A Study on the Functions of Ubiquitin Metabolic System Related Gene FBG2 in Gastric Cancer Cell Line

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Citation

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Abstract

Objective To investigate the influence of FBG2 on the growth, proliferation, apopatosis , infiltration and cell cycle of the gastric cancer line MKN45. Methods A critical component ubiquitin-protein ligase complex FBG2 cDNA was subcloned into a constitutive vector PCDNA3.1 followed by transfection in MKN45 by using liposome. Then stable expression clones were selected and appraised. The apoptosis and cell cycles were detected using flow cytometry. The growth and proliferation were analyzed by making cell growth curves and colony formation assay respectively. The ability of infiltration were tested using cancer cell migration assay. The MKN-FBG2 group and two control groups were detected Results MKN-FBG2 grew faster than MKN45 and MKN-PC. The cell counts of MKN-FBG2 in the forth, fifth, sixth and seventh days were significantly more than that of others($P<0.05$).Cell cycle analysis showed that MKN-FBG2 proliferated faster, proportions of cells in G2-M and S were different significantly($P<0.05$). Results of colony formation assay showed that the colony formation rate of MKN-FBG2 was higher than that of control groups ($P<0.05$). Results of cell migration assay were negative. Conclusion FBG2 can promote the growth and proliferation of gastric cancer cells and help tumor cell maintain malignant phenotype. But it can have a negative influence on the apoptosis or the ability of infiltration of gastric cancer cells.

FBG2 (F-BOX6) gene is an important member in ubiquitin metabolic system F-BOX family [1,2] , and forms E3 complex with the other members in the family. It has been proved by previous researches that ubiquitin metabolic system is an important way for the catabolism of some protein molecules in cells, such as many oncogenes and anti-oncogenes [3,4,5] , and these protein molecules enter metabolic system through the identification by F-BOX family in E3 complex. The changes in the expression of FBG2 gene in cells may affect the level of some oncogenes or anti-oncogenes so as to influence some biological characters of cells to some degree. Our department [6,7] used cDNA gene chip to detect the difference in gene expression between adenocarcinoma of stomach and the normal mucous membrane tissues near carcinoma, and found that the expression level of FBG2 gene in carcinoma tissues was higher than that in normal tissues. However, there has been no report on the functions of this gene in stomach cancer cells both at home and abroad at present. In this research, gene transfection method was used to introduce FBG2 gene into gastric adenocarcinoma cell strain MKN45 and screen out the cell strains with stable expression. Then the changes in the related biological characters of the cell strain were detected in order to perform a preliminary analysis on the functions of this gene in gastric cancer.

MATERIALS AND METHODS

MATERIALS

Gastric adenocarcinoma cell line MKN45 was provided by Shanghai Institute of Biotechnology and preserved by our department. FBG2 monoclonal antibody was purchased from Abcam company (USA), PCDNA3.1 vector was preserved by our department, common cell culture plates were purchased from Orange Company, Transwell cell culture plates were purchased from Castar Company, and Watrigel gel was purchased from BD company (USA). AnexinV-FITC apoptosis detection kit was purchased from Biosea Biotechnology Co., Ltd. All the primers used in this research were synthesized by Shanghai Boya Biotechnology Co., Ltd.

METHODS

DETECTION OF EXPRESSION OF FBG2 GENE IN GASTRIC ADENOCARCINOMA CELL STRAIN MKN45

RT-PCR and immunocytochemical method were used to detect the expression of FBG2 in cells, and the results

showed that this cell strain was a FBG2 defective cell strain, which was suitable for transfection experiment research.

CONSTRUCTION AND IDENTIFICATION OF PC-FBG2 VECTOR

The cDNA obtained by RT-PCR from total RNA of human gastric adenocarcinoma mucous membrane tissues was used as templet. Inner and external primers were respectively synthesized:

Figure 1

S: 5' GGGGTACCCCAGGCCATGGATGCTC 3' 129

A: 5' CGGGATCCAACCGGGGCAGGAGTCG 3' 1104

S□ 5' GGGGTACCATGGATGCTCCCCACTC 3' 136

A□5' CGGGATCCATGGACAGCTGTCAGAA 3' 1024

With the templet, nested PCR method was used to obtain the FBG2 gene CDS double strand DNA fragments with KpnI and BamHI restriction sites in the two ends in two cycles of reactions. KpnI and BamHI were used to incise the double strand fragments and PCDNA3.1 vector. After the incised products were purified, they were kept at 16 over night for ligation under the actions of T4 ligase. Then the ligated products were used to transform $DH_{5□}$ competent cells, and routine antibiotic screening was performed. PCR identification was conducted to select positive clones. After amplification culture, positive clones were identified by KpnI and BamHI incision. The confirmed positive clones were sent for sequencing, and eukaryon vectors PC-FBG2 with expression of FBG2 gene of completely correct sequence were obtained.

PC-FBG2 VECTOR TRANSFECTED MKN45 CELLS

DMEM culture medium added with 10% fetal calf serum was used to culture the MKN45 cells in 12-well cell culture plates until the cells covered 90%-95% of the area. Serum-free DMEM culture medium was used instead for culture over night. Lipoinfectamine2000 liposome transfection kit was used. According to the directions for use, liposome and PC-FBG2 vector DNA were mixed and added into each well. PCDNA3.1 empty vector transfection group and blank control group (only liposome was added, and there was no vector DNA) were used. Transfection was completed after 24 hours' transfection.

SCREENING OF CELL STRAINS WITH STABLE EXPRESSION OF FBG2

Limited dilution method was used to dilute the transfected cells into 24-well culture plates according to the proportion of 1:20. Then G418 pressure screening method was used. G418 concentration was based on the results of preliminary tests (800μg/ml, the concentration at which there were no surviving cells at 7 days after the time when MKN45 cells covered 90% of the area of the wells in 6-well culture plate). 31 days was used for screening. 12 and 7 positive clones were respectively obtained in the PC-FBG2 vector transfection group and PCDNA3.1 empty vector transfection group. One positive clone was taken for identification and other positive clones were frozen for future use.

IDENTIFICATION OF CELL STRAINS WITH STABLE EXPRESSION

RT-PCR and Western blotting were respectively used to detect the mRNA and proteins of FBG2, and immunocytochemical method was used to detect the expression of FBG2 proteins in situ.

DETECTION OF THE INFLUENCE OF FBG2 GENE ON THE GROWTH OF CELLS USING CELL GROWTH CURVE METHOD

FBG2 gene stable expression cell group, PCDNA3.1 empty vector transfection group and blank cell control group were used. The cells in each group were inoculated into 24-well culture plate for culture using the concentration of 5×10^{4} /ml. After the cells completely adhered to the wall, the cells in 5 wells in each group were completely digested, the mean values were calculated, and growth curves were plotted. In addition, blank cell control and stable transfection cell control of PCDNA3.1 empty vector group were used.

DETECTION OF CELL CYCLES AND APOPTOSIS USING FLOW CYTOMETRY

FBG2 gene stable expression cell group, PCDNA3.1 empty vector transfection group and blank cell control group were used. When the cells covered 70% of the area of cell culture flask in each group, serum-free culture medium was used for culture for 24 hours instead for synchronization. After 24 hours' continuous culture, the cells were digested and fixed with anhydrous alcohol. After one night, 30 minutes' staining was performed, and flow cytometer was used to detect the cell cycles. After synchronization and 24 hours' continuous culture, the cells were digested, PI and AnexinV-FITC double staining was performed, and flow cytometry was used to detect the apoptosis of cells. 3 replicate test groups were used in each group, the average values were calculated, and comparison was conducted.

DETECTION OF CELL MULTIPLICATION USING PLATE CLONING METHOD

FBG2 gene stable expression cell group, PCDNA3.1 empty vector transfection group and blank cell control group were used. 1000 cells/plate in each group were respectively inoculated into five 9cm cell culture dishes. After 18 days' culture with DMEM containing fetal calf serum, the number of clones with more than 50 cells in each well was counted under microscope (clone formation rate = number of clones in each plate / 1000×100%). 5 wells were selected from each group, and the mean value of 5 wells was calculated for comparison.

DETECTION OF INVASIVENESS OF CELLS BY CELL MIGRATION TEST

Similarly, the above 3 groups were used. According to the method of He Huiying et al. [8] , 1×10^4 cells from each group were inoculated into Transwell cell culture plate (filter membranes treated with Watrigel biogel had been placed, and 3 wells were inoculated for each group). After 24 hours' culture with complete medium, the filter membranes were rinsed, and the adhering cells in both sides were digested and counted. Comparison was performed on the cell migration rate [= number of cells permeating through the membrane (number of cells in the reverse side of membrane) / total number of cells].

STATISTICAL TREATMENT

SPSS11.0 statistical software was used.

Two-factor and one-factor analysis of variance was used for statistical analysis.

RESULTS

DETECTION OF THE EXPRESSION OF FBG2 GENE IN GASTRIC ADENOCARCINOMA CELL STRAIN MKN45 WITH RT-PCR AND IMMUNOCYTOCHEMICAL METHODS

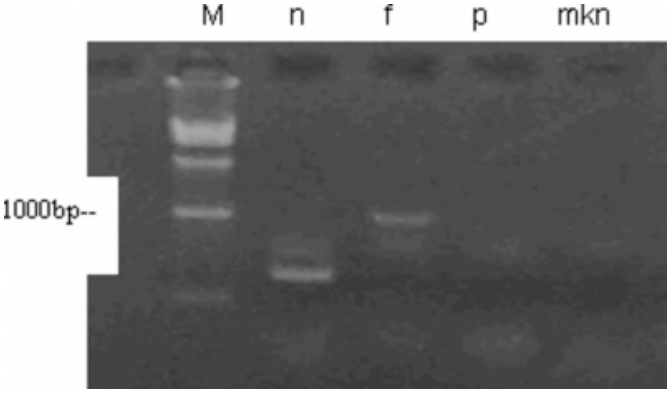
All the results were negative, indicating there was no expression of FBG2 gene in untreated MKN45 cells.

DETECTION OF THE EXPRESSION OF FBG2 GENE IN MKN45 CELLS WITH STABLE TRANSFECTION USING RT-PCR, WESTERN BLOTTING AND IMMUNOCYTOCHEMICAL METHODS

The results showed that when compared with the untreated MKN45 cells and the MKN45 cells transfected with PCDNA3.1 empty vectors, the expression of FBG2 gene significantly increased. The immunocytochemical test results showed that the expression of FBG2 gene in cells was mainly distributed in cytoplasm and there was no obvious positive signal in cell nucleus and cell membrane (Figures 1, 2, 3, 4).

Figure 2

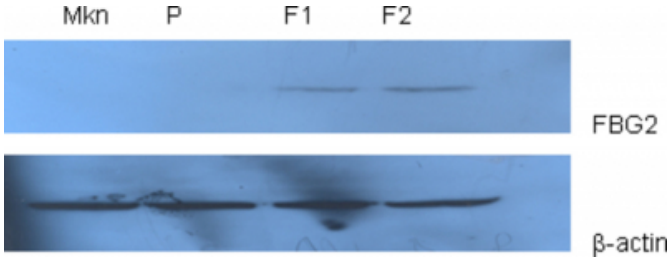
Figure 1: The result of RT-PCR



(Note: n was β-actin negative control, f was the MKN45 cells with stable transfection of FBG2, P was the MKN45 ells transfected with empty vectors, and Mkn was blank cells)

Figure 3

Figure 2: The result of Western bloting



(Note: F1F2 were the MKN45 cells with stable transfection of FBG2, P was the MKN45 cells transfected with mpty vectors, and Mkn was blank cells)

Figure 4

Figure 3: The Immunohistochemistry result of MKN-FBG2 group ×200

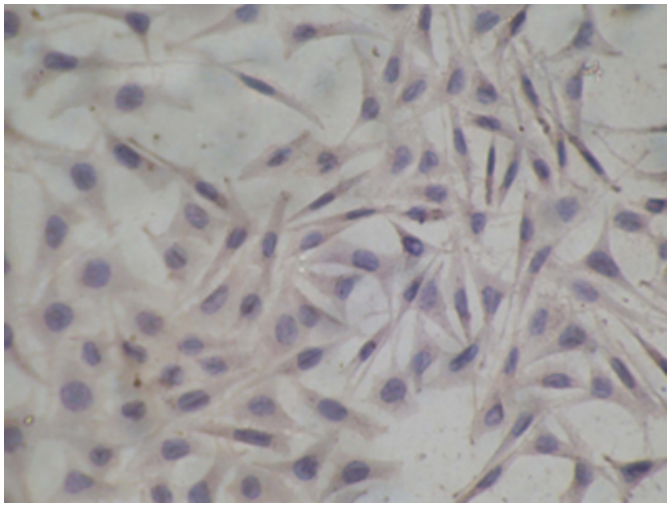
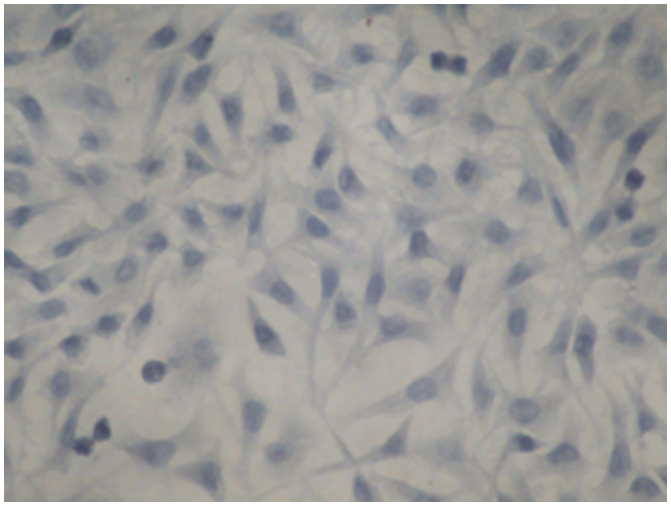


Figure 5

Figure 4: The Immunohistochemistry result of MKN-PC group ×200

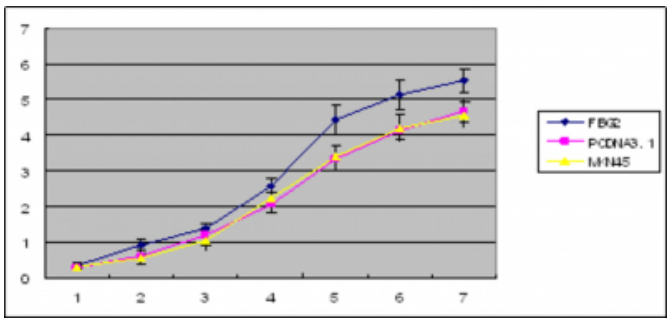


DETECTION RESULTS OF THE INFLUENCE OF FBG2 GENE ON THE GROWTH OF CELLS USING CELL GROWTH CURVE METHOD

Two-factor analysis of variance showed that the growth of the cell line MKN-FBG2 with stable transfection of FBG2 gene was significantly faster than that of the untreated MKN45 cells and the MKN45 cells transfected with PCDNA3.1 empty vectors (P<0.05), and there was no significant difference between the latter two groups (Figure 5). At 4, 5, 6 and 7 days after inoculation of the same amount, the average cell count results of MKN-FBG2 group were respectively 2.58×10^{5} , 4.42×10^{5} , 5.14×10^{5} and 5.54×10^{5} , which were significantly higher those of the other two groups (P<0.05).

Figure 8

Figure 7: The result of cancer cell migration assay



(notethe unit of vertical axis was$\times10^{5}$ horizontal axis was the number of days)

DETECTION OF CELL CYCLES USING FLOW CYTOMETRY

The results showed that the proportion of cells in G2-M phase in the MKN-FBG2 group was significantly higher than that of the untreated MKN45 cells and the MKN45 cells transfected with PCDNA3.1 empty vectors (P<0.05), the proportion of cells in S phase was significantly lower than that of the other two groups (P<0.05), and there was no significant difference in the proportion of cells in other phases between them (Table 1).

Figure 7

Table 1: The different cell cycle of MKN-FBG2, MKN-PC and MKN45 group

Group	n	G0—G1□%□	G2—M□%□	S□%□
FBG2	3	46.21±4.43	16.52±2.33	37.27±0.56
PC3.1	3	44.33±3.45	2.79±0.44 [1]	52.88±6.43 [1]
MKN45	3	47.90±2.39	3.12±5.37 [1]	48.98±4.22 [1]

note compare with the group of MKN-FBG2 [1] denoting P<0.05

DETECTION OF APOPTOSIS USING FLOW CYTOMETRY

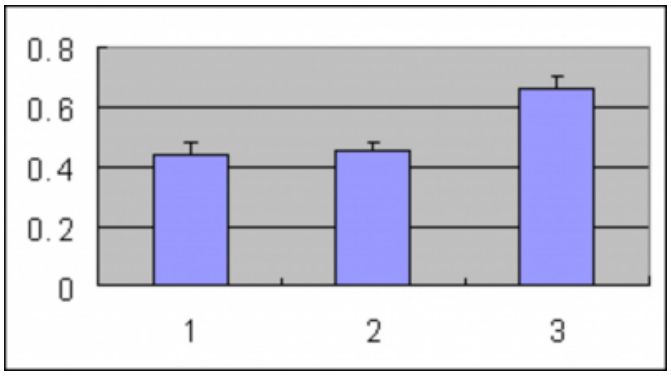
The results showed that the average apoptotic rates of the cells in MKN-FBG2 group, the MKN45 cells transfected with PCDNA3.1 empty vectors and the untreated MKN45 cells were respectively 0.78±0.04%, 0.85±0.03% and 0.81±0.07%, and there was no statistical significant difference between them (P>0.05).

DETECTION OF CELL MULTIPLICATION USING PLATE CLONING METHOD

The plate clone formation rate of the MKN-FBG2 group (0.66±0.04) was significantly higher than that of the untreated MKN45 cells (0.44±0.04) and the MKN45 cells transfected with PCDNA3.1 empty vectors (0.45±0.03) ($P<0.05$) (Figure 6).

Figure 9

Figure 7: The result of cancer cell migration assay



(Note:1 was blank cells, 2 was the MKN45 cells transfected with empty vectors, 3was the MKN45 cells with stable transfection of FBG2.)

DETECTION OF INVASIVENESS OF CELLS BY TRANSWELL MEMBRANE CELL MIGRATION TEST

The results showed that the migration rate of each group of cells was about 0.3 (Figure 7) and there was no statistically significant difference between the groups ($P>0.05$).

{image:9}

(Note: 1 was the blank cell group, 2 was the group of MKN45 cells transfected with empty vectors, and 3 was the group of MKN45 cells with stable transfection of FBG2)

DISCUSSION

FBG2 and the other members in F-BOX family participating in the metabolism of ubiquitin play a role in many functions of cells, but there is still lack of researches both at home and abroad for the moment. Some researches [9] showed that F-BOX family participated in the degradation of some anti-oncogenes including P53. The other researches by Wu Qingming, Zhang Weiguo et al [10,11] also showed there was a close relation between the metabolic system of ubiquitin and the proliferation and apoptosis of gastric cancer cells, so it was suspected that the overexpression of the genes of this family might be concerned with the formation of development of gastric cancer cells. The results of gene chip research by our department also preliminarily confirmed the upregulation of FBG2 in gastric adenocarcinoma tissues. The gene clone technique used in this research further verified its functions in gastric cancer cells. First, liposome mediated gene transfection method and G418 pressure screening method were used to obtain cell strains with stable expression of FBG2 genes, which were verified by immunocytochemistry, RT-PCR and Western blotting methods. Growth curve method, plate clone formation test and flow cytometry were used for the functional verification on the gastric cancer cell strains with stable expression of FBG2. The results of growth curve method and plate formation test showed that the growth and proliferation of the cell strains with stable expression of FBG2 were significantly faster than those of the cells transfected with empty vectors and blank control cells. Therefore, FBG2 gene could accelerate the growth and proliferation of cells. The reasons might be as follows: (1) The gene products promoted the activities of the metabolic system of ubiquitin so as to enhance the metabolism of some protein molecules in cells and accelerate the growth and proliferation of cells. (2) It might accelerate the degradation of proteins inhibiting the growth and proliferation of cells so as to promote the growth and proliferation of cells. The results of flow cytometry showed that the proportion of cells in G2-M phase in the cell strain with stable expression of FBG2 was higher than that of the control cells and the proportion of cells in S phase was lower than that of the control cells. There was no significant difference in the apoptotic rate between each group of cells. These results indicated that for the cell strain with stable expression of FBG2, many were in the division stage, so FBG2 gene could accelerate the growth and proliferation of cells. However, this gene did not affect the apoptosis of cells perhaps because FBG2 gene and the metabolic system of ubiquitin had little influence on the key genes concerned with apoptosis. The results of Transwell membrane cell migration test showed that there was no significant difference in the migration capacity, which reflected the invasiveness of cells, between the groups of cells and the cause was to be further investigated. A probable cause was that there was no close relation between the gene products (proteins) concerned with invasion and FBG2 gene and the metabolic system of ubiquitin. The main conclusion of this research is as follows: FBG2 gene can significantly promote the growth and proliferation of cells. There were still many deficiencies in our research. For example, only single cell line was used. In future researches,

cell lines with high expression of FBG2 gene will be used for RNAi or antisense and ribozyme expression inhibition test in order to further verify the functions. Animal experiment will also be used to indepthly investigate the relation between FBG2 gene (even the whole F-BOX family and the metabolic system of ubiquitin) and the occurrence and development of gastric cancer.

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