

RESEARCH ARTICLE

Anti-proliferation Effects of Interferon-gamma on Gastric Cancer Cells

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Abstract

IFN- γ plays an indirect anti-cancer role through the immune system but may have direct negative effects on cancer cells. It regulates the viability of gastric cancer cells, so we examined whether it affects their proliferation and how that might be brought about. We exposed AGS, HGC-27 and GES-1 gastric cancer cell lines to IFN- γ and found significantly reduced colony formation ability. Flow cytometry revealed no effect of IFN- γ on apoptosis of cell lines and no effect on cell aging as assessed by β -gal staining. Microarray assay revealed that IFN- γ changed the mRNA expression of genes related to the cell cycle and cell proliferation and migration, as well as chemokines and chemokine receptors, and immunity-related genes. Finally, flow cytometry revealed that IFN- γ arrested the cells in the G1/S phase. IFN- γ may slow proliferation of some gastric cancer cells by affecting the cell cycle to play a negative role in the development of gastric cancer.

Keywords: Gastric cancer cells - IFN- γ - cell cycle - AGS - gene Chip

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Introduction

Gastric carcinoma is one of the main diseases of humans and is the fourth most common cancer throughout the world. The annual incidence of gastric cancer is 930,000, and the mortality rate is the second among all cancers in the world (Parkin et al., 2005). In 2002, 700,000 people worldwide died from stomach cancer (Parkin et al., 2005). The incidence of gastric cancer in Asia is higher than that in other areas. World Health Organization data show that the incidence is increasing.

About 75% of non-cardia gastric carcinoma is caused by *Helicobacter pylori*, and gastric mucosa infected by *H. pylori* show a high level of IFN- γ (Karttunen et al., 1995). Among all T helper cell type 1 (Th1) cytokines, IFN- γ is an important inflammatory factor involved in immune regulation. It has direct and indirect anti-tumor effects (Gerber et al., 2013; Tagawa et al., 2013), as well as anti-virus effects.

IFN- γ has a direct effect on tumor cells and a negative role in cancer cell proliferation. IFN- γ may directly or indirectly affect apoptosis of some cancer cells (Wagner et al., 1997; Tu et al., 2011; Ni et al., 2013), such as glioma cells (Rajiv et al., 2008), multiple myeloma cells and cervical cancer cells (Lee et al., 2005; Lindkvist et al., 2006). It can also induce aging of human endothelial cells, thus leading to cell cycle change (Kim et al., 2009), and

affect the expression of genes such as Secretoglobin 3A1 (SCGB3A1) (Yamada et al., 2009), osteopontin (OPN) (Xinfang et al., 2003), CXCR3 binding chemokine IP-10 (CXCL10) (Yeruva et al., 2008), and the phosphorylation of signal transducer and activator of transcription (STAT1), for example (Mitchell et al., 2004). IFN- γ regulates cell apoptosis and the viability of gastric cancer cells (Shimako et al., 2002) and indirectly lowers the invasion of gastric cancer cells (Kuga et al., 2003). Clinical data show that IFN- γ is associated with gastric epithelial cell apoptosis (Frank et al., 2002). However, whether IFN- γ affects gastric cancer cell aging, cell cycle, and gene expression is unknown.

Because IFN- γ has an effect on gastric cancer cell proliferation, we examined the colony formation ability of gastric cancer cells exposed to IFN- γ . We used flow cytometry to determine whether IFN- γ affects gastric cancer cell apoptosis and cell cycle and β -gal staining to determine its effect on cell senescence, as well as microarray assay of the mRNA expression of affected genes.

Materials and Methods

Cell and cultivation

The gastric cancer cell line AGS was cultured in F12 (Gibco, USA), and the lines GES-1 and HGC-27 were

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cultured in RPMI1640 (Gibco, USA) with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/ml penicillin, 2 mM L-glutamine, at 37 °C with 5% CO₂.

Colony formation experiments

AGS, GES-1 and HGC-27 cells subcultured for 16 h were exposed to IFN- γ (0.065, 0.65, 6.5 ng mL⁻¹) (Kai Mao, China) for 2 h, then digested with trypsin (Gibco, USA), collected by centrifugation and diluted 300 cells per well in 6-well plates for culture for 10 days. When cell colonies were visible by the eye, cells were fixed for 5-10 min with 500 μ l methanol per well, then washed with phosphate buffered saline (PBS) and stained for 10 min with 1 ml Giemsa dye. The number of colonies with more than 50 cells was counted. Control cells were exposed to normal sodium as above.

Flow cytometry of cell apoptosis

AGS cells were exposed to IFN- γ (6.5 ng mL⁻¹) for various times, then digested with trypsin and washed 3 times with PBS, then suspended with 900 μ l PBS. Cells were added to 100 μ l Hoechst 33342 (100 mg mL⁻¹) for 7-10 min at 37 °C, centrifuged at 1000 rpm for 5 min at 4 °C, then incubated with 1.0 ml PI dye for 15 min at 4 °C. Cells were filtered by use of a 400 screen filter and analyzed by flow cytometry (BD, USA).

β -gal staining of cell senescence

AGS cells were exposed to IFN- γ (6.5 ng mL⁻¹) for various times and rinsed with PBS, then fixed in 3% formaldehyde for 5 min. Cells were washed with PBS, then incubated with 500 μ l β -gal dye (X-gal, 1.0 mg mL⁻¹; citric acid, 40.0 mM; potassium ferrocyanide, 5.0 mM; potassium ferricyanide, 5.0 mM; NaCl, 150.0 mM; MgCl₂, 2.0 mM) in 6-well plates that were sealed with film for 24 h at 37 °C without CO₂. Senescent cells were observed under an inverted microscope.

Microarray assay

Microarray assay involved the Human 12 x 135K Gene Expression Array (Roche NimbleGen). About 45,033 genes were collected from data sources including NCBI. After being exposed to IFN- γ (6.5 ng mL⁻¹) for 2 h, AGS cells were harvested and RNA was isolated by use of TRIzol reagent (Invitrogen, USA); the RNA concentration was measured at A260 nm. Double-stranded cDNA (ds-cDNA) was synthesized from 5 μ g total RNA by use of the SuperScript ds-cDNA synthesis kit (Invitrogen, USA) with 100 pmol oligo dT primers. ds-cDNA was labeled in accordance with the Nimblegen Gene Expression Analysis protocol (Nimblegen Systems, Madison, WI, USA). Briefly, ds-cDNA was incubated with 4 μ g RNase A for 10 min at 37 °C and cleaned with phenol: chloroform: isoamyl alcohol (25:24:1), then ice-cold absolute ethanol precipitation. The purified cDNA was quantified by use of a nanodrop ND-1000. For Cy3 labeling of cDNA, the One-Color DNA labeling kit was used (Nimblegen Systems). In total, 1 μ g ds-cDNA was incubated for 10 min at 98 °C with 40 μ l Cy3-9 mer primer. Then, 100 pmol deoxynucleoside triphosphate and 100 U Klenow fragment (New England Biolabs, USA) were

added for incubation for 2 h at 37 °C. The reaction was stopped by adding 0.1 volume of 0.5 M EDTA, and the labeled ds-cDNA was purified by isopropanol: ethanol precipitation. Microarrays were hybridized at 42 °C for 16 to 20 h with 4 μ g Cy3-labelled ds-cDNA in hybridization buffer/hybridization component A (Nimblegen Systems) in a hybridization chamber, then washed by use of the Wash Buffer kit (Nimblegen Systems) in an ozone-free environment; slides were scanned by use of the Axon GenePix 4000B microarray scanner (California, USA).

Raw data were extracted as paired files by use of GenePix pro V6.0. Images and quantitative data of expression levels were analysed by use of Agilent GeneSpring v11.0.

Quantitative real-time RT-PCR (qRT-PCR)

According to microarray results, we randomly chose 7 genes (with expression fold change ≥ 2 , $p \leq 0.05$) for examination of mRNA expression. Primers are in Table I. RNA isolation and cDNA production are described above. An amount of 20 μ l PCR reaction volume contained SYBR Premix Ex Taq™ and ROX Reference Dye (both TaKaRa, Japan), 100 ng cDNA, and 500 nM each of forward and reverse primers. The PCR protocol was one cycle at 95 °C for 10 sec, then 40 cycles at 95 °C for 5 sec and 55 °C for 31 sec. PCR products were detected with use of Prism7000 (ABI). β -actin gene was used as the endogenous control.

Flow cytometry of cell cycle

AGS cells were exposed to IFN- γ (6.5 ng mL⁻¹) for 2 h and digested by trypsin. Cells were centrifuged at 1000 rpm for 8 min and washed twice with ice-cold PBS, then centrifuged at 1000 rpm for 4 min. Cells were suspended with ice-cold PBS and separated into single cells, fixed with 70% ethanol pre-cooled at -20 °C, then rested overnight at 4°C. Cells were centrifuged at 800 rpm for 5 min and washed twice with PBS, then stained with 0.5 ml PI dye (PI, 5 mg; RNase, 2 mg; 1.0% Triton X-100, 0.25 ml; physiological saline, 65 ml; sodium citrate, 100 mg; per 100 ml PI dye) and incubated for 30 min away from the light at 4 °C. Cell cycle was detected by flow cytometry (BD, America) with standard procedures, and 20,000 to 30,000 cells were counted. Results were analyzed by use of ModFit vLT 3.1.

Data analysis

All experiments were performed in triplicate. Statistical analysis involved Student's t test and SPSS 19.0 (SPSS Inc., Chicago, IL). $p < 0.05$ was considered statistically significant.

Results

Effect of IFN- γ on colony formation ability, apoptosis and aging of gastric cancer cells

The colony formation ability and thus proliferation of gastric cancer cells was decreased with IFN- γ (6.5 ng mL⁻¹) ($p < 0.05$) (Figure 1). Cell apoptosis is programmed cell death regulated by genes and reduces the colony formation ability of gastric cancer cells. We found no

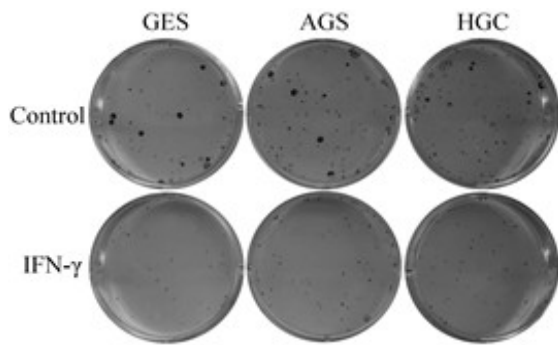


Figure 1. Colony Formation Ability of Gastric Cancer Cells Exposed to Interferon γ (IFN- γ ; 6.5 ng mL⁻¹) for 2 h, and the Control Cells Were Exposed to Normal Sodium

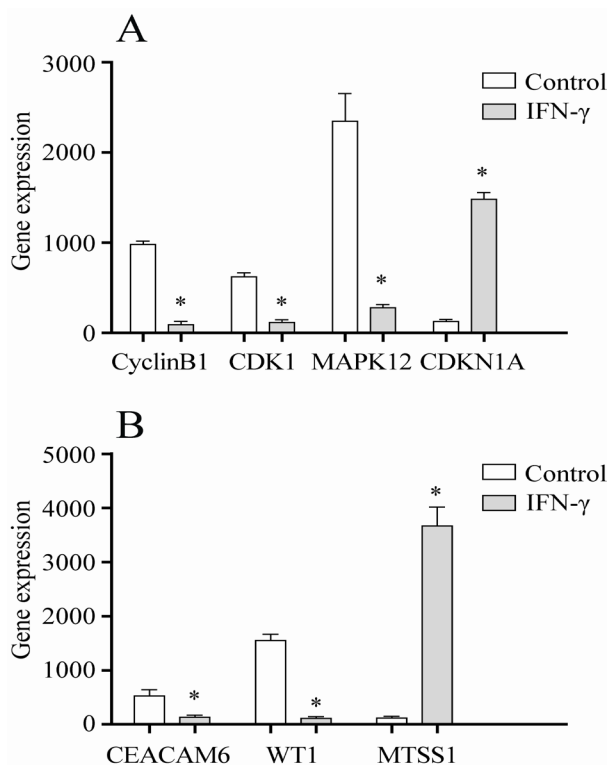


Figure 2. Quantitative Real-time PCR Analysis of Differential Expression of Genes Related to the Cell Cycle (A) and Cell Proliferation and Migration (B) on Treatment with IFN- γ (6.5 ng mL⁻¹) for 2 h. * $p < 0.05$

effect of IFN- γ on apoptosis of gastric cancer cells (data not shown). Cell aging represents decreased cell proliferation capacity and physiologic function and is a barrier to cancer development; however, we found no effect of IFN- γ on aging of AGS cells (data not shown).

Microarray assay of genes in AGS cells exposed to IFN- γ

Because IFN- γ reduced the colony formation ability of gastric cancer cells but did not induce apoptosis or aging of AGS cells, we examined the effect on gene expression in gastric cancer cells by microarray assay. In total, 283 genes showed more than two-fold difference in expression as compared with controls: 172 with more than two-fold upregulated expression and 111 with more than two-fold downregulated expression. Some genes with differential expression are in Table II. IFN- γ altered

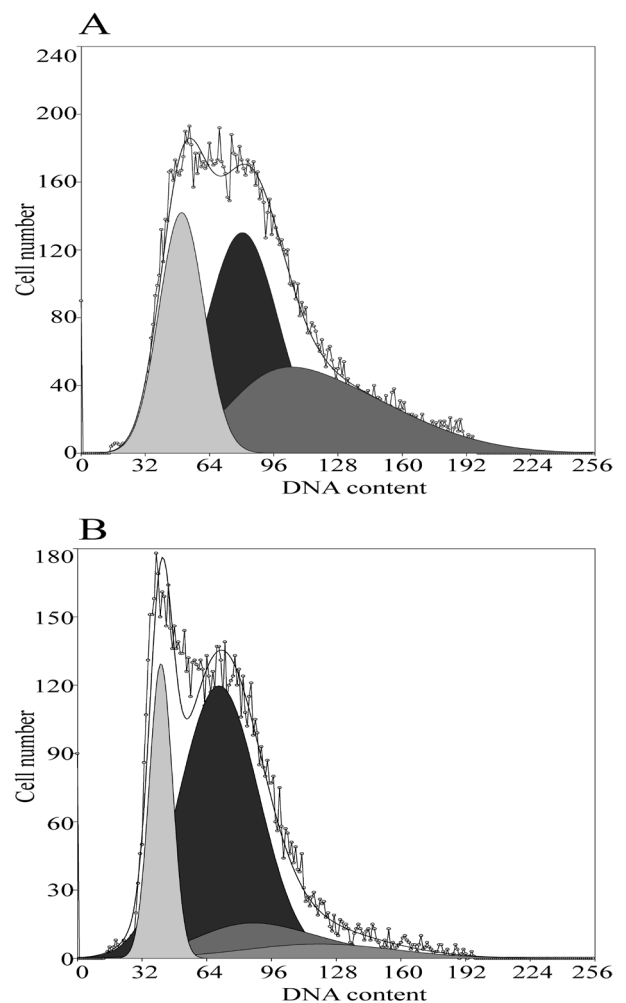


Figure 3. Flow Cytometry of Cell Cycle of AGS Gastric Cancer Cells with (A) Control Treatment and (B) IFN- γ Treatment (6.5 ng mL⁻¹) for 2 h

the expression of genes related to the cell cycle, and cell proliferation and migration, as well as chemokines and chemokine receptors, immunity-related genes and genes with unknown function.

qRT-PCR confirmation of gene expression

qRT-PCR analysis of the up- and downregulated mRNA expression of genes agreed with microarray assay results. Of 7 genes examined, 4 are closely associated with the cell cycle: cyclin B1 (Yuan et al., 2004; Yuan et al., 2006), cyclin-dependent protein kinase 1 (CDK1) (Li et al., 2000; Xiao et al., 2009), and mitogen-activated protein kinase 12 (MAPK12) (Marinissen et al., 2001; Tortorella et al., 2003; Hou et al., 2010) were downregulated and cyclin-dependent kinase inhibitor 1A (CDKN1A) was upregulated (Cazzalini et al., 2010; Gao et al., 2010; Takeuchi et al., 2010; Wei et al., 2010). Other genes closely associated with cell proliferation and migration were differentially regulated: carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) (Duxbury et al., 2004; Mahata et al., 2006; Poola et al., 2006; Blumenthal et al., 2007; Lasa et al., 2008) and Wilms tumor 1 (WT1) (Sakamoto et al., 2009; Rushing et al., 2010) were downregulated, and metastasis suppressor 1 (MTSS) was upregulated (Figure 2) (Liu et al., 2010).

Change in cell cycle of AGS cells exposed to IFN- γ

We further examined whether IFN- γ affected the cell cycle of AGS gastric cancer cells. With control treatment, 55.19% of cells were in the G0/G1 phase and 44.81% were in the S phase; however, with IFN- γ treatment, 76.55% of cells were in the G0/G1 phase, 7.66% were in the G2/M phase, and 15.79% were in the S phase ($P < 0.05$) (Figure 3). IFN- γ caused cell cycle arrest during the G0/G1 phase and therefore affected the cell cycle of gastric cancer cells.

Discussion

IFN- γ affects apoptosis of gastric cancer cells (Shimako et al., 2002) and clinical study suggested that IFN- γ has a close relation with the apoptosis of gastric epithelial cells (Frank et al., 2002). We investigated the effect of IFN- γ on gastric cancer cell proliferation through colony formation ability representing cell proliferation ability. IFN- γ decreased the colony formation ability of gastric cancer cells but did not affect their apoptosis, which disagrees with Shimako et al. (2002). The differences might result from different cell strains used. IFN- γ was found to cause aging of endothelial cells through P53-dependent DNA damage (Xinfang et al., 2003), but we found no effect of IFN- γ on aging of gastric cancer cells, which again may indicate cell-specific effects.

To investigate the mechanism of IFN- γ decreasing the colony formation ability of gastric cancer cells, we used microarray assay of gene expression and found cell-cycle-related genes downregulated (cyclin B1, CDK1, MAPA12) or upregulated (CDKN1A) and confirmed the expression changes by Quantitative real-time PCR.

Cell division follows a cycle of G1 \rightarrow S \rightarrow G2 \rightarrow M \rightarrow G1. The cycle is regulated mainly by cyclin, CDK and CDKIs at 2 key points, G1/S and G2/M. Cyclin B1, an important member of the cyclin family, is synthesized at the S phase, locates in cytoplasm at the G2 phase and enters the nucleus at the beginning of the M phase. Cyclin B1 binds to CDK1 and induces Thr160/Thr161 phosphorylation, forming heterologous dimers of maturation-promoting factor, which enable the G2/M transition and promote the M phase to accelerate the cell cycle. Cyclin overexpression causes abnormal cellular multiplication and cell transformation and is seen in many tumors cancers as gastric, liver, breast, cervical, and ovarian cancer (Yang et al., 2008; Chen et al., 2008; Feng et al., 2009; Huang et al., 2010). It is related to the occurrence and development of tumor and directly to the classification, differentiation, intrusion and metastasis of tumor (Yang et al., 2008; Chen et al., 2008; Feng et al., 2009; Huang et al., 2010). It is considered a necessary molecule of tumor cell survival and malignant proliferation (Li et al., 2009). Cyclin B1 and its related molecules are potential new antitumor therapy targets. siRNA knockdown of cyclin B1 in tumor cells suggested that tumor cell apoptosis was induced with its expression, and proliferation was inhibited (Yuan et al., 2004). Cyclin B1 knockout increased the sensitivity of mammals to antitumor drugs and induced cell cycle arrest (Yuan et al., 2006). Antisense full-length cyclin B1 cDNA interferes with its expression, and HeLa cell proliferation was inhibited and apoptosis induced with its increased

expression (Li et al., 2009). The Chinese crude drug Huang Lian Su degrades cyclin B1 and inhibits tumor cell growth, so it may be an effective drug for treating tumor (Li et al., 2000). Another Chinese crude drug, Allicin, downregulated cyclin B1 expression in the human gastric cancer cell line BGC823, which was arrested in the G2/S phase, thus inhibiting cell proliferation (Liu et al., 2005). We found that IFN- γ downregulated cyclin B1 in AGS gastric cancer cells, and the cell cycle was arrested, with decreased colony formation. IFN- γ may suppress gastric cancer cell colony formation by decreasing cyclin B1 expression.

IFN- γ downregulated the expression of CDK1 in gastric cancer cells. CDK1 is an important member of the CDK family. CDK1 alone has no activity until binding to cyclin B1 and being phosphorylated. It is expressed highly in many solid tumors (Huang et al., 2004; Zhao et al., 2009; Chen et al., 2009; Zhang et al., 2009; Xia et al., 2010). As the main biological initiator of gastric cancer, *H. pylori* induces CDK1 expression; thus, CDK1 upregulation by *H. pylori* induces gastric cancer (Xia et al., 2010). Both CDK1 and cyclin B1 are highly expressed in tumor (Huang et al., 2004; Zhao et al., 2009; Chen et al., 2009; Xia et al., 2010), and CDK1 and cyclin B1 are positively correlated in esophageal and glioma cancer (Zhao et al., 2009; Chen et al., 2009). The 2 molecules synergetically promote the occurrence and development of esophageal cancer (Zhao et al., 2009). CDK1 is a target of cancer treatment. Inhibiting CDK1 and CDK2 in HeLa cells induced cell cycle arrest and apoptosis (Xiao et al., 2009). IFN- γ may induce cell cycle arrest by regulating CDK1.

At present, we lack results of MAPK12 affecting the cell cycle and cell proliferation. MAPKs belong to the serine-threonine kinase family and show high homology (Tortorella et al., 2003). MAPK12 is also called ERK3, ERK6, p38 γ or SAPK3. Protein tyrosine phosphatase H1 (PTPH1) is the specific phosphatase of p38 γ . PTPH1 binding directly to p38 γ plays an important role in the growth of Ras-mediated malignant tumor (Hou et al., 2010). At present, 2 signaling pathways of p38 γ induce tumor: ATM \rightarrow MKK6 \rightarrow p38 γ \rightarrow Cdk1 \rightarrow CDK1 \rightarrow G2, which causes cell proliferation (Zeng et al., 2005); and the small molecule GTP-binding protein RhoA \rightarrow PKN \rightarrow MKK6 \rightarrow ERK6 \rightarrow MEF2, jAP1 \rightarrow c-jun. We found downregulated MAPK12 expression in gastric cancer cells exposed to IFN- γ . IFN- γ may affect gastric cancer cell proliferation by regulating MAPK12, which requires further study into whether the regulation affects the cell cycle.

The N-terminal region of CDKN1A, also called p21 or Cip1, contains a region binding to CDK, which inhibits the activity of cyclin D-dependent protein kinase and inhibits the cell cycle from the G1 to S phase. CDKN1 directly inhibits DNA replication, which arrests the cell cycle at the G1 phase, so cells stop dividing and begin to repair DNA or die (Pan et al., 2009; Cazzalini et al., 2010; Jin et al., 2010). Transfection of the dsRNA of p21 in lung cancer cells upregulated p21 expression, suppressed cell growth and enhanced sensitivity to cisplatin, an important anticancer drug (Wei et al., 2010). p21Waf1/Cip1 and

p16INK4a may synergistically facilitate the aging of skin cancer cells and play an anti-tumor role (Takeuchi et al., 2010). We found CDKN1A upregulated in gastric cancer cells exposed to IFN- γ . Thus, IFN- γ may affect the G1/S phase transition by increasing CDKN1A level, which arrests the cell cycle and affects the growth of gastric cancer cells. Recently, CDKN1A was used as a target for cancer therapy. Oridonin were injected into BALB/C nude mice with tumor and promoted p21 expression, which arrested the cell cycle of colon carcinoma cells and led to cell aging and apoptosis, and tumor growth was inhibited (Gao et al., 2010). IFN- γ has a similar mechanism as Oridonin, and we found that IFN- γ also caused cell cycle arrest and inhibited cell proliferation.

Gastric cancer cells exposed to IFN- γ resulted in expression change of cell cycle-related genes, so we examined whether IFN- γ affected their cell cycle by flow cytometry. Gastric cancer cells stayed in the G0/G1 period, which reduced the number of cells in the G1/S period. Presumably this is the main reason why IFN- γ reduces the colony formation ability of gastric cancer cells, so IFN- γ may play an unfavorable role in the development of gastric cancer.

In conclusion, we found that IFN- γ decreases the proliferation of gastric cancer cells by affecting the cell cycle, so it may have an indirect anti-cancer role in the development of gastric cancer in addition to its immunoregulatory role. Also, our microarray results revealed 283 genes with differential expression on treatment with IFN- γ , which provides basic data and a research direction for further investigating the effect of IFN- γ on gastric cancer cells.

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