# EFFECTS OF IFN-α COMBINED WITH IL-6 ON GROWTH AND EXPRESSION OF THE GENES RELATED TO CELL-GROWTH AND APOPTOSIS OF BONE MARROW CELLS FROM PATIENTS WITH CML

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## ABSTRACT

**Objective: To investigate the effects of interferon-α (IFN-α) and IFN-α combined with interleukin-6 (IL-6) on growth and expression of bcr-abl, bcl-2 and c-myc genes in the mononuclear cells (MNCs) from bone marrow (BM) of patients with chronic myelogenous leukemia (CML). Methods: MNCs were collected from BM of the patients with CML in chronic phase by centrifugation in lymphocyte separation medium and cultured in liquid with IFN-α (200U/ml) or IFN-α (200U/ml) plus IL-6 (100 ng/ml). The growing cells were counted every day. The expression levels of β-actin, bcr-abl, bcl-2 and c-myc genes in the MNCs incubated for 24 h were detected by reverse transcriptase-polymerase chain reaction (RT-PCR) and relatively quantitative analysis of the amplified fragments by optical density scanning for the bands on gel. Results: The cell growth was markedly suppressed by IFN-α but the degree of cell-growth inhibition was slightly decreased by IL-6 on the basis of IFN-α effect. The expression of bcr-abl chimeric gene was intensely inhibited by IFN-α or IFN-α plus IL-6. The expression of bcl-2 gene was suppressed by either IFN-α or IFN-α plus IL-6, whereas that of c-myc gene was also inhibited by IFN-α but strongly elevated by IL-6 on the basis of IFN-α action. Conclusions: Both IFN-α and IFN-α plus IL-6 can inhibit the expression of anti-apoptosis gene such as bcr-abl and bcl-2 and regulate the expression of the gene related to cell proliferation and differentiation such as c-myc. Either IFN-α or IFN-α combined with IL-6 will serve as a trustful strategy of clinical treatment for CML.**

**Key words: Chronic myelogenous leukemia, Combination therapy, Interferon-α Interleukin-6, Gene expression, Apoptosis**

CML is a clonal myeloproliferative disorder. The Philadelphia chromosome (Ph) is the cytogenetic hallmark of this disease.[1] The Ph chromosome translocation [t(9; 22) (q34; qll)] results in the creation of a hybrid bcr-abl fusion gene and forms an abnormal 210-kD protein ($p210^{bcr-abl}$) with increased tyrosine kinase activity.[2,3] These molecular changes were thought to play a significant role in leukemogenesis. $p210^{bcr-abl}$ confers a growth advantage on ph-positive CML cells over normal hematopoietic precursors and induces the suppression of apoptotic cell death that results in the accumulation of myeloid cells to a greater extent.[4] Several clinical trials have demonstrated that administration of IFN-α is an effective treatment for CML in chronic phase. In approximately 15%–20% of patients under IFN-α therapy, cytogenetic remission can be induced and long-term survival is improved as compared with conventional chemotherapy.[5] IFN-α and IFN-γ exerted dose-dependent and synergistically suppressive effects on the growth of a human leukemia cell line, KT-1 cells. Furthermore, IFN-α suppressed the expression of bcr-abl fusion gene in these cells, and induced apoptotic cell death.[6] IL-6 is primarily a monocyte-derived cytokine and a myeloid differentiation-inducing factor. These previous studies demonstrated that the monocyte function was impaired and cytokine such as IL-6 secretion was

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deficient in CML patients.[7] Murine M1 myeloid leukemia cells induced by IL-6 exhibited morphological and functional alterations, and ceased to divide and differentiated into macrophages or towards commitment.[8, 9] In order to further explore the strategies of combination therapy with IFN-α and cytokines and the molecular mechanisms for CML treatment, cell counting and relatively quantitative RT-PCR methods were used to measure the effects of IFN-α and IFN-α combined with IL-6 on the cell-growth and expression of the genes related to cell-growth and apoptosis of MNCs from CML patients in chronic phase.

## METHODS

### Specimen Collection

BM samples were collected from 14 patients (aged 17–58 y) with CML whose diagnoses were confirmed by PCR for detection of the chimeric bcr-abl gene. All of the CML patients were in chronic phase and untreated until the time of specimen puncture.

### BM Cell Separation

BM 2.5 ml was aspirated from the posterior iliac crest and transferred into RPMI-1640 medium (GIBCO/BRL) supplemented with 20 U/ml heparin immediately. A single-cell suspension was obtained by pipette to remove large cell clumps. This cell suspension was layered over lymphocyte separation medium (Shanghai Reagent Com.) and centrifuged at 400 g (4°C) for 30 min. MNCs were isolated by collecting the interface cells, washed twice in RPMI-1640 medium and resuspended in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS, Shanghai) until the cell density was achieved at $5\times10^5$/ml.

### Cell Culture

The different factors were added into 10 ml of the MNCs suspension for each experimented group: group 1 (control)--without any factors, group 2--with 200 U/ml IFN-α (Mega gene) and group 3--with 200 U/ml IFN-α plus 100 ng/ml IL-6 (Promega). The cultures were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ in air. After culture for 24 h, the cells were separated by centrifugation for preparing total RNA. 2 ml of the MNCs suspension for each experimented group were also incubated under the same culture conditions mentioned above for 6 days and the viable cells were counted by trypan blue dye exclusion test every day. All cultures were performed in duplicate. The growth-curves of the cells cultured in different condition were drawn using the average viable cell-number of 14 cases.

### Extraction, Purification and Concentration Measurement of Total RNA

The collected cells after culture for 24 h were lysed in 1 ml of Trizol reagent (GIBCO/BRL). Total RNA was isolated according to the manufacture's protocols and dissolved in 90 μl of diethyl pyrocarbonate (DEPC, Serva)-treated water. 10 μl of 10×multi-core digestion buffer and 1.5 U of RNase-free DNase I (Boehringer Mannheim) were added into the RNA sample. The probably residual DNA in the RNA sample was digested at 37°C for 30 min. RNA was re-extracted by phenol/chloroform method and dissolved in 20 μl DEPC-treated water. RNA concentration was determined by spectrophotometry.

### RT-PCR Analysis

2 μg of RNA was reverse-transcribed using an oligo $(dT)_{15}$ as a primer for 60 min incubation at 42°C in 30 μl of a mixture containing 50 mmol/L KCl, 10 mmol/L $MgCl_2$, 50 mmol/L Tris-HCl (pH 8.3), 500 μmol/L spermidine, 10 mmol/L DTT, 1 mmol/L (each) deoxynucleoside triphosphate, 30 pmol/L of primer, 30 U of RNasin (Promega) and 10 U of AMV reverse-transcriptase (Promega). Four independent amplifications were performed using four separate sets of primers which were showed as follows:

human β-actin: 5'-TGACGGTCAGGTCATCACTATCG-GCAATGA-3' (upstream) and 5'-TTGATCTTCATGGT-GVTAGGAGCGAGGGCA-3', V=A, G or C (downstream);

bcr-abl: 5'-ATTCGCTGACCATCAATAAG-3' (upstream) and 5'-GGCGTGATGTAGTTGCTTGG-3' (downstream);

bcl-2: 5'-CGACGACTTCTCCCGCCGCTACCGC-3' (upstream) and 5'-CCGCATGCTGGGGCCGTACAGT-TCC-3' (downstream);

c-myc: 5'-TTCTCTCCGTCCTCGGATTC-3' (upstream) and 5'-GTAGTTGTGCTGATGTGTGG-3' (downstream).

All of the oligonucleotide primers were synthesized by GIBCO/BRL. Each reaction mixture contained 5 μl of the reverse-transcription mixture, 20 pmol of each primer, 200 μmol/L each dNTP, 5 μl of 10×PCR buffer (Promega), and water up to 50 μl. The reaction mixture was heated to 94°C for 5 min before the addition of 2.5 U of Taq polymerase (Promega). The amplification profiles comprised 35 cycles for bcr-abl, bcl-2 and c-myc, and 30 cycles for β-actin respectively. Each cycle consisted of 30 sec at 94°C, 40 sec at 56°C and 40 sec at 72°C for β-actin, 30 sec at 94°C, 40 sec at 56°C and 50 sec at 72°C for bcr-abl, 1 min at 94°C and 1 min at 72°C for bcl-2, and 1 min at 94°C, 50 sec at 55°C and 1 min at 72°C for c-myc cDNA amplification respectively. The final cycle included a further 5 min at the extension temperature (72°C). The amplified fragments were separated by

electrophoresis on 2% agarose gel and visualized by ethidium-bromide staining. The bands with expected molecular size from β-actin (260 bp), bcr-abl (372 bp from b3a2 fusion mRNA or 297 bp from b2a2 fusion mRNA), bcl-2 (318 bp) and c-myc (282 bp) mRNA RT-PCR amplification fragments were scanned and quantitated by using Eagle Eye II image recognition and analysis system (Strata gene). The signals strength ratio of bcr-abl/β-actin, bcl-2/β-actin and c-myc/β-actin was calculated and the differences between control and several treated groups were statistically compared using T-test in pairs.

## RESULTS

### Effects of IFN-α and IFN-α plus IL-6 on the Growth of MNCs from Patients with CML in Chronic Phase

The addition of IFN-α (200 U/ml) suppressed the growth of MNCs from patients with CML. The degree of growth inhibition in MNCs decreased with supplementation of IL-6 (100 ng/ml) on the basis of IFN-α effect (Figure 1).

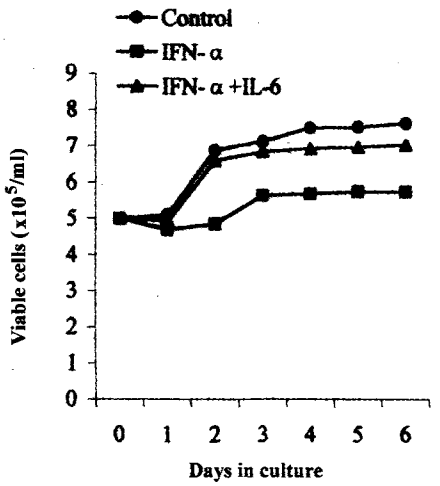


Fig. 1. Growth-suppressive effects of IFN-α (200 U/ml) and IFN-α (200 U/ml) plus IL-6 (100 ng/ml) on MNCs from patients with CML in chronic phase.

### Effects of IFN-α and IFN-α Combined with IL-6 on the Expression of bcr-abl, bcl-2 and c-myc Genes in MNCs from Patients with CML in Chronic Phase

The PCR amplification signals of β-actin, bcr-abl, bcl-2 and c-myc cDNA were detected in the expected sizes of 260 bp, 372/297 bp, 318 bp and 282 bp respectively (Figure 2 to Figure 4).

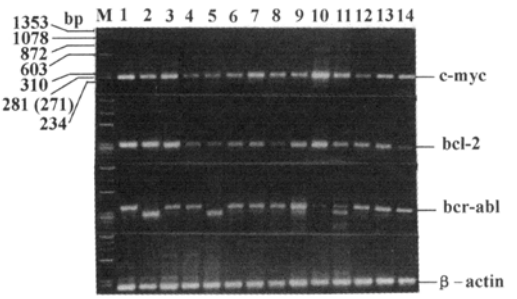


Fig. 2. Expression of the detected mRNA in MNCs cultured without any additional cytokines. Bands represent the products of RT-PCR for c-myc, bcl-2, bcr-abl and β-actin (upper gel to lower gel) from MNCs of patients (case No. 1 to 14) with CML in chronic phase.

M: ϕ x174/HaeIII DNA molecular size markers

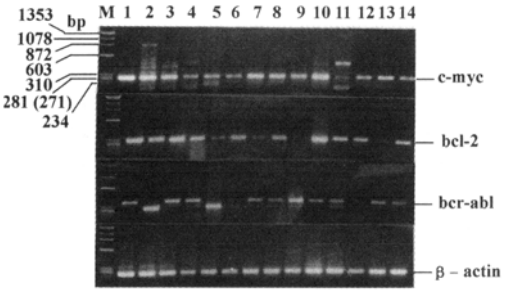


Fig. 3. Expression of the detected mRNA in MNCs cultured with IFN-α. Bands represent the products of RT-PCR for c-myc, bcl-2, bcr-abl and β-actin (upper gel to lower gel) from MNCs of patients (case No. 1 to 14) with CML in chronic phase.

M: ϕ x174/HaeIII DNA molecular size markers

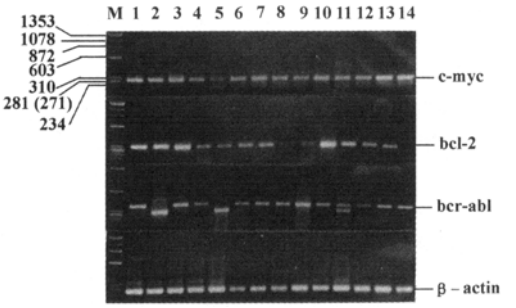


Fig. 4. Expression of the detected mRNA in MNCs cultured with IFN-α plus IL-6.. Bands represent the products of RT-PCR for c-myc, bcl-2, bcr-abl and β-actin (upper gel to lower gel) from MNCs of patients (case No. 1 to 14) with CML in chronic phase.

M: ϕ x174/HaeIII DNA molecular size markers

The ratios of bcr-abl, bcl-2 or c-myc mRNA with β-actin mRNA showed the meaningful results (Table 1). Either the single use of IFN-α (200 U/ml) or the

combination of IFN-α (200 U/ml) with IL-6 (100 ng/ml) inhibited very significantly the expression of bcr-abl chimeric gene. IFN-α (200 U/ml) decreased the expression levels of both bcl-2 and c-myc genes but there were no statistically significant differences between them and that of the control. The addition of IL-6 (100 ng/ml) further suppressed the expression of bcl-2 gene and markedly promoted the expression of c-myc gene.

*Table 1. Effects of IFN-α and IFNα- plus IL-6 on the expression of mRNA in MNCs from patients with CML in chronic phase ($\bar{x}\pm s$, n=14)*

| Culture condition | c-myc/β-actin | bcl-2/β-actin | bcr-abl/β-actin |
|---|---|---|---|
| Control | 0.746±0.165 | 0.869±0.327 | 0.863±0.183 |
| IFN-α | 0.699±0.215 | 0.701±0.319 | 0.564±0.172* |
| IFN-α+IL-6 | 0.826±0.111* | 0.699±0.355 | 0.628±0.292* |

*$P<0.05$ (compared with IFN-α addition group) *$P<0.01$ (compared with control group)

## DISCUSSION

The leukemic cell expansion in CML must in some way result from perturbed cell proliferation, differentiation and/or cell death including apoptosis during hematopoietic cell development. Reduced apoptosis has been considered as a possible criterion for the myeloid expansion in CML.[10] It is convinced that $p210^{bcr\text{-}abl}$ is the direct hallmark of CML pathogenicity.[4,11] The products of bcl-2 and c-myc oncogenes are involved in the regulation of proliferation, differentiation and apoptosis for CML cells.[12] The bcl-2 protein is a strong inhibition factor of apoptosis. The product of c-myc gene can either promote cell proliferation or initiate apoptotic cell death according to the enrichment of growth factors or without.

IFN-α treatment and HLA-identical sibling BM transplantation are routine therapies for CML in chronic phase worldwide. An interesting advance in the treatment of CML is actually the introduction of IFN-α therapy. IFN-α is a clinically useful cytokine for treating a variety of cancers. It has been used to treat CML patients with beneficial results, including the overall median survival rate and the emergence of Ph-negative, presumably normal hematopoiesis in up to 15%–20% of the patients.[13,14] Several investigators have addressed to the question how IFN-α may exert its beneficial effects. There is considerable interest in understanding the mechanisms by which IFN-α can induce hematological and cytogenetic remission and prolong survival in patients with CML. There is evidence that IFN-α can restore susceptibility of CML cells to apoptosis through fas-mediated downmodulation of $p210^{bcr\text{-}abl}$.[15]

In the current study, we observed the effects of IFN-α and IFN-α combined with IL-6 on cell growth and expression of the genes related to cell-growth and apoptosis to further demonstrate the molecular mechanisms of IFN-α therapy and combination therapy for CML. Our results show that both IFN-α and IFN-α in combination with IL-6 inhibited the cell growth of MNCs from BM of the patients with CML in chronic phase. Based on the reported anti-apoptotic activity of bcr-abl, we hypothesized that the inhibitory effects of IFN-α or IFN-α combined with IL-6 on CML cell growth could be related to modulation of bcr-abl and expression of other apoptosis-related oncoproteins in CML cells. RT-PCR analysis confirmed this inference that IFN-α inhibited bcr-abl, bcl-2 and c-myc gene expression. These data further suggest that bcl-2 and c-myc gene expression are internally correlated to bcr-abl chimeric gene expression in pathogenesis of CML, and inhibition of the anti-apoptosis gene expression would be the direct evidence of CML cells undergoing apoptosis in response to IFN-α and the molecular background of malignant clone reduction in IFN-α treated CML patients in chronic phase.

IL-6 is a monocyte-derived cytokine and assumes the potential role of inducing myeloid differentiation. It has been used in the study of gene therapy for acute and chronic myeloid leukemia through gene transfer in mice.[18] IL-6 can also regulate immune function and enhance the susceptibility of malignant cells to chemotherapeutic agents. Our results revealed that IL-6 sharply decreased the expression levels of bcr-abl and bcl-2 genes synergistically with IFN-α, and significantly enhanced c-myc gene expression based upon IFN-α action. Together with other recent studies, we illustrate that IL-6 could suppress the growth of CML cells by synergistically regulating the expression of the genes related to cell-growth and apoptosis with IFN-α.

The exact mechanisms of the actions of IFN-α and IFN-α plus IL-6 could collectively touch upon gene expression, apoptosis induction, immune response and interleukin stimulation. It merits further study *in vivo* in this field by experiments of transgenic mice[16] and clinical investigation.

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## REFERENCES

[1] Rowley JD. A new consistent chromosomal abnormality in chronic myelogenous leukemia identified by quinacrine fluorescence and giemsa staining. Nature 1973; 243:290.

[2] Ben-Neriah Y, Daley GQ, Mes-Masson AM, et al. The chronic myelogenous leukemia-specific p210 protein is the product of the bcr-abl hybrid gene. Science 1986; 233:212.

[3] McWhirter JR, Wang JYJ. Activation of tyrosine kinase and microfilament-binding functions of c-abl by bcr sequences in bcr/abl fusion protein. Mol Cell Biol 1991; 11:1553.

[4] McGahon A, Bissonette R, Schmitt M, et al. BCR-ABL maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death. Blood 1994; 83:1179.

[5] Tura S, Baccarani M, Zutfa E, et al. Interferon alfa-2a as compared with conventional chemotherapy for treatment of chronic myeloid leukemia. N Engl J Med 1994; 330:820.

[6] Yanagisawa K, Yamauchi H, Kaneko M, et al. Suppression of cell proliferation and the expression of a bcr-abl fusion gene and apoptotic cell death in a new human chronic myelogenous leukemia cell line, KT-1, by interferon-α. Blood 1998; 91:641.

[7] Anand M, Chodda SK, Parikh PM, et al. Abnormal levels of proinflammatory cytokines in serum and monocyte cultures from patients with chronic myelogenous leukemia in different stages, and their role in prognosis. Hematol Oncol 1998; 16:143.

[8] Tsuberi B, Naparstek E. Modification of Ml cells by exogenous introduction of IL-6 gene: a model for gene therapy of acute and chronic myeloid leukemia in mice. Leukemia 1995; Suppl 1:s93.

[9] Cambier N, Zhang Y, Vairo G, et al. Expression of BCR-ABL in Ml myeloid leukemia cells induces differentiation without arresting proliferation. Oncogene 1999; 18:343.

[10] Selleri C, Sato T, Vecchi LD, et al. Involvement of Fas-mediated apoptosis in the inhibitory effects of interferon-α in chronic myelogenous leukemia. Blood 1997; 89:957.

[11] Enright H, McGlave PB. Biology and treatment of chronic myelogenous leukemia. Oncology 1997; 11:1295.

[12] Vaux DL, Strasser A. The molecular biology of apoptosis. Proc Natl Acad Sci USA 1996; 93:2239.

[13] Kantarjian HM, Smith TL, O'Brien S, et al. Prolonged survival in chronic myelogenous leukemia after cytogenetic response to interferon-α therapy. Ann Intern Med 1995; 122:254.

[14] Talpaz M, McCredie K, Kantarjian H, et al. Chronic myelogenous leukemia: hematological remissions with alpha interferon. Br J Hematol 1986; 64:87.

[15] Selleri C, Jaroslow P, Maciejewski, et al. Fas-mediated modulation of bcr/abl in chronic myelogenous leukemia results in differential effects on apoptosis. Blood 1998; 92:981.

[16] Hanchun Chen, Andrew Pierce, Tony Whetton. Study on the treatment of MT/p210$^{bcr\text{-}abl}$ transgenic mice *in vivo* by 5-fluorouracil. Life Science Res 1999; 3:36.