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RAPID COMMUNICATION

Changes in gene-expression profiles of colon carcinoma cells induced by wild type K-ras2

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Abstract

AIM: To further elucidate the possible molecular biological activity of wild type K-ras2 gene by detecting changes in wild type K-ras2 gene-induced gene-expression profiles of colon carcinoma cells using cDNA microarray techniques.

METHODS: Total RNA was isolated from peripheral blood of health volunteers. Reverse transcription of RNA and polymerase chain reaction were used to synthesize wild type K-ras2 cDNA. K-ras2 cDNA fragment was cloned into a T easy vector and sequenced. A eukaryotic expression vector pCI-neo-K-ras2 was constructed and transfected to Caco2 cell line using the liposome method. Finally, mRNA was isolated, reverse-transcribed to cDNA from pCI-neo-K-ras2 or pCI-neo blank vector-transfected Caco cells, and analyzed by cDNA microarray assay.

RESULTS: Restriction enzyme analysis and DNA sequencing verified that the constructed expression vector was accurate. High-quality RNA was extracted and reverse transcribed to cDNA for microarray assay. Among the 135 genes, the expression was up-regulated in 24 and down-regulated in 121. All these differentially expressed genes were related to cell proliferation, differentiation, apoptosis and signal transduction.

CONCLUSION: Differentially expressed genes can be successfully screened from wild type K-ras2-transfected colon carcinoma cells using microarray techniques. The results of our study suggest that wild type K-ras2 is related to the negative regulation of cell proliferation, metabolism and transcriptional control, and provide new clues to the further elucidation of its possible biological activity.

Key words: Wild type K-ras2; Colon cancer; Microarray

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INTRODUCTION

Carcinogenesis and progression of human colon carcinoma result from abnormal expression of many tumor-associated genes. Activation oncogenes and deactivation antioncogenes are known as one of their important mechanisms[1,2]. Ras gene which is closely related to carcinogenesis and progression of colon carcinoma consists of three members: Hras1, Nras and K-ras2[3,4]. It is traditionally believed that activated Ras gene plays a dominant role as an oncogene in the pathogenesis of colon carcinoma. About 30% of tumors display mutations of Ras gene members, the most frequent mutation is found in K-ras2[5,6], and the relatively high frequency of K-ras2 mutation is observed in colon, pancreas and lung carcinomas[7-9]. Recent studies indicate that frequent loss of wild type Ras gene occurs in human and mouse lung adenocarcinomas, thus questioning the dominant role of Ras gene in the pathogenesis of tumors[10]. Loss of heterozygosity on chromosome 12p12-13 in K-ras2 gene has also been found in non-small-cell lung cancer[11,12]. We have reported that frequent loss of heterozygosity occurs in this domain during carcinogenesis and progression of colon carcinoma[13]. By observing the changes in growth curve and cell cycle of colon carcinoma cells transducted with wild type K-ras2 gene, we found that wild type K-ras2 gene could step down the growth and cell cycle of colon carcinoma cells manifested as significantly increased stage G0-G1 cells and decreased stage G2-M cells, suggesting that resting cells with proliferation activity are inhibited to advance into proliferating cell cycle[14]. In the present study, in order to study the biological activity of wild type K-ras2 gene, we constructed a eukaryotic expression vector of wild type K-ras2 gene phenotype, screened differentially expressed genes of colon carcinoma cells transfected by wild K-ras2 gene with cDNA microarray, and detected the effect of wild K-ras2 on the gene-expression profiles

of colon carcinoma and its function *in vivo*. The results provide new clues to the exploration of the pathogenesis of colon carcinoma and the functions of K-ras2 gene.

MATERIALS AND METHODS

Cells and cDNA array

Human colon adenocarcinoma cell line Caco-2 was obtained from the ATCC. DMEM, FBS, Trizol RNA isolation kit, pCI-neo mammalian expression vector and Lipofectamine2000 were purchased form Invitrogen (Carlsbad, CA). A commercial human expression cDNA array was obtained from Shanghai Biochip Company (Shanghai, China). The array includes 8568 known genes, which can be categorized into cell division, cell signaling, cell structure, gene and protein expression, metabolism and pseudogene, *etc*.

Transfection of Caco-2 cells

Total RNA was isolated from peripheral blood of health volunteers using Trizol RNA isolation kit. Reverse transcription of RNA and polymerase chain reaction were used to synthesize the full-length sequence of wild K-ras2 cDNA. Primers containing Mul1 and Sal1 restrictions (Y1: 5'-ACCCACGCGTATGACTGAATATAAAC-3'; Y2: 5'-AACGTCGACTTACAT AATTACACACT-3') were synthesized by Shanghai Ouke Biotech Company (Shanghai, China). The PCR products were inserted into pGEM-T Easy vector (Promega) to generate pGEM-T-Ras, and positive clones were identified by blue/white color screening followed by sequencing. pGEM-T-Ras and pCI-neo eukaryotic expression vector were digested in Mul1 and Sal1 restriction enzymes and ligated using T4 DNA ligase (Promega) to produce pCI-neo-K-ras2. The recombinant pCI-neo-K-ras2 and empty pCI-neo-K-ras2 were transfected into Caco-2 cells (ATCC) using Lipofectamine2000 according to the manufacturer's instructions, and the positive clones were selected from G418 (Amresco).

cDNA microarray analysis

Total RNA was extracted from pCI-neo-K-ras2 (transfection group) and empty pCI-neo-K-ras2 (control group) using Trizol RNA isolation kit. The purity of RNA was confirmed by agarose gel electrophoresis and absorbance (A) ratio (A260/A280). To make cDNA probes, approximately 5 μg of total RNA was labeled with Cy3-dUTP (control) or Cy5-dUTP (transfection group) by reverse transcription. The probes were precipitated using ethanol and dissolved in 5 × SSC + 2 g/L SDS at 20℃. The microarray and probes were denatured in 95℃ water bath for 5 min. Hybridization was performed at 60℃ for 15-17 h. Microarray was washed with 2 × SSC + 2 g/L SDS and 1 × SSC + 2 g/L SDS for 10 min respectively and dried at room temperature. Scanning was performed with ScanArray3000 (General Scanning, Inc.). The acquired image was analyzed using ImaGene 3.0 software (BioDiscovery, Inc.). The intensities of Cy3-dUTP and Cy5-dUTP were normalized by a coefficient according to the ratio of housekeeping genes. The positively expressed genes were as follows: Cy5-dUTP: Cy3-dUTP signal ratio > 2.0, red fluorescent displaying up-regulated expression; Cy5-dUTP: Cy3-dUTP signal ratio < 0.5, green fluorescent displaying down-regulated expression.

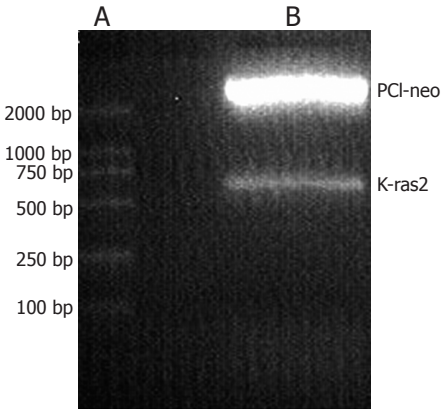

Figure 1 Restriction enzyme analysis of PCI-neo with K-ras2 genes showing a 576 bp K-ras2 gene and a 5600 bp carrier.

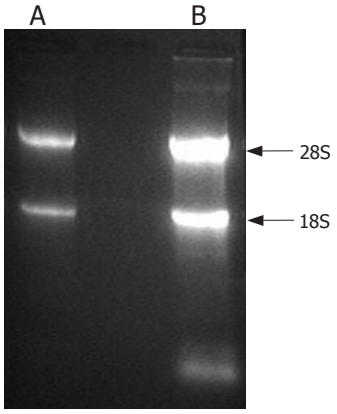

Figure 2 Electropherogram of total RNA from control (A) and transfection (B) groups.

Statistical analysis

Data on gene expression were analyzed by Student's *t* test using SPSS 10.0 software and $P < 0.05$ was considered statistically significant.

RESULTS

Validation of wild type K-ras2 and RNA

Restriction enzyme analysis and nucleotide sequencing of eukaryotic expression vector pCI-neo-K-ras2 showed that its sequence containing an integrity open reading frame was accurate (Figure 1). A 260/A280 of total RNA ranging from 1.9 to 2.1 and 28S/18S of about 2 indicated that RNA was not degraded and could be used for preparation of hybridization probe (Figure 2).

Results verified by microarray hybridization system

To monitor the whole process of microarray hybridization, we set up 6 negative controls and 10 positive controls. Scanning of hybridization array and report of array detection showed that hybridization array and sample RNA were intact with good background value and well-distributed noise. The hybridization reaction system was normal and the results were reliable. To compare gene profiles between transfection and control groups, a scatter

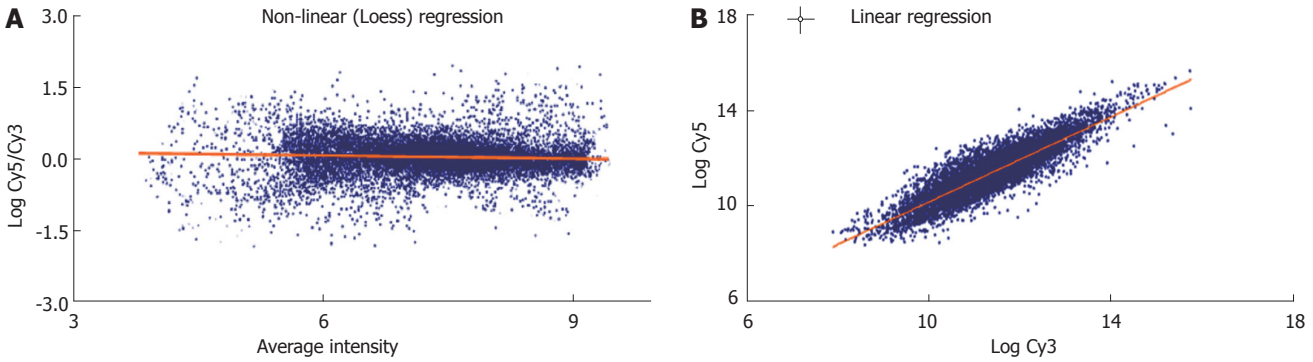

Figure 3 Scatter bar of gene-expression profiles of Caco2 cells transfected (**A**) and non-transfected (**B**) with wild-type K-ras2 gene.

Table 1 Biological function of genes with down-regulated expression

Gene	Chromosomal localization	Biological function	Cy5/Cy3
NDP	Xp11.4	Cross-cell signal transmission, signal transduction, NS development	4.509
SSX4	Xp11.23	Transcription regulation, immunoreaction	4.044
CASP1	11q23	Positive regulation of I-kappaB kinase/NF-kappaB cascadereaction, signal transduction, apoptosis	3.754
HPRT1	Xq26.1	Cytolysis, lymphocyte proliferation, purine nucleotide synthesis	3.715
TM4SF2	Xp11.4	ECM	3.554
DXS1283E	Xp22.3	Unkown	3.418
DPYS	8q22	NS development, signal transduct-ion, nucleotide metabolism	3.386
TYRP1	9p23	Cell metabolism, melanin synthesis	3.358
CADPS	3p21.1	Calcium-regulated Exocytosis	3.238
MCF2	Xq27	Cytokeleton	3.187
COL4A6	Xq22	ECM and ECM synthesis	3.201
F13A1	6p25.3-p24.3	Transcriptional control	3.108
HTR2C	Xq24	IP3-induced signal pathway	3.061
IL13RA1	Xq24	ECM	3.050
OGT	Xq13	Signal transduction	3.030
TOSO	1q32.1	Defence reaction, anti-apoptosis	2.994
MAB21L1	13q13	Positive regulation of cell proliferation, visual development	2.990
CYBB	Xp21.1	Cross-cell signal transmission, chemotaxis, inflammation, signal transduction	2.972
STK9/CDKL5	Xp22	Microduct skeleton and its synthesis, histogenesis	2.946
PTPRG	3p21-p14	PTK signal pathway	2.878
ELAVL2	9p21	Transcriptional control	2.853
GLA	Xq22	Osteoclast differentce regulation,bone resorption, cell adhere	2.851
APXL	Xp22.3	Channel protein of sodium ion	2.839
SERPINA7	Xq22.2	ECM, transport of TH	2.833

profile was plotted for the probe signal values, showing that most genes were distributed around the regression line, and their expression in two tissue samples was similar, but a few genes had a different expression. When the difference in gene expression increased, the number of differently expressed genes decreased (Figure 3). The data were confirmed by the low hybridization signal of these genes. Cy5 fluorescein (red) and Cy3 fluorescein (green) were used to mark the probes of experimental and control groups, and the difference in color was expressed as the difference in gene expression between the two groups. Yellow indicated no expression difference. According to the experimental protocol, the expression of 24 genes with their cy5/cy3 $\geqslant$ 2 (Table 1) was up-regulated, accounting for 17.76% of all the differentially expressed genes, and the expression of 121 genes with their cy5/cy3 $\leqslant$ 0.5 was down-regulated, accounting for 89.63% of all the differentially expressed genes. The top 30 down-regulated genes are listed in Table 2.

Biological function classification of differentially expressed wild type K-ras2 genes Biological function classification of differentially expressed wild type K-ras2 genes was performed based on the biological classification of genes in Affymetrix gene ontology database. Eleven subtypes were found to be closely related to carcinogenesis (Table 3).

DISCUSSION

Carcinogenesis and progression of colon cancer represent its phases from normal mucosa to atypical hyperplasia (including intestinal metaplasia) of adenoma and adenocarcinoma, involving multiple genes and factors[15]. K-ras2 gene plays a dominant role as an oncogene in promoting carcinogenesis because of point mutation[16,17]. In the present study, in vitro experiments demonstrated that carcinogenic agents used in the treatment of loss of heterozygosity in mice with wild type K-ras2 gene facilitated the development of cancer but not in those with normal wild phenotype K-ras2 gene. Moreover, the

Table 2 Biological function of genes with up-regulated expression

Gene	Chromosomal localization	Biological function	Cy5/Cy3
SZF1	3p21	Transcriptional control	0.181
MMP14	14q11-q12	Incision enzyme, proteolysis	0.186
WIT-1	11p13	Cell proliferation	0.189
LOC253012	7q21.3	Gas exchange, cell adhere, early nerve difference and axonogenesis	0.201
OPCML	11q25	Cell adhere, nerve identification	0.239
PLAU	10q24	Chemotaxis, proteolysis, transduction	0.258
MYC	8q24.12-q24.13	Positive-regulated cell proliferation, inhibit cell cycle, balance of iron ion, mRNA synthesis control	0.274
SNTB1	8q23-q24	Muscle contraction, skelet-matrix adhere	0.281
SERPINESL	7q21.3-q22	Ser incision enzyme inhibitor, cell component, angiogenetic regulation	0.290
C20A1	2q11-q14	PCD transport, cell component	0.294
SPTBN1	2p21	Cytokeleton	0.298
GNAS	20q13.2-q13.3	Energy metabolism, G-protein signal pathway, signal transduction	0.302
PCOLCE	7q22	Proteolysis, cell component	0.303
ENPP2	8q24.1	Cell movement, G-protein signal pathway, phosphorylation metabolism, lytic activity p'tase activity, transcription	0.309
CDK6	7q21-q22	Proliferation, cell cycle, protein conjunct	0.315
HIST2H2BE	1q21-q23	Chromosome component and synthesis	0.323
ZNF137	19q13.4	Transcription control, ion binding	0.332
IL1B	2q14	Inflammation, proliferation, chemotaxis	0.336
IL1A	2q14	Apoptosis, proliferation, transduction, chemotaxis	0.343
MTSS1	8p22	Actin component and synthesis, cell adhere, cell movement, PTK signal pathway, muscle and NS development, internalization	0.346
ACHE	7q22	Muscle contraction, transduction	0.353
EPHB4	7q22	Proliferation, organ-formation, angiogenesis	0.368
AZGP1	7q22.1	Cell member component	0.376
PDE4DIP	1q12	Protein synthesis, actin component	0.377
PITPN	17p13.3	PHL transport	0.379
MEOX1	17q21	Growth	0.386
GNB2L1	5q35.3	Intercellular signal cascade reaction, protein localization, transduction, PKC	0.394
MERTK	2q14.1	Transduction, cross-cell signal transmission, protein phosphorylation	0.405
CUTL1	7q22.1	Transcription control	0.419
FCGR2A	1q23	Immune reaction, signal switch, defence reaction, B cell proliferation	0.428
PAX8	2q12-q14	Organ-formation, transcription control, metanephros development, mRNA synthesis	0.442

size of poorly-differentiated adenocarcinoma in mice with loss of heterozygosity was significantly larger than that of adenoma in mice with abnormal wild phenotype K-ras2 gene. It was reported that cell lines activated by wild type K-ras2 gene-transfected ras can inhibit cell growth, clone formation and tumorigenesis in nude mice, indicating that wild type K-ras2 gene may be a potential anti-oncogene[9,18]. Changes in gene-expression of Caco2 cells induced by wild type K-ras2 gene were found in our study, showing the possible biological activity of wild type K-ras2 gene.

In our study, genes related to signal transduction, transcription control and cell differentiation were dominant, accounting for 33.33% of the total up-regulated genes. The top 30 down-regulated genes related to cell proliferation accounted for 24.79% of the total up-regulated genes. The expression of genes related to cell metabolism, cell cycle and transcription control was up-regulated. Wild type ras may inhibit cell proliferation by promoting differentiation. In fact, it has long been known that Ras proteins can induce differentiation of some cell types, such as neurons, under certain conditions[19]. Our findings suggest that K-ras2 can negatively regulate cell proliferation, metabolism and transcription control, and inhibit the growth of colon carcinoma.

The expression of NDP is most significant. As a genetic locus, its mutation may give rise of genetic-correlated Norrie disease caused by two molecular defects in NDP gene. One is 265 C>G missense mutation in the 97th codon by changing arginine into praline, the other is excalation in 3'-non-translated region of the third exon[20]. It was reported that patients with gene excalation present relatively severe symptoms, whereas patients with gene mutation display relatively mild symptoms[21]. The SSX4 gene (a member of the node point protein family) whose expression was significantly up-regulated in our study, can inhibit cell transcription, cause humor- and cell-mediated immune reaction, and may be a very valuable target for vaccine therapy of tumors[22]. Caspase-1 encoding

Table 3 Functional classification of differentially expressed genes

Type of gene	Down-regulated genes (*n*)	Rate of down-regulated genes (%)	Up-regulated genes (*n*)	Rate of up-reguleted genes (%)
Metabolism-associated	18	14.88	3	12.50
Cell proliferation	30	24.79	3	12.50
Cell cycle	16	13.22	0	0
Signal transduction	18	14.88	8	33.30
Cytokelet	8	6.61	1	4.17
Transcription control	18	14.88	4	16.67
Cell adhere	11	9.09	1	4.17
Cell apoptosis	8	6.61	2	8.33
Cell differentiation	14	11.57	4	16.67
Immune-associated	14	11.57	2	8.33
ECM	6	4.96	1	4.17

apoptosis-associated thioserinase (a member of the caspase family) can lead to proteolysis and activate pro-IL-1, thus playing an important role in cell apoptosis[23]. Its up-regulated expression in wild-type K-ras2-transducted cells may be related to apoptosis of tumor cells, suggesting that caspase-1 is one of the human p53-dependent cell modulators[24].

The myc gene whose expression was most significantly up-regulated in our study, is closely related to tumors. It is adjusted by many factors, and can promote cell mitosis and make target cells proliferate and immortalize. This gene involving cell apoptosis is related to tumorigenesis and progression of diverse tumors[25]. Amplification of correlated sequence of myc has been observed in diverse human tumor cell lines including cell lines of granulocytic leukemia, retinoblastoma, neuroblastoma, breast and lung cancer, as well as in human colon carcinoma cell line[26,27]. The *MMP14* gene (MT1-MMP) is a member of the matrix metalloprotease family. Its function is modified and regulated by O-glycosylation, interaction with CD44, internalization and recycling, depending on its proper expression on the cell surface[28]. It can invade tumors by activating MMP2 protein. It was reported that up-regulated expression of MMP2 and MT1-MMP is related to invasion of glioblastoma[29], while the expression of MT1-MMP is related to local invasion of and metastasis to lymphonodes of oral squamous cell carcinoma[30], supporting its function in colon carcinoma LoVo cells[31]. The WIT-1 gene is localized in the upstream of Wilm's tumor gene sharing the same promoter. Methylation of the WIT-1 gene is related to chemotherapy-resistant tumors and acute leukemia[32]. The EPHB4 gene whose expression was remarkably down-regulated in our study is a member of the biggest receptor tyrosine kinase (RTK) family. Its encoding protein, a receptor of ephrin-B2, promotes microvascular endothelial cell migration and/or proliferation, thus playing an important role in angiogenesis of tumors[33]. It has been shown that EPHB4 expresses in diverse tumors such as prostate carcinoma and astrocytoma, and involves phenotype transformation post-metastasis[34,35].

In summary, K-ras2 seems to have a dual function. On the one hand, it promotes cancer development as a gain of function oncogene. On the other hand, it inhibits cancer as a loss-of-function tumor suppressor gene. There are some interesting parallels between the Trp53 tumor suppressor gene and the unfolding story of K-ras2. Trp53 was initially described as an oncogene carrying point mutations in tumors. Later, it was found that it is in fact the wild type copy of the gene that functions as a tumor suppressor gene and is capable of reducing cell proliferation. In this case, the Trp53 mutation may, in a sense, also be considered an activating (but not necessarily gain-of-function) mutation in that it produces a dominant-negative effect over the wild type p53 protein. The two major players in human cancer have more in common than they were previously thought[18].

COMMENTS

Background

Studies indicate that mutation of the K-ras2 gene plays an important role in carcinogenesis and progression of tumors. It was reported that K-ras2 gene not only induces tumors but also inhibits tumor growth. We have reported that loss of heterozygosity on chromosome 12p12-13 K-ras2 gene occurs in colon carcinoma and wild type K-ras2 gene can effectively inhibit its growth. This study was to construct a eukaryotic expression vector of the wild type K-ras2 gene phenotype, screen differentially expressed genes of colon carcinoma cells transfected by the wild K-ras2 gene with cDNA microarray, and detect the effect of wild K-ras2 gene on the gene-expression profiles of colon carcinoma and its function *in vivo*.

Research frontiers

Based on the results of recent studies, it is hypothesized that K-ras gene plays a role both in carcinogenesis and in inhibition of cancer. It is a poto-oncogene in normal physiological conditions. However, when mutations occur, the activated K-ras2 gene changes into an oncogene and wild type K-ras2 gene becomes an anti-oncogene. This is what we want to prove in this study.

Innovations and breakthroughs

Since the inhibitory effect of wild type K-ras2 gene on tumors was reported by Zhong-Qiu Zhang, its role in suppressing long cancer has been extensively studied by foreign scholars. This is the first time to study the inhibitory effect of wild type K-ras2 gene on colon carcinoma in China.

Applications

The possible molecular pathway of K-ras2 gene in suppressing tumor cell proliferation found in this study may contribute to finding the genes closely related with colon cancer.

Peer review

This interesting paper investigated the importance of K-ras cascade at mRNA level. The major finding of this study is that wild-type K-ras results in both complex induction and more common inhibition of several genes, and may have a dual role in carcinogenesis. The new association elucidated herein may provide further insight into the carcinogenesis and may identify potentially important therapeutic targets.

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