

## Original Research Article

# Mir-204 regulates the biological behavior of childhood leukemia cells by binding to 3'UTR end of target gene and reducing the level of the gene

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Sent for review: 22 December 2023

Revised accepted: 6 May 2024

### Abstract

**Purpose:** To study the regulatory impact of mir-204 on the biological behavior of childhood leukemia cells and to elucidate its mechanism of action.

**Methods:** A total of 112 children with leukemia who were treated in Three Gorges University Yichang Central People's Hospital, Yichang, China from July 2018 to June 2020 were randomly divided into study and control groups (n = 56 each). The expression levels of mir-204 and hepatocyte growth factor (HGF) were determined using real-time polymerase chain reaction (RT-PCR) while the cell proliferation potential was determined using the CCK-8 method. Apoptosis and cell cycle progression of each group were evaluated using flow cytometry while changes in cell invasion and migration were assessed by Transwell assay. Luciferase reporter assay was used to determine the binding of mir-204 and HGF to 3'UTR.

**Results:** The expression level of mir-204 in study group was significantly lower than that in control group, while expression level of HGF was significantly higher ( $p < 0.01$ ) compared to control group. There was no significant change in cell proliferation capacity at 24 h. The expression level of mir-204 was significantly decreased, while the expression level of HGF was significantly increased ( $p < 0.05$ ). Luciferase activity of wild-type HGF 3'UTR in mir-204 overexpression group was significantly decreased, relative to mir-204 negative control group ( $p < 0.01$ ). There was no significant difference in luciferase activity in mutant HGF gene between the two groups ( $p > 0.05$ ).

**Conclusion:** Mir-204 reduces HGF expression level via HGF 3'UTR end, thereby inhibiting cell proliferation, invasion and migration, while promoting apoptosis, blocking cell cycle and regulating biological behavior of childhood leukemia cells.

**Keywords:** miR-204, Target gene, Leukemic cells in children, Biological behavior

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## INTRODUCTION

Leukemia is a systemic disease of the blood, and it is characterized by fever, anemia, pathological bleeding and leukemic cell infiltration. It usually occurs in adolescents and children under 18 years old, especially in children [1]. Statistics

have shown that China ranks high amongst countries with high incidence of leukemia, with over 15,000 new cases yearly. With advances in medical science and technology and continuous progress in techniques used in molecular biology and genetics, significant progress has been made in the treatment of leukemia and

prognostic risk stratification. However, the subjects still have low degree of long-term survival. Drug resistance and recurrence are still big problems in the leukemia treatment [2].

MicroRNAs (miRNAs) are non-coding, single-stranded RNAs that block the expressions of gene targets through interaction with 3'UTRs of these genes, thereby regulating post-transcriptional gene expression [3]. A member of the miRNA family, mir-204, targets and regulates functional protein genes in a variety of signaling pathways, thereby participating in the pathogenesis of bladder cancer, gastric cancer and other diseases [4].

Therefore, a study on the influence of mir-204 and associated genes on childhood leukemia may provide an important lead for therapy of the cancer.

In this study, the impact of mir-204 on human Jurkat cells and the target genes that may bind mir-204 were determined. In addition, the process associated with the regulation of childhood leukemia by mir-204 was determined so as to identify new markers and targets for the evaluation and treatment of childhood leukemia.

## METHODS

### Subjects

A total of 112 children with leukemia who received treatment in the Department of Pediatrics at the Three Gorges University Yichang Central People's Hospital from July 2018 to June 2020 were selected as the study and control cohorts, with 56 subjects in each cohort. Approval for this study was obtained from the Ethics Authority of Three Gorges University Yichang Central People's Hospital, Yichang, China (approval no. TGUYC2023002) in line with Helsinki Declaration [5]. Human leukemia cell line (Jurkat cell) was obtained from Shanghai Hongshun Biotechnology Co. Ltd.

### Inclusion criteria

All included subjects aged 2 to 14 years met the diagnostic criteria for leukemia, using cytological morphology and immunology. Moreover, those who gave informed permission supported by their families, were included.

### Exclusion criteria

Subjects with infectious ailments or illnesses of the blood; those who did not agree to participate

in the study, and patients with malignant tumors, were excluded.

### Procedures

Bone marrow samples (3-mL portions) were taken from members of the study and control cohorts. The expression levels of mir-204 and HGF were measured using RT-fluorescence quantitative PCR. The bone marrow specimens were centrifuged in the cold using Model 5418R centrifuge (Shanghai Aiyuan Biotechnology Co. Ltd.), and 6 mL of lymphocyte separation solution was added to each of the supernatants. The bone marrow mononuclear cells were separated using density gradient centrifugation, and the cells were subsequently lysed with lysing buffer in 6-well plates.

After standing in 1.5 mL EP tubes at laboratory temperature, 1 mL of chloroform was added, mixed by turning the EP tube upside down, and allowed to stand before centrifugation. The supernatant was mixed with 500  $\mu$ L isopropyl alcohol, and after standing, the mixture was centrifuged to obtain RNA precipitate. The precipitate was washed with 75 % ethanol, centrifuged, and the supernatant was discarded. The RNA was dissolved in 15  $\mu$ L of DEPC water and its level and purity were measured spectrophotometrically. Reverse transcription was performed to produce cDNA, and PCR amplification was performed.

### Cell culture

The Jurkat cells were cultured at 37 °C in a 5 % CO<sub>2</sub> incubator (Shanghai Rundu Biotechnology Co. Ltd., Model: Herocell 180) in DMEM containing 10 % fetal bovine serum. When cell culture reached about 75 % confluence, cells in logarithmic growth stage were removed from the suspension, centrifuged, and suspended in complete medium.

### Cell transfection

Cells in logarithmic phase were seeded in 6-well plates at a concentration of  $1 \times 10^4$  cells per well and cultured in a cell incubator until the cell density reached about 75 % of the starting density. Then, mir-204 interference plasmids, mir-204 overexpression plasmids and mir-204 control plasmids were transfected into different groups.

The transfected cells were maintained in complete serum medium for 48 hours, with the medium replaced every 4 hours. Then, the cells were divided into mir-204 negative control, mir-

204 overexpression, and mir-204 low-expression groups, with 5 multiple wells set for each group.

### Determination of cell proliferation

After cell transfection, Jurkat cells in logarithmic phase were digested with 0.25 % trypsin. After neutralization in 1640 medium, the cells were counted, placed in 96-well plates at the density of  $2 \times 10^4$  cells/mL in a constant-temperature incubator, and incubated for 24, 48 and 72 h. Then, 20  $\mu$ L of CCK-8 solution was added to each of the wells incubated at the various time points, followed by incubation for 2 and 4 h in the dark, after which the culture medium in each well was discarded. Then, 100  $\mu$ L of DMSO was added to each well and the solution was gently rocked on a shaker. The data were analyzed and recorded using microplate reader.

### Apoptosis

Cells washed in phosphate buffer solution (PBS) were used. TRIzol reagent (cell apoptosis detection kit (Thermo Fisher Scientific (China) Co. Ltd.) was directly added to the culture plate to lyse the cells. The lysed cells were fully homogenized, and 5  $\mu$ L of Annexin V-FITC was added to each well, followed by addition of 5  $\mu$ L of 7-aminoactinomycin D (7-AAD). After standing for  $\frac{1}{4}$  h under laboratory conditions, flow cytometric analysis was done in a flow cytometer.

### Cell cycle

Cell transfection was followed by rinsing thrice in chilled PBS solution before centrifugation. After the supernatant was discarded, the cells were re-suspended in PBS. The cells were adjusted to a concentration of  $1 \times 10^5$  cells/mL, and fixed with 75 % ethanol. Then, the cells were centrifuged for 1 hour and incubated in the dark with RNase, after which cells were placed in a 37 °C water bath for  $\frac{1}{2}$  h. Thereafter, full staining of cells was done with 400  $\mu$ L of PI in a dark place for 30 min. The proportion of cells in each phase of the cell cycle was determined in a flow cytometer.

### Cell invasion

Single suspension of cells in logarithmic growth phase was made. Then, 400  $\mu$ L single cell suspension was added to the upper chamber of Transwell, while the lower chamber contained 400  $\mu$ L of serum-deficient DMEM laced with 10 % FBS and matrix glue. After culturing at 37 °C in a 5 % CO<sub>2</sub> atmosphere for 24 h, invaded cells were gently wiped off and stained with crystal violet dye, rinsed with phosphate buffer and

examined under a light microscope (biological microscope; Shanghai Batuo Instrument Co. Ltd., Model: XSP-19C).

### Cell migration

Single cell suspension (400  $\mu$ L) was put in Transwell chamber upper compartment, and 400  $\mu$ L DMEM with 10 % FBS was put in the lower compartment. The other steps were the same as in the cell invasion test.

### Investigation of the target link between HGF and mir-204

The online bioinformatics prediction software, Targetscan and MirDB, were used to preliminarily study how these 2 factors are related. The levels of mir-204 and HGF were measured using RT fluorescence q-PCR and protein imprinting method (PCR kit; Tianjin Biochip Technology Co. Ltd). All experiments were repeated 3 times to reduce errors.

### Evaluation of parameters/indices

#### *Real-time fluorescence quantitative PCR for mir-204 and HGF expression levels*

Bone marrow mononuclear cells were isolated from subjects in both the study and control groups. Total RNA was extracted using standard protocols. Subsequently, reverse transcription was performed to synthesize complementary DNA (cDNA) from the extracted RNA templates. Real-time fluorescence quantitative PCR assays were conducted using specific primers and fluorescent probes designed for mir-204 and HGF.

Amplification was carried out in a real-time PCR instrument, and the resulting fluorescence signals were monitored and recorded. Data analysis involved normalization of expression levels relative to appropriate internal controls.

#### *CCK-8 assay for cell proliferation*

Cell proliferation assays were performed using the Cell Counting Kit-8 (CCK-8) method. Cells from both study and control groups were put in 96-well plates at predetermined densities and cultured as usual. Following the designated incubation period, CCK-8 solution was added to each well, and absorbance measurements were taken at specified time points using a microplate reader. The absorbance values obtained were proportional to the metabolic activity of the cells and were used to assess cell proliferation rates.

### Flow cytometry for apoptosis and cell cycle analysis

Cell cycle distribution and apoptosis were analyzed with flow cytometry. Cells were harvested and processed according to standard protocols. Subsequently, they were stained with fluorescent dyes targeting DNA and apoptotic markers. Flow cytometric analysis was performed using a suitable flow cytometer equipped with appropriate lasers and filters. Data acquisition was followed by analysis using specialized software to determine the proportions of cells in different phases of the cell cycle and the percentage of apoptotic cells.

### Transwell assay

Cell invasion and migration were evaluated using Transwell chambers with porous membranes. Cells were seeded onto the upper chamber in serum-free medium, while medium containing chemoattractant was placed in the lower chamber.

Following incubation, non-migratory cells on the upper surface of the membrane were removed, and migratory cells on the lower surface were fixed, followed by staining, microscopic enumeration, and comparison of population of migrated cells between the experimental groups.

### Protein imprinting method for mir-204 and HGF expression levels

Protein extracts were prepared from cells in both study and control groups. These extracts were subjected to protein imprinting, a technique involving the immobilization of specific antibodies or probes targeting mir-204 and HGF. After incubation and washing steps, bound proteins were determined and quantified using appropriate methods such as fluorescence detection and enzyme-linked immunosorbent assay (ELISA). Quantitative analysis was performed to compare the expression levels of mir-204 and HGF between the study and control groups.

### Statistical analysis

The SPSS 23.0 software package was used for statistics. Measurement data are presented as mean  $\pm$  standard deviation (SD). Multi-group comparison was done with single-factor ANOVA and multiple comparison methods. Comparison between two groups was done with independent sample *t*-test. Significance of difference was assumed at  $p < 0.05$ .

## RESULTS

The results showed that HGF is likely gene target of mir-204. To verify whether HGF was the target gene of mir-204, the reporter plasmid of HGF gene wild type in 3'UTR location and reporter gene mutant of binding center of mir-204 and HGF region were constructed, and the above vectors were co-transfected into mir-204 control and mir-204 overexpression cells, and cultured for 24 h. The two groups were assessed for luciferase activity.

### Expression levels of mir-204 and HGF in bone marrow mononuclear cells

Compared with control cells, mir-204 expression level in mononuclear cells in study group was significantly reduced. Conversely, the expression level of HGF in mononuclear cells in the study group was significantly increased ( $p < 0.01$ ; Table 1).

**Table 1:** Expression levels of mir-204 and HGF in bone marrow mononuclear cells (n=56)

Group	miR-204	HGF
Study	1.04 $\pm$ 0.33	1.85 $\pm$ 0.31
Control	2.15 $\pm$ 0.84	1.00 $\pm$ 0.26
<i>T</i>	9.204	15.721
<i>P</i> -value	<0.001	<0.001

### Proliferation potential of cells

Table 2 shows that there was no significant change in cell proliferation at 24 h. However, from 48 to 72 h, the proliferation potential of mir-204 over-expression group was significantly decreased, while the proliferation potential of mir-204 under-expression group was markedly up-regulated.

### Apoptosis potential

As shown in Table 3, the apoptosis potential in mir-204 over-expressed group was markedly increased, relative to mir-204 negative control. In contrast, the mir-204 under-expressed group has marked reduction in apoptosis.

### Cell cycle changes

The population of cells in the G1 stage in mir-204 over-expression group was significantly increased, when compared with mir-204 negative control cells, while the proportion of S phase cells was significantly decreased ( $p < 0.05$ ). There was marked reduction in proportion of G1 phase cells in mir-204 low-expression group, but S phase cell number was markedly raised ( $p < 0.05$ ; Table 4).

**Table 2:** Proliferation capacity of each group (n=5)

Group	Cell proliferation (OD)		
	24h	48h	72h
miR-204 negative control	0.26±0.04	0.52±0.04	0.85±0.03
miR-204 over-expression	0.23±0.03	0.37±0.05 <sup>a</sup>	0.44±0.06 <sup>a</sup>
miR-204 under-expression group	0.28±0.02	0.71±0.03 <sup>ab</sup>	0.97±0.05 <sup>ab</sup>
<i>F</i>	3.28	87.10	165.50
<i>P</i> -value	0.073	<0.001	<0.001

<sup>a,b</sup>*P* < 0.05, <sup>a</sup>vs. mir-204 -ve control; <sup>b</sup> vs. mir-204 overexpression

**Table 3:** Apoptosis potential in each group (n = 5)

Group	Cell apoptosis (%)
miR-204 negative control	12.93±1.63
miR-204 over-expression	28.62±2.12 <sup>a</sup>
miR-204 under-expression	6.34±1.02 <sup>ab</sup>
<i>F</i>	239.88
<i>P</i> -value	<0.001

<sup>a,b</sup>*P* < 0.05, <sup>a</sup>vs. mir-204 -ve control; <sup>b</sup> vs. mir-204 overexpression

**Table 4:** Cell cycle changes in each group (n = 5)

Group	G1 phase	S phase
miR-204 negative control	59.32±1.15	24.27±1.45
miR-204 over-expression	72.68±1.53 <sup>a</sup>	19.11±1.24 <sup>a</sup>
miR-204 under-expression	51.13±1.86 <sup>ab</sup>	29.68±1.85 <sup>ab</sup>
<i>F</i>	249.18	31.77
<i>P</i> -value	<0.001	<0.001

<sup>a,b</sup>*P* < 0.05, <sup>a</sup>vs. mir-204 -ve control; <sup>b</sup> vs. mir-204 overexpressed cells

### Changes in invasion and migration potential of cells

As presented in Table 5 and Figure 1 and Figure 2, the mir-204 over-expressed group had significantly reduced number of cells involved in invasion and migration than mir-204 negative control cells, while mir-204 low-expression group had significantly increased number of cells involved in invasion and migration.

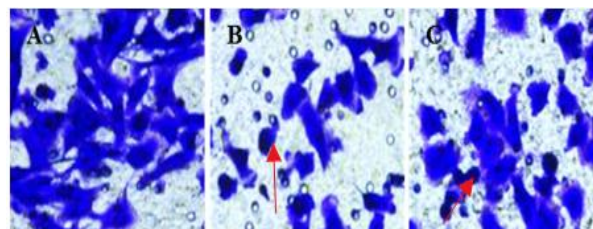
### Expression levels of mir-204 and HGF

The mir-204 overexpressed group had significantly increased mir-204 expression level and significantly decreased HGF expression

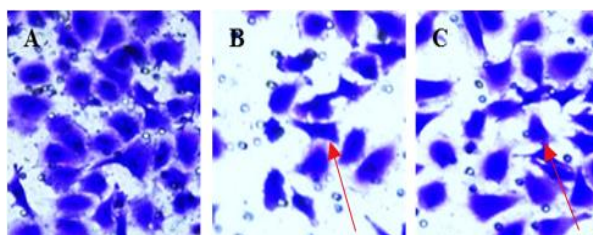
level, relative to mir-204 negative control (*p* < 0.05). However, mir-204 was markedly down-regulated in low expression cells, while HGF was markedly up-regulated (Table 6 and Figure 3).

### Luciferase activity at the 3'UTR end of mir-204 and HGF

The activity of luciferase at 3'UTR end of wild-type HGF in mir-204 overexpression group was significantly decreased, relative to the mir-204 negative control group (*p* < 0.01). Luciferase activity at 3'UTR end of mutant HGF was comparable in both groups (*p* > 0.05, Table 7).



**Figure 1:** Invasiveness of each group of cells. (A) miR-204 negative control cells, (B) miR-204 overexpression group, (C) miR-204 low expression group.



**Figure 2:** Cell migration potential of each group of cells. (A) miR-204 negative control, (B) miR-204 overexpression group, (C) miR-204 low expression group

**Table 5:** Changes in invasion and migration potential of cells in each group (n=5)

Group	Number of invaded cells	Number of migrating cells
miR-204 negative control	213.58±14.51	158.95±22.18
miR-204 over-expression	71.26±19.36 <sup>a</sup>	74.39±13.28 <sup>a</sup>
miR-204 under-expression	153.63±23.74 <sup>ab</sup>	123.31±17.49 <sup>ab</sup>
<i>F</i>	66.66	27.30
<i>P</i> -value	<0.001	<0.001

<sup>a,b</sup>*P* < 0.05, <sup>a</sup>vs. mir-204 -ve control; <sup>b</sup> vs. mir-204 overexpression

**Table 6:** Expressions of mir-204 and HGF in each group (n=5)

Group	miR-204	HGF
miR-204 negative control	1.02±0.03	0.98±0.12
miR-204 over-expression	8.54±0.77 <sup>a</sup>	0.41±0.04 <sup>a</sup>
miR-204 under-expression	0.37±0.02 <sup>ab</sup>	1.84±0.67 <sup>ab</sup>
F	520.54	16.69
P-value	<0.001	<0.001

**Note:** <sup>a,b</sup>P < 0.05, <sup>a</sup>vs. mir-204 -ve control; <sup>b</sup> vs. mir-204 overexpression

**Table 7:** Dual-luciferase reporting assay for luciferase activities of mir-204, HGF and 3'UTR terminal

Group	Wild type	Mutant type
miR-204 negative control	1.00±0.14	0.99±0.16
miR-204 over-expression	0.47±0.02	1.01±0.18
T	8.380	0.186
P-value	<0.001	0.857

## DISCUSSION

Leukemia is usually a childhood ailment with a severe degree of malignancy and high death rate. With advances in science, significant progress has been made in the clinical treatment of leukemia. However, leukemia subjects have low long-term degree of survival due to poor prognosis of the disease. Therefore, it is important to study the pathogenesis of acute lymphoblastic leukemia and to identify more effective treatment strategies for the disease. Mir-204, a member of the miRNA family, is located on the q21 transient receptor potential ion channel protein 3 gene of human chromosome 9. It targets the protein expressions of functional genes in a variety of signaling pathways and it is regulated by a variety of signaling routes and transcription factors. Studies have shown that mir-204 plays an important role in the occurrence and development of non-small cell lung cancer, gastric cancer, pulmonary hypertension and other diseases [6]. In colon cancer, conversion protein 2 $\beta$  promotes apoptosis by binding to the 3'UTR binding sites of mir-204 and Bcl-2 [7]. It has been reported that miRNAs are crucial in the pathogenesis of leukemia [8]. Hepatocyte growth factor (HGF) is a double-chain glycoprotein that regulates cell proliferation and morphology, and it promotes cell renewal [9]. In the present investigation, mir-204 expression level was markedly decreased, while HGF level was significantly increased in bone marrow mononuclear cells of children with leukemia. Thus, mir-204 and HGF play crucial roles in the pathogenesis of leukemia. The malignant biological behaviors of cells are mainly manifested as changes in proliferation, apoptosis, invasion and migration [10]. In this study, the cells were transfected with mir-204

interference plasmid or mir-204 overexpression plasmid, resulting in low or overexpression of mir-204, and changes in cell proliferation potential in each group were determined using the CCK-8 method, with a view of ascertaining if mir-204 expression level regulated the biological behavior of leukemia cells. Apoptosis and cell cycle distribution were measured flow cytometrically, while invasion and migration were measured with Transwell assay. Spontaneous and abnormal proliferation of a variety of blood cells is a vital risk factor for leukemia. Large numbers of immature leukemia cells with abnormal morphology accumulate in the bone marrow and other tissues of patients, thereby crowding out normal blood cells and inhibiting normal blood cell growth and proliferation [11]. Invasion and migration are important biological characteristics of malignant tumors, and they are complex and multi-step cascade processes with important link to the prognosis of cancers [12]. Transwell invasion and migration assay is a relatively effective method for the comprehensive assessment of tumor invasion and migration potential as well as analysis of tumor cell adhesion and degradation. The results of this study showed that up-regulation of mir-204 expression inhibited cell proliferation, invasion and migration. Moreover, it enhanced cell apoptosis and arrested cells in G1 phase. The 5' end of the 3'UTR of miRNA has seed sequences of 6-8 bases; miRNA modulates the expression of target genes by binding to the 3' UTR regions, thereby regulating biological behaviors [13]. In this study, the use of bioinformatics software revealed that HGF was the downstream target gene of mir-204, and that mir-204 negatively regulated HGF expression. Luciferase reporter gene was used to construct the 3'UTR of the target miRNA target gene into the 3'UTR of the luciferase gene, followed by transfection of the existing luciferase gene expression vector into cells to change the miRNA expression level. Then, the expression level of luciferase was assayed to determine the miRNA target in 3'UTR [14,15]. In this study, luciferase reporter assay showed that the luciferase activity at the 3'UTR end of the wild-type HGF gene was significantly decreased after transfection of the wild-type HGF gene reporter plasmid in 3'UTR area, and mutant gene reporter plasmid at the binding site of mir-204 and HGF region with mir-204 overexpressed plasmid. These results suggest that mir-204 may reduce HGF expression by binding to the 3'UTR end of HGF.

### Limitations of this study

One limitation of this study is the reliance on *in vitro* experiments to elucidate the mechanism of

action of mir-204 on childhood leukemia cells. While *in vitro* studies provide valuable insights into cellular mechanisms, they may not fully capture the complexities of the tumor microenvironment and interactions with other cell types present *in vivo*. Thus, further studies utilizing animal models or clinical samples are needed to validate the findings and assess the translational relevance of mir-204 as a likely treatment target in childhood leukemia.

## CONCLUSION

Mir-204 inhibits cell proliferative, invasive and migratory potential, and it enhances cell apoptosis, blocks cell cycle progression and regulates the biological properties of childhood leukemia cells through interaction with the 3'UTR end of target gene HGF.

## DECLARATIONS

### Acknowledgements

The University of Nigeria in Nsukka, Nigeria, Departments of Microbiology, Zoology and Environmental Biology, Pharmaceutical Microbiology & Biotechnology, and Pharmaceuticals are gratefully acknowledged by the writers. The authors would also like to express their gratitude to the University of Nigeria, Nsukka, and its Department of Veterinary Pathology & Microbiology, for granting access to their diagnostic laboratory, where this study was conducted in part. Professors Adikwu and Esimone are also acknowledged.

### Funding

None provided.

### Ethical approval

None provided.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

We declare that this work was performed by the authors named in this article, and all liabilities

pertaining to claims relating to the content of this article will be borne by the authors. Wei Lu designed the study, supervised the data collection, and analyzed the data. Hong Kou and Ting Kong interpreted the data and prepared the manuscript for publication. Huifei Lu supervised the data collection, analyzed the data and reviewed the draft of the manuscript.

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