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 Caco2 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.
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 from 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 healthy 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'-AACGTCGACTTACATAATTACACT-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 Cy5-dUTP (transfection) or Cy3-dUTP (control) by reverse transcription. The probes were precipitated using ethanol and dissolved in 5 × SSC + 2 g/L SDS at 20°C. The microarray and probes were denatured in 95°C water bath for 5 min. Hybridization was performed at 60°C 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.

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 DNA 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
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 ≥ 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 ≤ 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[^13^]. 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

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**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 β-kappaB kinase/NF-κappaB cascadereaction, signal transduction, apoptosis | 3.754   |
| HRPT1| Xq26.1                   | Cytolysis, lymphocyte proliferation, purine nucleotide synthesis | 3.715   |
| TM4SF2| Xp11.4                  | ECM                                                   | 3.554   |
| DDX126E| Xp22.3                 | Unknown                                               | 3.418   |
| DYS1| Xq22                     | NS development, signal transduction, nucleotide metabolism | 3.386   |
| TYR1| 9p23                     | Cell metabolism, melanin synthesis                    | 3.358   |
| CADPS| Xp21.1                   | Calcium-regulated Exocytosis                          | 3.238   |
| MCF2 | Xq27                     | Cytokeleton                                           | 3.187   |
| COLA4| Xq22                     | ECM and ECM synthesis                                  | 3.201   |
| F13A1| 6p25.3-p24.3             | Transcriptional control                                | 3.108   |
| HTR2C| Xq24                     | IP3-induced signal pathway                             | 3.061   |
| IL18RA1| Xq24                | ECM                                                   | 3.050   |
| OCT  | Xq13                     | Signal transduction                                   | 3.030   |
| TOSO | 1q32.1                   | Defence reaction, anti-apoptosis                      | 2.994   |
| MAR21L1| 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 difference 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   |

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size of poorly-differentiated adenocarcinoma in mice with loss of heterozygosity was significantly larger than that of adenoma in mice with abnormal wild type 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 coding region, correlated Norrie disease caused by two molecular defects in NDP gene. One is 265 C>G missense mutation in the codon 97th by changing arginine into praline, the other is excitation in 5’-non-translated region of the third exon. It was reported that patients with gene excitation present relatively severe symptoms, whereas patients with gene mutation display relatively mild symptoms. 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 humoral and cell-mediated immune reaction, and may be a very valuable target for vaccine therapy of tumors. Caspase-1 encoding
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-transduced 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 metalloproteinase 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 lymphnodes 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].

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 [33].

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