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RAPID COMMUNICATION

Growth inhibitory effect of wild-type *Kras*2 gene on a colonic adenocarcinoma cell line

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Abstract

AIM: To observe the growth inhibitory effect of wild-type *Kras*2 gene on a colonic adenocarcinoma cell line Caco-2.

METHODS: Recombinant plasmid pCI-neo-*Kras*2 with wild type *Kras*2 open reading frame was constructed. The Caco-2 cells were transfected with either pCI-neo or pCI-neo-*Kras2* using Lipofectamine 2000. The expression of wild type *Kras*2 was examined by Northern blot analysis. And the expression of wild type *Kras*2 protein was examined by Western blot analysis. The effects of wild-type *Kras*2 on cell proliferation were analyzed by monotetrazolium (MTT) assay, meanwhile analyses of cell cycle and spontaneous apoptosis rate were carried out by flow cytometry (FCM).

RESULTS: The plasmid of pCI-neo-*Kras*2 was successfully established. The growth rate of cells transfected with pCI-neo-*Kras*2 was significantly lower than the control cells transfected with the empty pCI-neo vector ($P < 0.05$). Cell cycle analysis revealed arrest of the pCI-neo-*Kras*2 transfected cells in G_0/G1 phases, decreased DNA synthesis and decreased fractions of cells in S phase. The proliferative index of cells transfected with pCI-neo-*Kras*2 was decreased compared with the control cells (49.78% *vs* 64.21%), while the apoptotic rate of Caco-2 cells with stable *Kras*2 expression increased (0.30% *vs* 0.02%).

CONCLUSION: The wild-type *Kras*2 gene effectively inhibits the growth of the colonic adenocarcinoma cell line Caco-2.

Key words: Colonic adenocarcinoma; Wild-type *Kras*2; Cell cycle; Apoptosis

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INTRODUCTION

A number of molecular studies have shown that colon carcinogenesis results from an accumulation of epigenetic and genetic alterations, including activation of the proto-oncogenes, and inactivation of the mutations of tumor suppressor genes or of DNA repair genes[1]. Previously extensive studies have been done on the activation of the *K-ras*2 proto-oncogene[2-4] and have revealed the association between colon carcinogenesis and the frequency of RAS mutations. Approximately 50% of colorectal tumors contain K-*ras*2 gene mutations, leading to activation of the *K-ras*2 oncogene, which is an early event in carcinogenesis. A recent study on *Kras*2, however, demonstrated that the wild-type *Kras*2 inhibited the growth, colony formation and *in vivo* tumor formation of lung cancer cells[5]. In addition, wild-type *Kras*2 has also been shown to inhibit colony formation and tumor development by transformed NIH/3T3 cells. These results indicated wild-type *kras*2 might exert its tumor suppressor effect in carcinogenesis. In the current study, we attempted to investigate the tumor suppressive effect of wild-type *Kras*2 on the proliferation of a colon cancer cell line. We transfected the wild-type *Kras*2 gene into Caco-2 cell, a human colon adenocarcinoma cell line, and established a stably transfected cell line by G418 selection. The effects of wild-type *Kras*2 on cell growth, cell cycle distribution and apoptosis were analyzed in Caco-2 cells.

MATERIALS AND METHODS

Cell culture

Human colon tumor cell line Caco-2 was purchased from American Type Culture Center (ATCC) (Rockville, MD, USA) and was maintained in MEM-NEAA medium supplemented with 10% FCS and 100 U/mL penicillin-

streptomycin in a humidified atmosphere with 5% CO_2 at 37℃.

Cloning of wild-type Kras2 gene and plasmid construction

Appropriate institutional approval for human tissue studies and the informed consent from all subjects were both obtained. The wild-type *Kras*2 cDNA fragment was amplified by PCR. Briefly, total RNA from human peripheral blood was extracted by Trizol reagent (Invitrogen) and the Reverse Transcription System kit (Promega) was used to synthesize the cDNA. The primer sets Y1: 5'-ACCCACGCGTATGACTGAATATAAAC-3' and Y2: 5'-AACGTCGACTTACATAATTACACACT-3' (Shanghai Oke Corp.) with *Mlu* I and *Sal* I enzyme sites respectively were used to amplify the wild-type *Kras*2 cDNA. The PCR product was harvested from 1% agarose gel, digested by *Mul* I and *Sal* I enzymes and inserted into the pCI-neo vector at *Mul I* and *Sal I* cloning sites to construct the pCI-neo-*Kras*2 expression vector. All ligation products were transformed into DH5 competent cells. The constructs were confirmed by restriction enzyme mapping and DNA sequencing.

Cell transfection and stable screening

For transfection experiment, Caco-2 cells were seeded into 100-mm culture dishes and grown until 80% confluence. The empty plasmid pCI-neo and the pCI-neo-*Kras*2 were stably transfected using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instruction. Caco-2 cells were selected with 1.2 mg/mL G418 for 2 wk and maintained in normal MEM medium containing 0.6 mg/mL G418 (Amresco USA).

RNA isolation and Northern blot analysis

A total of 1.5×10^5 viable Caco-2 cells transfected with the pCI-neo empty vector or *Kras*2 stably expressing cells were plated into one well of six-well plates with 5 mL of culture medium and cultured until 90% cell confluence. Total RNA was isolated in 2 mL of TRIZOL reagent according to the manufacturer's instruction. Twenty micrograms of total RNA were electrophoresed through a 1% agarose gel containing formaldehyde and were transferred to a Hybond N^+ membrane (Amersham). The membrane was hybridized with a [^{32}P]-labeled *Kras*2 cDNA probe in 5 mL hybridization buffer containing 50% deionized formamide, 0.5 × Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), and 1 mg of salmon sperm DNA (Sigma). After hybridization overnight at 50℃, the membrane was washed according to the manufacturer's instruction and exposed to BioMax-MS film (Eastman Kodak) for 2 d at -80℃. The autoradiographic bands were performed in quantitative analysis using Image software.

Monotetrazolium (MTT) assays

The cancer cells with stable expression of wild-type *Kras*2 and the control cells were separately seeded at the same time into 96-well culture plates and then routinely cultured for 6 d. MTT was added to the culture wells and the 570 nm wave-length absorption value (*A* value) was assayed at 5 h, 1, 2, 3, 4, 5 and 6 d after seeding of cells. The cell growth curves were protracted using the following formula, GI = Nc-Nt/Nc × 100%. Nc and Nt represent the *A* value of the control and the experimental group cells. All experiments were performed in triplicate and repeated 3 times.

Cell cycle analysis

Cell cycle analysis was performed by propidium iodide (PI) staining. Briefly, Caco-2 cells were firstly seeded into 10-cm dishes at 5×10^5. After further cultured for 2 d, the cells were trypsinized and harvested in PBS followed by two washes with PBS. After that the cells were resuspended in PBS at 1-2 × 10^6/mL and fixed with 70% cool ethanol for at least 1 h. Next, the cells were washed twice and centrifuged at 1000 × *g* for 3 min. The pelleted cells were resuspended in 1 mL PBS and added with 50 μL of RNase A stock solution (10 g/mL) and mixed well, and then incubated for 3 h at 4℃. Finally, the cells were pelleted and added with 1 mL of PI staining solution (3.8 mmol/L sodium citrate, 50 μg/mL PI in PBS) and incubated for 1 h at room temperature. After that the cells were analyzed by flow cytometry on an FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) exciting at 488-nm, and the DNA-linked red fluorescence (PI) was measured through a 600-nm wavelength filter. This experiment was performed three times.

Western blot analysis

A total of 50 μg of proteins in whole cell lysates was separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore, Bedford, MA). After blocking with 5% (w/v) nonfat milk, the blots were incubated with the first anti-*Kras*2 antibody (F234, sc-30; SantaCruz Biotechnology, Santa Cruz, CA; 1:100 dilution in a blocking solution, 2 h at room temperature) or with the anti-GAPDH antibody (Sigma Chemical, St. Louis, MO; 1:2000 dilution in a blocking solution, 1 h at room temperature). The blots were then washed with PBST and incubated with a secondary antibody (Amersham Biosciences, Buckinghamshire, UK). Positive signal bands were detected using ECL plus (Amersham Biosciences).

Statistical analysis

The results for MTT assay and cell cycle analysis were presented as the mean ± SD of three replicate culture wells. Analysis was performed using Statview software. All data were evaluated for paired variables to compare between two groups. $P < 0.05$ was considered to be statistically significant.

RESULTS

Identification of the expression vector of Kras2 gene

The plasmids including pCI-neo and pCI-neo-*Kras*2 were digested with *Mul* I and *Sal* I enzymes and the 576 bp and 5472 bp products were observed. The 576 bp *Kras*2 cDNA was inserted into the pCI-neo vector. And the inserted fragment was verified by sequencing.

Transcription of the Kras2 gene in Caco-2 cells

To measure the expression level of *Kras*2 gene in Caco-2 cells, northern blot analysis was used to detect the *Kras*2

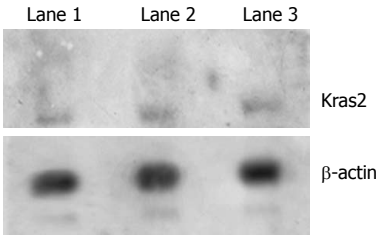

Figure 1 mRNA expression of wild-type *Kras*2 in Caco-2 cell.

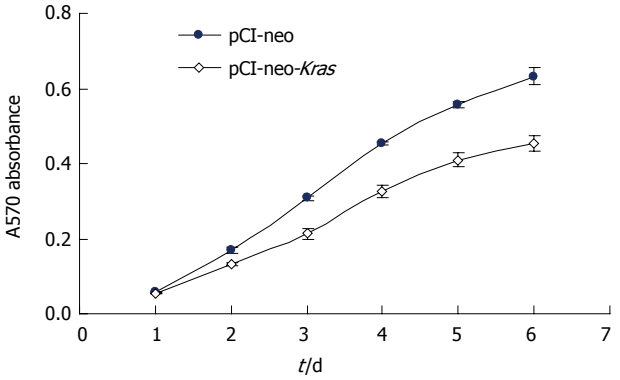

Figure 2 Growth inhibitory effect of wild-type *Kras*2 on Caco-2 cell.

mRNA. β-actin mRNA was also detected as an inner control. Consistent with the information provided by ATCC, the *Kras*2 transcript was not detected in Caco-2 cells (Figure 1).

Suppression of Caco-2 cell growth by wild-type Kras2

To determine the effect of wild-type *Kras*2 on the proliferative capacity of Caco-2 cells, a cell growth curve was generated by MTT assay. As shown in Figure 2, the growth rate of cells transfected with pCI-neo-*Kras*2 was significantly lower than the control cells transfected with the empty pCI-neo vector beginning from the third day after cell seeding.

Effect of wild-type Kras2 on cell cycle and apoptosis of Caco-2 cells

To detect the effect of wild-type *Kras*2 on cell differentiation and apoptosis of Caco-2 cells, cell cycle distribution and the apoptosis rate were analyzed by flow cytometry. The proliferative index of cells transfected with pCI-neo-*Kras*2 was decreased, compared with the control cells (49.78% *vs* 64.21%) (Table 1). As shown in Figure 3, the pCI-neo-*Kras*2 transfected cells were arrested in G_0/G1 phases, compared with control cells, and the apoptotic rate of Caco-2 cells with stable *Kras*2 expression was increased (0.30% *vs* 0.02%).

Expression of wild-type Kras2 gene

To determine whether the expression level of the *Kras*2 protein was different between the cells transfected with pCI-neo-*Kras*2 and the control cells transfected with the empty pCI-neo vector, we performed Western blot analysis on both cell lines using a monoclonal *Kras*2 antibody. The results showed that the Caco2 cell line without pCI-neo-*Kras*2 transfection expressed a faint protein product (Figure 4, lane A), and pCI-neo-*Kras*2 stably transfected cell line (lane B) expressed a 21 kDa product of *Kras*2. GAPDH was used as an internal control for loading volume.

Table 1 Influence of wild-type *Kras*2 gene on cell cycle of Caco-2 cells (%)

Cell lines	G0/G1 phase	S phase	G2/M phase	Proliferative index
pCI-neo	35.79	40.04	24.17	64.21
PCI-neo-*Kras*2	50.22	32.88	16.9	49.78

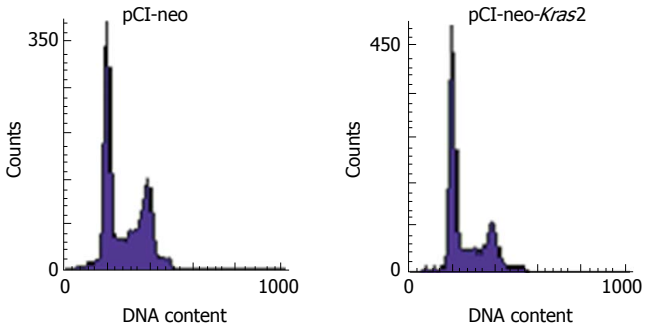

Figure 3 Effect of the wild-type *Kras*2 on cell cycle of Caco-2 cells.

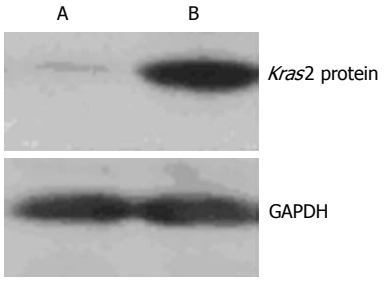

Figure 4 Expression of the wild-type *Kras*2 protein in cell lines.

DISCUSSION

Human colon carcinogenesis and tumor progression are considered to be the result of multigene alterations. Activating point mutations in *ras* gene are widely correlated with human colon carcinogenesis and progression, and have been detected in more human tumor types and at a higher frequency (25%-30% of all human tumors) than other oncogenes[6-9]. For example, *ras* mutations are detected in 40%-50% of colon carcinomas[10,11], 80% of pancreatic carcinomas[12-15], and 30%-50% of lung adenocarcinomas[16-20]. *Kras*2 mutations account for > 90% of the activating *ras* mutations observed in these tumor types. The oncogenic alleles of *ras* genes are generally perceived to be dominant because their transforming ability exists when normal alleles are also expressed[21]. However, recent evidence from both *in vivo* and *in vitro* studies suggested that the dominance of the *ras* oncogene might result from overexpression of the mutant *ras* allele or from deletion of the wild-type allele. Previous studies also suggested that the wild-type *Kras*2 allele could suppress the oncogenic potential of the mutant

*Kras*2 allele[5]. Heterozygous *Kras*2 deficient mice were highly susceptible to chemically induced lung tumors compared to wild-type littermates. All tumors displayed an activated *Kras*2 because of a chemically induced mutation. Experimental evidence demonstrated that wild-type *Kras*2 inhibited cell growth, colony formation and tumor development in a murine lung tumor cell line containing an activated *Kras*2 allele. In addition, the loss of wild-type *Kras*2 was found in 67%-100% of chemically induced mouse lung adenocarcinomas harboring a mutant *Kras*2 allele[5]. Moreover, results from epidemiological investigations showed that loss of heterozygosity (LOH) of chromosome 12p was detected in nearly 50% of human lung adenocarcinomas and large cell carcinomas, and *Kras*2 mutations were detected at codon 12 in about 40% of human lung cancers[22]. We also observed that high frequent loss of heterozygosity in this domain occurred in carcinogenesis and progression of colon carcinoma[23]. These findings suggest that wild-type *Kras*2 is a tumor suppressor and is frequently lost during tumor progression.

Several mutational events common to tumor cells in colorectal cancer have been identified. It is not known whether the accumulation of these mutations occurs in a specific sequence. However, certain stages in the disease development do correlate with the presence of particular mutations[24]. In the genetic model of human colon tumorigenesis, activation of *Kras*2 mutation has been identified to promote colon carcinogenesis and progression, meanwhile, masking the potential tumor suppressor effect of the wild-type *Kras*2. To elucidate whether the wild-type *Kras*2 is a tumor suppressor in more tumor types, we hypothesized that the *K-ras2* plays double roles of both "oncogenic" and "anti-oncogenic" effects. In physiological state, *K-ras2* is a proto-oncogene, which is transformed to an oncogene by activating point mutation, whereas the wild-type *K-ras* can still maintain its tumor-suppressor function. To explore the biological role of the wild-type *K-ras* gene, it is necessary to exclude the interference of activated mutations of *K-ras*. Therefore, it is reasonable to select Caco-2 cells as the cell model in this study[25]. To our knowledge, this is the first report on the relationship between the presence of wild-type *Kras*2 and the growth and apoptotic properties of human colon cancer cells. Our results demonstrated that the wild-type *Kras*2 effectively inhibited the proliferation, and meanwhile promoted apoptosis of colon cancer cells. Ras is a GTP/GDP binding protein, affecting cell growth and proliferation *via* several signaling pathways[25]. In this study, wild-type *Kras*2 inhibited the growth of Caco-2 human colon cancer cells mainly through cell cycle arrest in G_0/G1 phase, decreased DNA synthesis, and decreased fractions of cells in S phase.

Aberrant apoptosis mechanism also exists in colon cancer. Previous studies have demonstrated that the activating mutation of Ras inhibited apoptosis in colon cancer cells[26]. Here, we showed that the exogenous expression of wild-type *Kras*2 increased the apoptotic rate of Caco-2 cells (0.30% *vs* 0.02%), possibly via a mechanism involving Ras G-proteins and Raf-1 kinase, which needs to be further investigated[27].

Taken together, through exogenous expression of the wild-type *Kras*2 gene in Caco-2 colon cancer cells, we firstly demonstrate that the wild-type *Kras*2 gene is a potential tumor suppressor in human colon cancer. Our study also implies that increase of the expression level of wild-type *Kras*2 gene might be a gene therapeutic strategy for human colon cancer.

COMMENTS

Background

Conventional viewpoints suggest *ras* gene is closely related to carcinogenesis and progression of colon carcinoma. About 30 percent of tumors display mutation of *ras* gene, in which the most frequent one is *Kras*2, and a relatively high frequency of *Kras*2 mutation is seen in colon carcinoma, pancreas carcinoma and lung carcinoma. Recent studies raised doubt about the dominant oncogene roles of *Kras*2 gene, and showed the frequent loss of wild type *Kras*2 in human and mouse lung adenocarcinomas, and that loss of heterozygosity on chromosome 12p12-13 in *Kras*2 gene existed in non-small-cell lung cancer. In addition, wild-type *Kras*2 has also been shown to inhibit colony formation and tumor development by transformed NIH/3T3 cells. These results indicate that wild-type *kras*2 probably has a tumor suppressor effect in carcinogenesis.

Research frontiers

We investigated the tumor suppressive effect of wild-type *Kras*2 on cell proliferation of a colon cancer cell line.

Innovations and breakthroughs

To our knowledge, this is the first report on the relationship between the presence of wild-type *Kras*2 and the growth and apoptosis properties of human colon cancer cells.

Applications

This study demonstrates that the wild-type *Kras*2 gene is a potential tumor suppressor in human colon cancer. Strikingly and of great potential clinical relevance, our results indicate that increase in the expression level of wild-type *Kras*2 gene might be a beneficial gene therapeutic strategy for human colon cancer.

Terminology

*Kras*2, namely v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, is on chromosome 12p12-13, and is one of the three members of the *ras* gene: *Hras*1, *Nras* and *K-ras*2. Apoptosis, also called programmed cell death, or "cell suicide": A form of cell death in which a programmed sequence of events leads to the elimination of cells without releasing harmful substances into the surrounding area. Apoptosis plays a crucial role in biological development and maintaining health by eliminating old cells, and unhealthy cells. Cell cycle, the sequence of stages that a cell passes through between one cell division and the next. A cell cycle can be divided into four main stages: the M phase, when nuclear and cytoplasmic division occurs; the G1 phase; the S phase, in which DNA replication occurs; and the G2 phase, a relatively quiescent period.

Peer review

This is an interesting paper that challenges dogma of role of *Kras*2 in CRC. The study with appropriate methods investigating the effect of re-introduction of wild-type *Kras*2 in colorectal cancer cell lines on cell growth, proliferation and apoptosis. A major finding of the study was that re-expression of wild-type *Kras*2 inhibited cell growth and proliferation along with induction of the apoptosis in the studied cell line. It is worthy of publication in *WJG*.

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