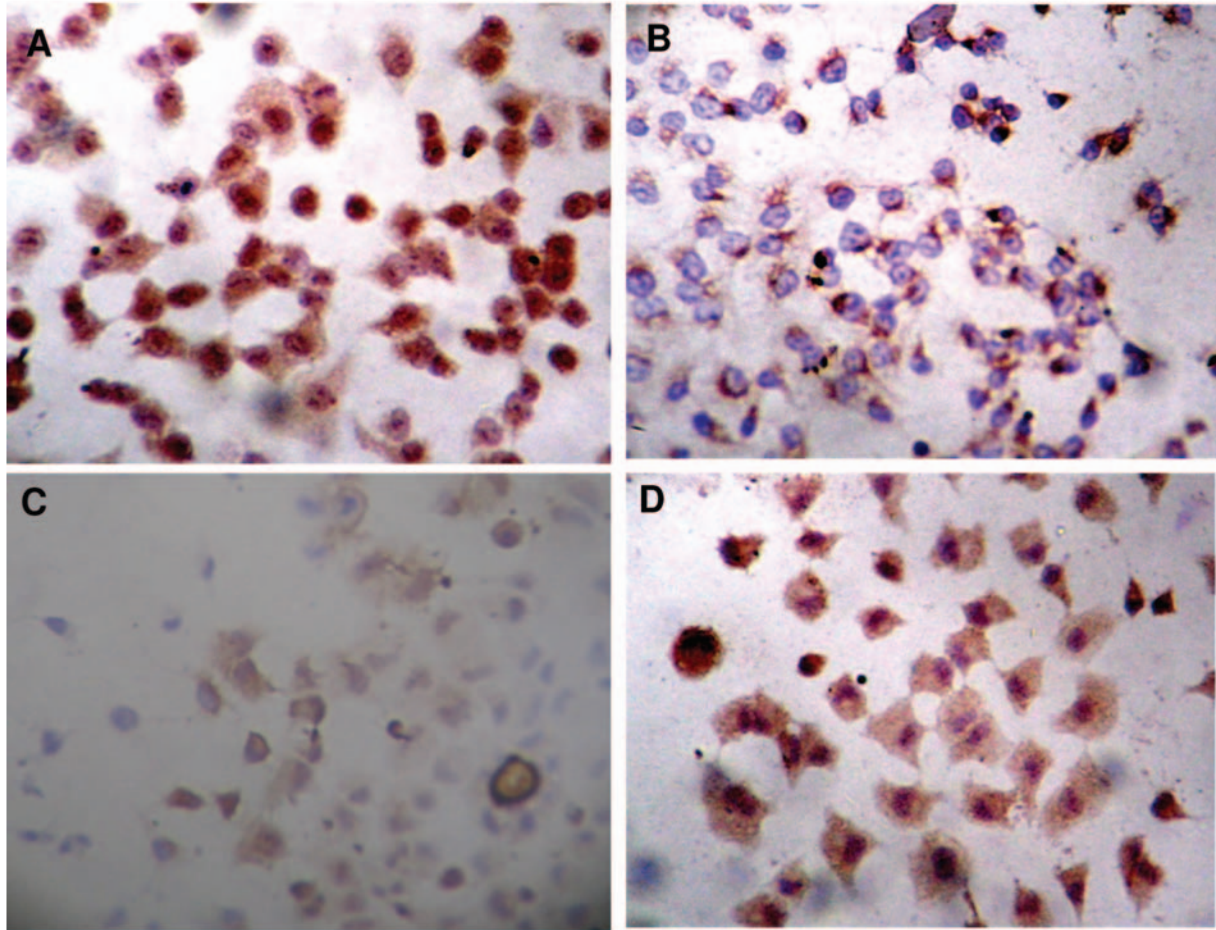

Figure 1. *Effect of verapamil on the expression of EGFR and nm23 in A549 cells. Cells were incubated with medium alone or with 40 μg/ml verapamil for 72 h. The expression of EGFR and nm23 was detected by immunocytochemical analysis (×200). A and B, EGFR expression; C and D, nm23 expression; A and C, medium only; B and D, 40 μg/ml verapamil. These figures are representative of three different experiments. (Magnification ×200.)*

mRNA and protein levels of EGFR expression in A549 human lung cancer cells. Furthermore, the expression of EGFR in lung cancer tissues was significantly higher than in normal lung tissues. These results suggest that verapamil might affect the expression of EGFR in lung cancer cells at the transcriptional levels and suggest that EGFR may be a promising target for molecular targeted therapy for lung cancer.

Nm23 is known to be associated with early onset of familial breast and ovarian cancer (16). Furthermore, an inverse relationship between nm23 expression and metastasis was also observed in various types of cancer (17). Although the mechanism by which nm23 regulates metastasis is not fully understood, the experimental data have shown that nm23 plays an important role in the regulation of metastasis in a number of types of human cancer (18). It was also reported that nm23 expression was a significant factor for predicting a favorable prognosis, suggesting an antimetastatic potential of nm23 in NSCLC (19, 20). In the present study, we found that treatment of A549 cells with verapamil significantly up-regulated protein levels of nm23, although there was no significant difference in the mRNA levels of nm23 between verapamil-treated and nontreated A549 cells. These results imply that verapamil might impact the expression of nm23 in lung cancer cells by posttranscriptional mechanisms and that nm23 may be involved in verapamil-mediated cytotoxity.

To our knowledge, this is the first report showing that verapamil down-regulates the expression of EGFR and up-regulates the expression of nm23 in human lung cancer cells. These results support a previous report that the inhibition of Ca^{2+} increase by the calcium chelator BAPTA inhibited EGFR transactivation in thrombin-stimulated vascular smooth muscle cells (21). Further study is needed to clarify whether verapamil induces cytotoxicity and apoptosis by down-regulation of EGFR and up-regulation of nm23 expression in other human lung cancer cell lines and primary lung cancer cells derived from patients. Nevertheless, the

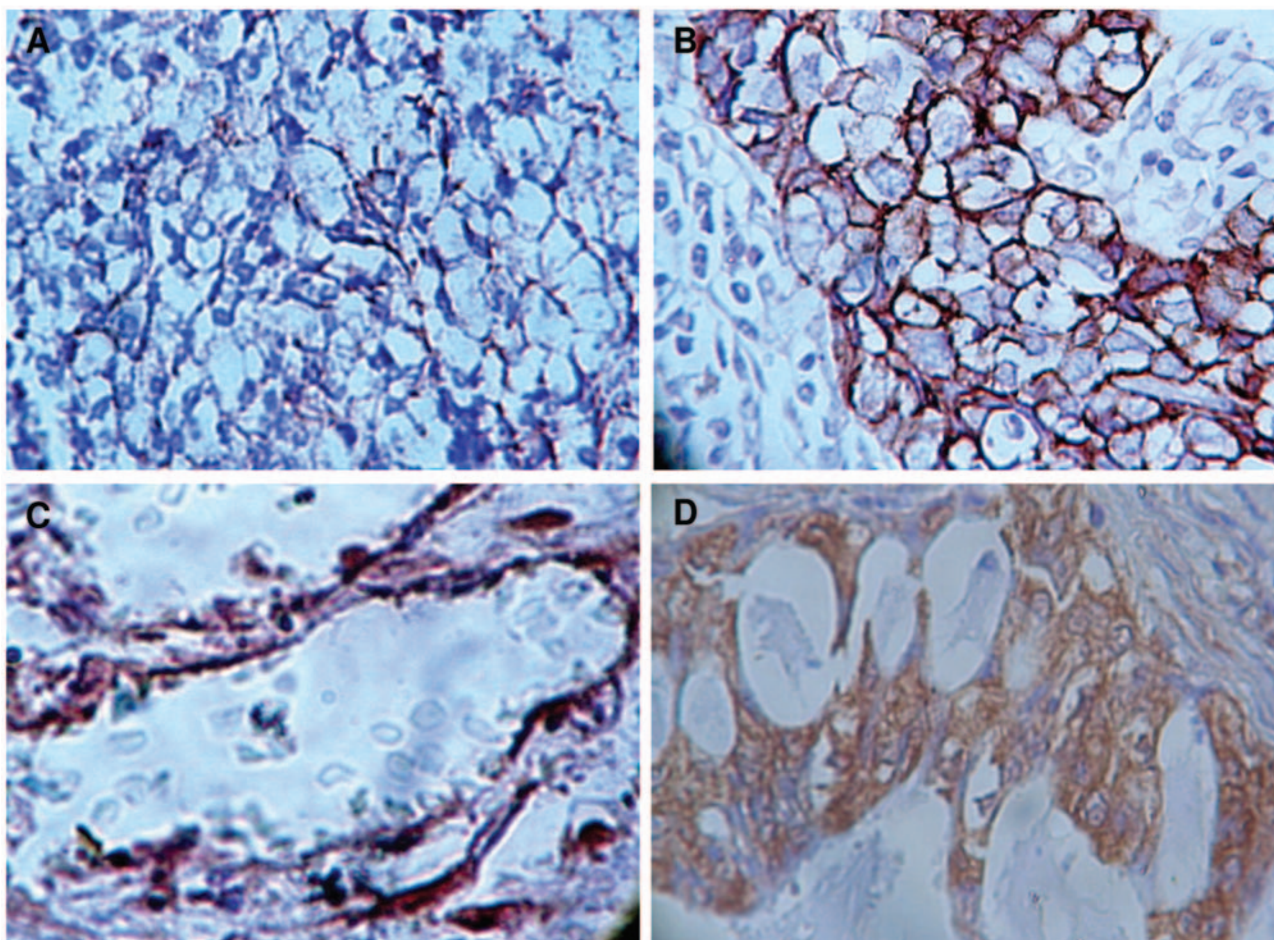

Figure 2. *Immunohistochemical staining for EGFR and nm23 in normal lung and lung cancer tissues. A, Negative EGFR staining in normal lung tissue; B, positive EGFR staining observed in the cytoplasm of lung cancer tissue; C, nm23 expression in normal lung tissue; D, nm23 expression in lung cancer tissue. These figures are representative of three different experiments. (Magnification ×200.)*

results obtained in this study suggest a new function of verapamil in cancer cells treated with verapamil.

The present study suggests that verapamil might regulate the expression of EGFR and nm23 in lung cancer cells by transcriptional and post-transcriptional levels, respectively. EGFR might be a promising therapeutic molecular target for lung cancer treatment using verapamil with chemotherapeutic agents.

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