·Basic Research·

Effects of E2F-1 overexpression on apoptosis of gastric cancer cells and expressions of apoptosis-related genes

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[Abstract] Background and Objective: IE2F transcription factor 1 (E2F-1) is an important transcription factor in cell cycle. This study was to investigate the effects of E2F-1 overexpression on apoptosis of gastric cancer MGC-803 cells and expressions of the downstream genes. **Methods:** The apoptotic rates were measured by flow cytometry in MGC-803/E2F-1 cells, MGC-803/EV cells or untransfected MGC-803 cells. The total RNA was extracted from MGC-803/E2F-1 cells or MGC-803 cells, and cDNA was obtained by RT-PCR. Fluorescent (fluorescence exchange clip) probes marked by Cy5 and Cy3 were hybridized with gene chips containing 21522 human genes. Subsequently, the two signal images were scanned by Lux Scan 10K/A dual pathways laser scanner and analyzed by LuxScan3.0 image analysis software. RT-PCR was used to verify the target genes. **Results:** The apoptotic rate of MGC-803/E2F-1 cells [(8.40±0.91)%] was higher than that of MGC-803/EV [(4.53±0.61)%] and MGC-803 cells [(4.97±0.47)%]. Fifteen differentially expressed apoptosis-related genes were detected, 4 of which were up-expressed and 11 were down-expressed genes, and the same results were verified by RT-PCR. **Conclusion:** Overexpression of E2F-1 accelerates apoptosis of gastric carcinoma MGC-803 cells, which may be related to the 15 differentially expressed genes.

Key words: gastric neoplasm, E2F-1, gene expression profile, differentially expressed genes, apoptosis

Apoptosis of tumor cells is a complex sequential process requiring multiple factors and multiple steps. It involves many genes and interactions among their products, as well as regulation of many signal pathways.[1] Currently, it is generally believed that induction of apoptosis in tumor cells will be a key basis for the successful treatment of tumors. E2F-1 is an important transcription factor regulating the cell cycle, and it is important in the process of apoptosis,[2, 3] but the underlying mechanism is still unclear. This study was to determine the influence of stable overexpression of E2F-1 on the apoptotic process in gastric cancer cells by flow cytometry. We used the gene chip technique, which has high throughput, to measure the variation in gene expression related to apoptosis in MGC-803 gastric cancer cells expressing E2F-1. This preliminary study investigated the possible molecular mechanism underlying the influence of E2F-1 overexpression on apoptosis in gastric cancer cells.

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Materials and methods

Materials

The human gastric cancer cell strain, MGC-803, was purchased from the Institute of Biochemistry and Cell Biology in Shanghai. A cell strain stably overexpressing E2F-1 (MGC-803/E2F-1) and a cell strain transfected with an empty plasmid (MGC-803/EV) were stored in our laboratory. The flow cytometer EPICS XL-MCL was purchased from the Beckman Coulter Company (United States), and Trizol was purchased from Invitrogen (United States). The gene chip used was the oligodeoxynucleotide chip with 22,000 human genes from CapitalBio Corporation. A dual-channel laser scanner, LuxScan 10K/A, was also purchased from CapitalBio. The following materials were also used: Lipofectamine 2000 transfection kit (Invitrogen); reverse transcription reagent (MBI Fermentas); RNA Clean-up Kit (MN) for purification (CapitalBio); TaKaRa Ex-Taq (TaKaRa); and 100-bp DNA Marker (Dongsheng Biotechnology).

Methods

Measurement of apoptosis by flow cytometry Cells in the logarithmic growth phase were selected and the supernatant was discarded. Cells were appropriately digested with trypsin and placed in culture medium. After centrifugation of the cells at 1 000 r/min for 5 min, the supernatant was discarded. PBS was

used to wash the cells, and the concentration of cells was readjusted to $5 \times 10^5 - 1 \times 10^6$/mL. One milliliter of cells was subjected to another 10 min of centrifugation at 1 000 r/min and 4℃, after which the supernatant was discarded. One milliliter of cold PBS was added and gently shaken to resuspend the cells. Another 10 min of centrifugation at 1 000 r/min and 4℃ were performed, and the supernatant was again discarded. The cells were resuspended in 100 μL binding buffer. Annexin V was added, and the solution was gently shaken and left to react for 30 min in the dark at 4℃. Propidium iodide (PI) was then added for 10 min. After another 400 μL binding buffer was added, the solution was subjected to flow cytometry within an hour. Flow cytometry was performed using a stimulating laser with a 488-nm wavelength, channel filters with wavelengths of 515 nm for detecting FITC fluorescence, and 560 nm for detecting PI.

Analysis of variance in gene expression by using a gene chip and the fluorescent hybridization test The Trizol single-step method was used to extract total RNA from cells expressing E2F-1 and cells expressing the empty vector. Total RNA was purified by using a NucleoSpinRNA® clean-up test kit and quantified by using a spectrometer. A quality check was performed on total RNA by using denaturation electrophoresis with formaldehyde. A cDNA synthesis kit was used to construct double-stranded cDNA with T7 Oligo (dT) primer as the primer. By using a T7 Enzyme Mix, double-stranded cDNA was transcribed *in vitro* to cRNA. CbcScript II reverse transcriptase was used for reverse transcription of randomly selected primers before purification. Then the cDNA was labeled by the Klenow enzyme with random primers. On the first gene chip, control samples were labeled with red Cy5 fluorescence and samples were labeled with green Cy3 fluorescence. On the second gene chip, control samples were labeled with green Cy3 fluorescence and samples were labeled with red Cy5. The cDNA probe and oligodeoxynucleotide chip, after processing, were hybridized overnight at 42℃ and then washed and dried. Lastly, the gene chip was scanned by using a dual-channel laser scanner, LuxScan 10K/A (CapitalBio Corporation).

Collection and data analysis of images of gene chips Images of gene chips were analyzed. Image signals were digitized with the LuxScan 3.0 imaging analysis software (CapitalBio). Using the global mean of the overall Cy5 and Cy3 signals, linear modification was performed between each chip. Data from the gene chips were then standardized by the Lowess method. Variation twice the standard, that is, when the ratio of Cy5/Cy3 was greater than 2.0 or less than 0.5, was considered to be a positive result that confirmed variation in gene expression.

Quality control for gene chips Positive controls, such as Hex, an external standard and an internal standard, showed positive signals; negative controls showed negative signals. Housekeeping genes in the chip had good repeatability, and the coefficient of variation (CV) of the ratio did not exceed 0.3. There was no sign of data contamination and the rate of missing signals did not exceed 3%. The detection rate was valid.

Verification of gene chips by RT-PCR

RNA was extracted by strictly following the requirements of the test kit. The purity of the acquired RNA was satisfactory. The primers for amplifying the target segment of human GAPDH were designed and synthesized according to the mRNA sequence data for the human GAPDH gene in GenBank (GenBank accession number: NM_002046) using primer design software premier 5.0 (GAPDH-U and GAPDH-L). The primers were synthesized by the Institute of Biochemistry and Cell Biology in Shanghai. Reverse transcription and PCR were performed in accordance with the manual in the test kit. The RT-PCR program included enzymatic activation for 5 min at 95℃; 30 cycles of denaturation for 45 s at 95℃, annealing for 30 s at 50–60℃ (the temperature depended on the primer status), and elongation for 15–30 s at 72℃ (the duration depended on the length of the product); and 10 min at 72℃. After the reaction, the computer automatically analyzed and calculated the quantitative information. The target segment was detected after RT-PCR amplification and the collected data were analyzed: 1–3 μL of product was used for 1.5% agarose gel electrophoresis and adjusted until bands were clearly visible. A gel imaging system was used to display a semiquantitative grayscale ratio to measure the expression level of each target gene.

Statistical methods

SPSS13.0 software was used for statistical analysis. Quantitative data were presented in form of mean (standard deviation). Intergroup comparisons were performed using one-way analysis of variance (ANOVA) or ANOVA of randomized complete block design. Alpha was set at 0.05.

Results

Measurement of apoptosis rate

We used MGC-803/E2F-1 (a cell strain expressing the E2F-1 target gene) and MGC-803/EV (a cell strain containing the empty vector). MGC-803 cells, without either the vector or the target gene, were used as the control group. As measured with flow cytometry with double staining, the rate of apoptosis was significantly higher in cells of the MGC-803/E2F-1 group than in cells of the MGC-803/EV and MGC-803 groups (Table 1). This result suggests that overexpression of the E2F-1 gene promotes apoptosis in cells.

Table 1 Comparison of apoptotic rates in different cells groups

Cell line	Apoptotic rate (%)	95% CI
MGC-803/E2F-1	8.40±0.91[a]	6.12–10.68
MGC-803/EV	4.53±0.61[b]	3.02–6.05
MGC-803	4.97±0.47[b]	3.79–6.14

[a]$P<0.01$, MGC-803/E2F-1 vs. MGC-803/EV or MGC-803; [b]$P>0.05$, MGC-803/EV vs. MGC-803.

Purity and integrity of total RNA

The extraction of total RNA from the MGC803/E2F-1 cells and untransfected MGC-803 cells yielded good results, and 80 to 170 μg of RNA was extracted from each group. Agarose gel electrophoresis showed clear bands of 28S RNA and 18S RNA,

with intensity values of greater than 1. The 5S RNA band was unclear, suggesting that the RNA was of good purity and integrity (Fig. 1) and could satisfy the needs in following experiments.

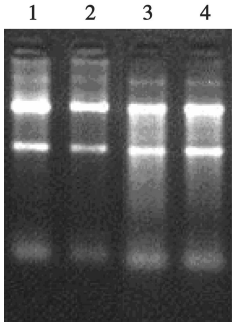

Figure 1 Agarose gel electrophoretogram of total RNA
Lanes 1,2, MGC-803; Lanes 3,4, MGC-803/E2F-1.

Computer analysis of graphic overlays of Cy3 and Cy5 signals

The chips were arranged in a matrix of 22 rows x 22 columns x 48 (sub-matrix). In the experimental group, green Cy3 was used as the label, whereas red Cy5 was used as the label for the negative control group. When the fluorescent signals overlapped at a specific point, the signal would appear green when the Cy3 signal was stronger (indicating down-regulation) and red when the Cy5 signal was stronger (indicating up-regulation). If both intensities were similar, a yellow color was visible (Fig. 2).

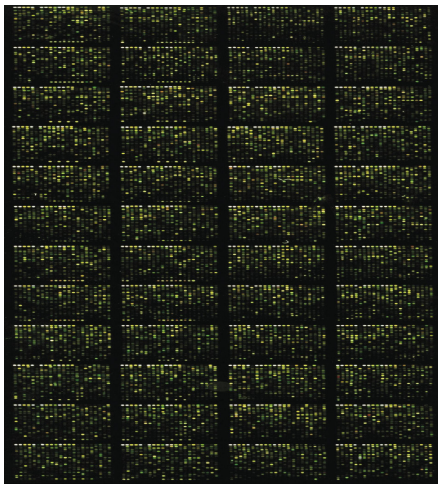

Figure 2 Bicolor florescence superposition chart of MGC-803/E2F-1 and MGC-803 cells

Signal intensity of hybridization between the MGC-803 and MGC-803/E2F-1 cells

The x-axis represents the foreground intensity value of fluorescent Cy3 in the experimental group, and the y-axis represents the foreground intensity value of fluorescent Cy5 in the negative control group. Each data point represents a hybridizing signal of a gene on the gene chip. Data shown in red and green represent Cy5/Cy3 ratios greater than 2 and less than 0.5, respectively, which reflect genes with variations in expression, with red suggesting up-regulation and green suggesting downregulation. Black indicates a ratio between 0.5 and 2, indicating no variation in expression (Fig. 3).

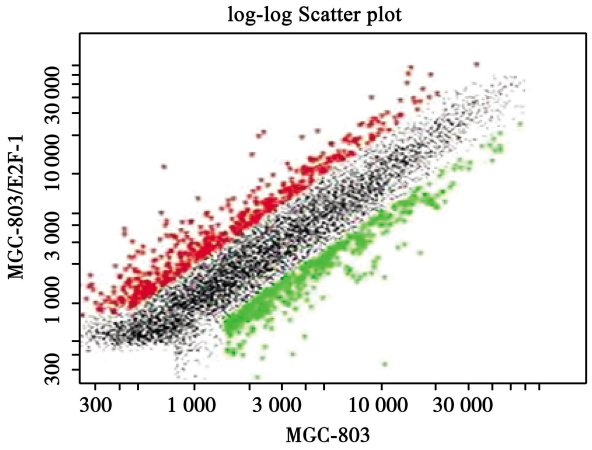

Figure 3 Scatter plot of cDNA microarray hybridization signals

Bioinformation analysis

Among the 21 522 genes included on the human genome chip, we detected 740 genes whose expression in MGC803/E2F-1 cells changed by at least 2-fold relative to their expression in untransfected MGC-803 cells. Of these 740, 15 genes were related to apoptosis, 4 of which were up-regulated and 11 of which were down-regulated (Table 2).

Verification of the target gene by RT-PCR

The Trizol single-step method was used to extract total RNA from the 3 groups of cells. After purification, electrophoresis showed 28S and 18S bands, while 5S band was unclear. Purified total RNA was used as a template for PCR of a housekeeping gene, GAPDH. A digestive reaction verified the purity of total RNA to satisfy the needs for following experiment. On this basis, multiple genes were verified, including MYC, IL-6, SSRP1, TIMP3, FN1, and PTEN. Results from the electrophoresis and grayscale intensities from the semiquantitative RT-PCR for the apoptosis-related gene MYC (Fig. 4) showed that expression of MYC was downregulated more in the MGC-803/E2F-1 group than in the MGC-803/EV and MGC-803 groups. The results of the verification were in accord with the result obtained from the gene chip. The directions of expression change in the verification of remaining multiple genes were the same as in the gene chips.

Discussion

E2F-1 is a member of the E2F family of transcription factors that is involved in regulating the cell cycle. E2F-1 also participates in the formation of a transcription factor complex and is an important regulatory factor for the apoptotic process. Our

Table 2 Differentially expressed genes related to apoptosis

Oligo	Ratio	Name	Description
H200011726	2.6514	RRAGC	Ras-related GTP binding C
H200004820	2.4581	MAP3K14	Mitogen-activated protein kinase 14
H200007187	2.1863	TRIAD3	TRIAD3 protein
H200000473	2.0404	TP53	Cellular tumor antigen p53
H200002757	0.4928	PDCD8	Programmed cell death 8
H200003690	0.4580	TRIB3	Tribbles homolog 3 (Drosophila)
H200002887	0.3958	SIAH2	Seven in absentia homolog 2 (Drosophila)
H200008094	0.3725	BID	Tumor antigen gene bid
H200006731	0.2815	SMNDC1	Survival motor neuron domain containing 1
H200003850	0.2680	MRPS30	Mitochondrial ribosomal protein S30
H200009671	0.2651	SGK	Serum/glucocorticoid regulated kinase
H200013961	0.2644	UBE1C	Ubiquitin-activating enzyme E1C
H200014835	0.2017	CHUK	Conserved helix-loop-helix ubiquitous kinase
H200006618	0.1875	Myc	Tumor gene myc
H200005162	0.1586	BAG2	bcl-2-associated athanogene 2
H200006853	0.1396	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)

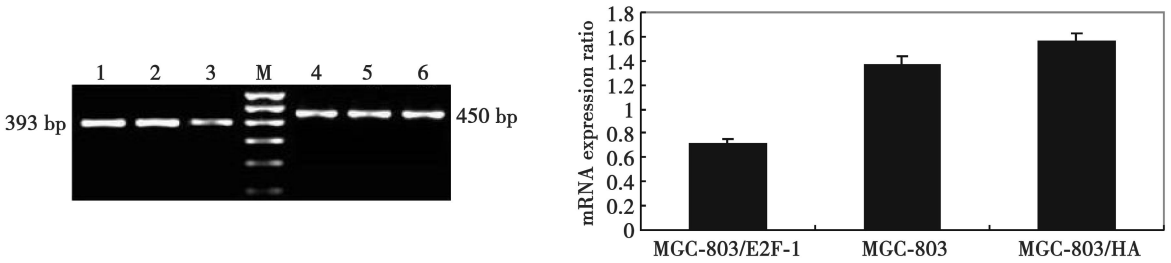

Figure 4 Agarose gel electrophoretogram of myc gene and GAPDH

Lane 1, MGC-803(myc); Lane 2, MGC-803/HA(myc); Lane 3, MGC-803/E2F-1(myc); lane 4, MGC-803(GAPDH); Lane 5, MGC-803/HA(GAPDH); Lane 6, MGC-803/E2F-1(GAPDH); M, marker.

experimental results showed the apoptosis rate was significantly higher in the MGC-803/E2F-1 group than in the MGC-803/EV and MGC-803 groups. (MGC-803/E2F-1 was the cell strain expressing the E2F-1 target gene and MGC-803/EV was the cell strain containing the empty vector. MGC-803 was the control group without either the vector or the target gene.) This result suggests that the overexpression of E2F-1 promotes apoptosis in gastric cancer cells. Li et al.[4] found that the overexpression of E2F-1 could induce apoptosis in tumor cells, as well as inhibiting tumor growth. When an adenovirus carrier was used to mediate the transfection of the E2F-1 gene, the overexpression of E2F-1 induced apoptosis in human gastric cancer cells on a large scale.[5] These results were the same as our findings in the current experiment, but the underlying induction mechanism for apoptosis by E2F-1 was still unclear.

The gene chip technique is a high-throughput screening technique that was developed for research on the human genome. It has characteristics of low power consumption and high sensitivity. This new technique has been widely applied to spectral analysis of the genomes of tumors[6] and has been successfully adapted for functional research on tumor-related genes,[7] the molecular classification of malignant tumors,[8] and research on chemotherapy and the underlying mechanisms of drug tolerance.[9] In this study, we found 15 genes with varying expression that were possibly related to apoptosis in gastric cancer cells with overexpressed E2F-1. There were 4 upregulated genes and 11 downregulated genes. TP53 is generally believed to be an important tumor suppressor gene. The overexpression of E2F-1 blocks the degradation of TP53 by activating the tumor suppressor gene p14ARF, which further guarantees the stability and activity of TP53.[10] Some scholars believe that the overexpression of E2F-1 could elevate the level of P53 protein without depending on p14ARF because the overexpression of E2F-1 elevates the level of P53 protein without elevating the expression of ARF.[11,12] Further research showed that the overexpression of E2F-1 could induce phosphorylation of multiple amino acid residues in P53. Phosphorylation of amino acid residues in P53, especially serine 20, prevents binding and degradation of P53 by mdm2.[13] Phosphorylation of amino residues in P53 induce auto-acetylation, which would further increase its binding to DNA to promote apoptosis.[14] Our experimental results suggest that the overexpression of E2F-1 in gastric cancer cells could upregulate the gene expression of TP53, raising the possibility that E2F-1 induces apoptosis through a TP53-dependent pathway.

BID is another key gene for apoptosis and is a member in the BCL gene family. It can elongate the lifespan of gastric cancer cells by inhibiting apoptosis, further increasing the chance that a tumor will occur and progress. BID protein has a direct regulatory effect on apoptosis and can inhibit apoptosis by many factors to ensure survival of cells.[15] BID can prevent the release of apoptotic cytokines, such as cytochrome C and

apoptosis-induced factor (AIF) from mitochondria. Increased expression of BID protein strengthens the inhibition of apoptosis. In our experiment, the expression of BIG was downregulated when E2F-1 was overexpressed. Thus, E2F-1 weakens the inhibition of apoptosis by BID, with an end result of apoptosis.

Myc is an apoptosis-related gene whose expression was negatively correlated with the overexpression of E2F-1, and it is an important oncogene. It is located on chromosome 8q24 and regulates the cell cycle. It is important in growth, differentiation, apoptosis, and transformation of tumors. The overexpression of E2F-1 reduced the expression of Myc, whereas normal levels of E2F-1 maintain the growth of cells. When its expression was altered, the induction of apoptosis increased. This alternation could also explain the increase in apoptotic gastric cancer cells during the overexpression of E2F-1. However, with overexpression of E2F-1, the expressions of many apoptotic factors, such as RRAGC, MAP3K14, TRIAD3, PDCD8, TRIB3, and SIAH2, also changed. This result suggests that E2F-1 may regulate apoptosis in gastric cancer cells through many other signal pathways and that there is complexity in the apoptotic process induced by E2F-1, in which many genes and pathways interact in a complex network. There have been reports from around the world on the expression of E2F-1 in many tumor tissues, but scholars have not agreed unanimously on the relationships between the variation in expression in different tumor cells or tumor tissues and the different levels of differentiation and the incidence or progression of tumors. Therefore, the importance of interactions among apoptotic genes for the induction of apoptosis still requires further investigation.

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