

Original Article

The mechanism of miR-15b inhibiting nasopharyngeal carcinoma cell proliferation

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Abstract: Nasopharyngeal carcinoma (NPC) accounts for the highest incidence in otolaryngology malignant tumors. MiRNAs can regulate malignant tumor growth as tissue sensitive tumor biomarkers. MiR-15b abnormally expressed in tumor and could be treated as biomarker. However, its expression and role in NPC cells is still unclear. This study aimed to investigate the mechanism of miR-15b in NPC occurrence and development. NPC cell line CNE2 was transfected with miR-15b mimics or inhibitor. Real time PCR was used to test miR-15b level. MTT assay was applied to determine cell proliferation. Transwell assay was performed to detect cell invasive ability. Real time PCR and Western blot were adopted to measure Bcl-2 expression. MiR-15b mimics transfection significantly promoted miR-15b overexpression and inhibited CNE2 cell proliferation ($P < 0.05$). Overexpressed miR-15b obviously downregulated Bcl-2 mRNA and protein expression ($P < 0.05$). On the contrary, miR-15b inhibitor markedly suppressed its level in NPC cells, promoted cell proliferation, and upregulated Bcl-2 mRNA and protein levels ($P < 0.05$). MiR-15b expression failed to impact tumor cell invasive ability ($P > 0.05$). MiR-15b can suppress NPC cell proliferation by downregulating Bcl-2 protein expression and promoting tumor cell apoptosis.

Keywords: Nasopharyngeal carcinoma, miR-15b, Bcl-2, miRNA, apoptosis

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors of otolaryngology with high incidence and younger trend [1, 2]. NPC oncogenesis shows regional, ethnic, and family heredity, and its incidence kept increase in our country [3, 4]. Southern coastal cities in our country present the high incidence of NPC, featured as regional aggregation outcome [5]. Because of the special and complex structure of nasopharynx, the pathogenic site is often conceal and deep. Its treatment strategy is often scarce without in individual specific choice. Chemotherapy drugs are difficult to choose and the treatment is difficult. Radiotherapy is the first choice at present. However, its curative effect shows large difference caused by different individual specificity, progression, clinical staging, clinical symptoms and immune status. Advanced patients with local metastasis present worse prognosis [6]. Up to now, 5-year survival rate and quality of life in

NPC patients failed to show obvious increase [7]. NPC oncogenesis is a process affected by various factors, including genetic factors, EB virus infection, dietary habit, and environmental factors. Its pathogenesis has not been fully elucidated [8].

MicroRNAs, also known as miRNAs, widely exist in animals and plants with regulatory biological functions. MiRNAs mainly regulate gene expression at posttranscriptional level by targeting and negatively regulating downstream target genes [9, 10]. MiRNAs participate in a variety of body growth mechanisms, leading to enhance the adaptive ability to the environment [11, 12]. Each miRNA can control more than 200 kinds of target genes, suggesting that at least one-third of human protein-coding genes are regulated by miRNAs [13]. Recent study found that miRNA played an oncogene or tumor suppressor gene role in tumor occurrence and development [14]. MiR-15 family contains miR-15a, miR-15b, and miR-192, of which miR-15a has

MiR-15b suppress NPC proliferation

Table 1. Primer sequence

Gene	Forward 5'-3'	Reverse 5'-3'
GADPH	AGTACCAGTCTGTGCTGG	TAATAGACCCGGATGTCTGGT
miR-15b	ATGAACTCTTTCTCTGTTGG	TCACCG CGGCCTCTTGTCACA
BCL-2	CTCCTCTGTCCACAGAAAG	GCATTACCTCTGTAATTGGGG

been proven to play an important role in multiple tumor regulation [15]. However, as a homologue to miR-15a, miR-15b expression and role in tumor have not been elucidated. This study aimed to explore the miR-15b expression and impact in NPC.

Materials and methods

Main instruments and reagents

NPC CNE2 cell line was purchased from ATCC cell bank. DMEM, FBS, EDTA, and penicillin-streptomycin were bought from Hyclone. DMSO and MTT powder were got from Gibco. Enzyme-EDTA was from Sigma. PVDF membrane was from Pall Life Science. Western blot related reagents were from Beyotime. ECL reagents were from Amersham Biosciences. Rabbit anti-human Bcl-2 primary antibody and mouse anti-rabbit HRP tagged IgG secondary antibody was from Cell Signaling. Transwell chamber was from Corning. MiR-15b mimics and inhibitor were from Genechem. RNA extraction kit and reverse transcription kit were from Axygen. Other reagents were from Sangon. Labsystem Version 1.3.1 microplate reader was from Bio-Rad.

CNE2 cell culture and grouping

CNE2 cell line preserved in liquid nitrogen was revived in 37°C water bath and 1000 RPM centrifugation for 3 min. The cells were resuspended in 5 ml medium and maintained at 37°C and 5% CO₂ for 24-48 h. CNE2 cells were seeded in 6-well plate at 1×10⁵/cm² and cultured in complete medium containing 10% FBS, 90% high-glucose DMEM medium, 100 U/ml penicillin, and 100 µg/ml streptomycin. The liquid was changed every other day, and the cells were passaged every 2-3 days. CNE2 cells at 2-8 generation in logarithmic phase were used for experiment. The cells were randomly divided into 4 groups, including mimics NC group, inhibitor NC group, miR-15b mimics group, and miR-15b inhibitor group.

Lipofectamine transfection of miR-15b mimics and inhibitor

MiR-15b mimics and inhibitor were transfected into CNE2 cells, respectively. MiR-15b mimics sequence was 5'-AGGUGCAAUCGGUGUUCA-3', miR-15b inhibitor sequence was 5'-AUCGGAGGUGCAUUCUA-3', miR-15b mimics negative control sequence was 5'-AUUCAGGUCGUGCAAUG-3', and miR-15b inhibitor negative control sequence was 5'-ACAGGUUUCGCAAGGUUG-3'. MiR-15b mimics and inhibitor as well as the negative control liposomes were added to 200 µl serum free medium and incubated at room temperature for 15 min, respectively. Then lipo200 was mixed with miR-15b mimics or inhibitor diluent and incubated at room temperature for 30 min. Next, the mixture was added to each well together with 1.6 ml serum free medium after removing the previous complete medium. The cells were incubated at 37°C and 5% CO₂ for 6 h. After changing the complete medium, the cells were further cultured for 48 h for use.

Real-time PCR

Total RNA was extracted from CNE2 cells using Trizol and reverse transcribed to cDNA according to the manual. The primers used in experiment were designed by Primer 6.0 and synthesized by Invitrogen (**Table 1**). Real time PCR consisted 55°C for 1 min, followed by 35 cycles of 92°C for 30 s, 58°C for 45 s, and 72°C for 35 s. GAPDH was selected as internal reference. Relative gene expression was semiquantitative analyzed by 2^{-ΔCt} method.

MTT assay

CNE2 cells in logarithmic phase were seeded in 96-well plate at 5×10³ cells for 24 h. After changing the medium, the cells were randomly divided into 4 groups, including miR-15b mimics negative control group (mimics NC group), miR-15b inhibitor negative control group (inhibitor NC group), miR-15b mimics group, and miR-15b inhibitor group, respectively. MTT at 20 µl was added to each group every 24 h with three replicates. After another 4 h incubation, the supernatant was removed and 150 µl DMSO was added for 10 min. The plate was read at 570 nm wavelength to calculate proliferation

MiR-15b suppress NPC proliferation

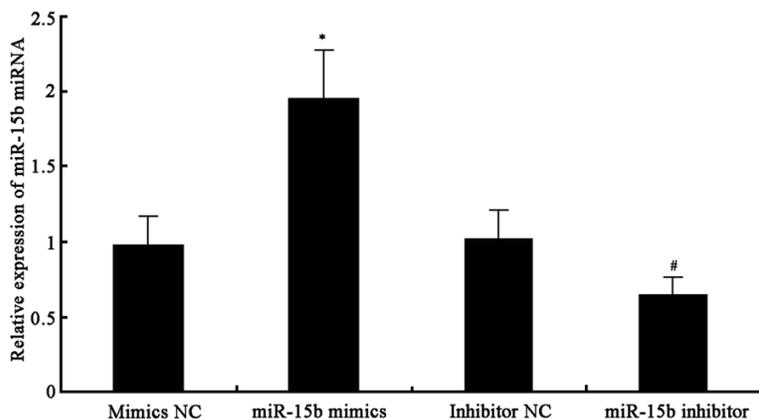


Figure 1. MiR-15b expression in CNE2 cells. * $P < 0.05$, compared with mimics NC group; # $P < 0.05$, compared with inhibitor NC group.

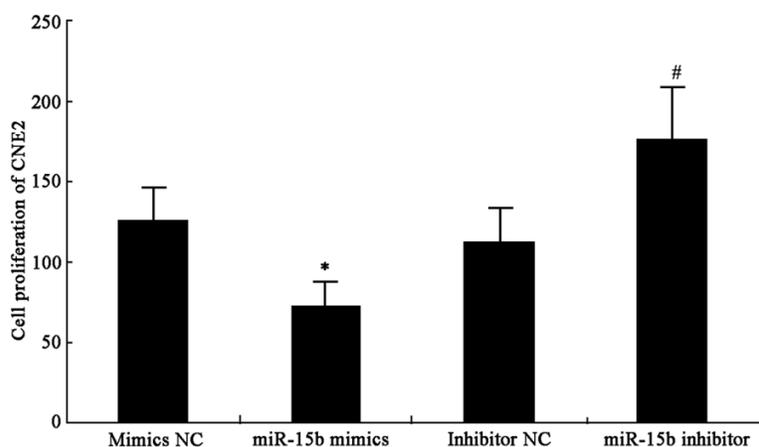


Figure 2. MiR-15b impact on CNE2 cell proliferation. * $P < 0.05$, compared with mimics NC group; # $P < 0.05$, compared with inhibitor NC group.

rate. The experiment was repeated for at least three times.

Transwell assay

The bottom and upper membrane of Transwell chamber were coated by 50 mg/L Matrigel diluent at 1:5. The chamber was dried at 4°C. Complete DMEM medium at 500 μ l and tumor cell suspension in serum free medium at 100 μ l were added to lower and upper chamber with three replicates, respectively. Cells in Transwell chamber without Matrigel were treated as control. After cultured for 48 h, the membrane was washed by PBS and fixed with ice ethanol. After stained by crystal violet, the cell number on the lower surface was counted. The experiment was repeated for three times.

Western blot

CNE2 cells were treated by RIPA solution (150 mM NaCl, 1% NP-40, 0.1% SDS, 2 μ g/ml Aprotinin, 2 μ g/ml leupeptin, 1 mM PMSF, 1.5 mM EDTA, 1 mM NaVanadate) for 15~30 min. Then the cells were ultrasonicated at 5 s \times 4 times and centrifuged at 4°C and 10 000 \times g for 15 min. The supernatant was stored at -20°C. The protein was separated by 10% SDS-PAGE and transferred to PVDF membrane. After blocked by 5% skim milk for 2 h, the membrane was incubated in primary antibody (1:1000) at 4°C overnight. Next, the membrane was further incubated in secondary antibody (1:2000) at room temperature for 30 min after washed by PBST. At last, the membrane was added with chemiluminescent agent for 1 min and developed. Protein image processing system software and Quantity one software were adopted for scanning and calculation. All the experiments were repeated for four times (n = 4).

Statistical analysis

All data were presented as mean \pm standard deviation ($\bar{x} \pm S$). SPSS11.5 was applied for data analysis. T-test or ANOVA was applied for comparison. $P < 0.05$ was considered as statistical significance.

Results

MiR-15b expression in CNE2 cells

Real time PCR was applied to test miR-15b expression in CNE2 cells after miR-15b mimics or inhibitor transfection. It was revealed that miR-15b mimics transfection obviously enhanced miR-15b expression in CNE2 cells ($P < 0.05$), while miR-15b inhibitor transfection effectively reduced its level in CNE2 cells ($P < 0.05$) (**Figure 1**).

MiR-15b suppress NPC proliferation

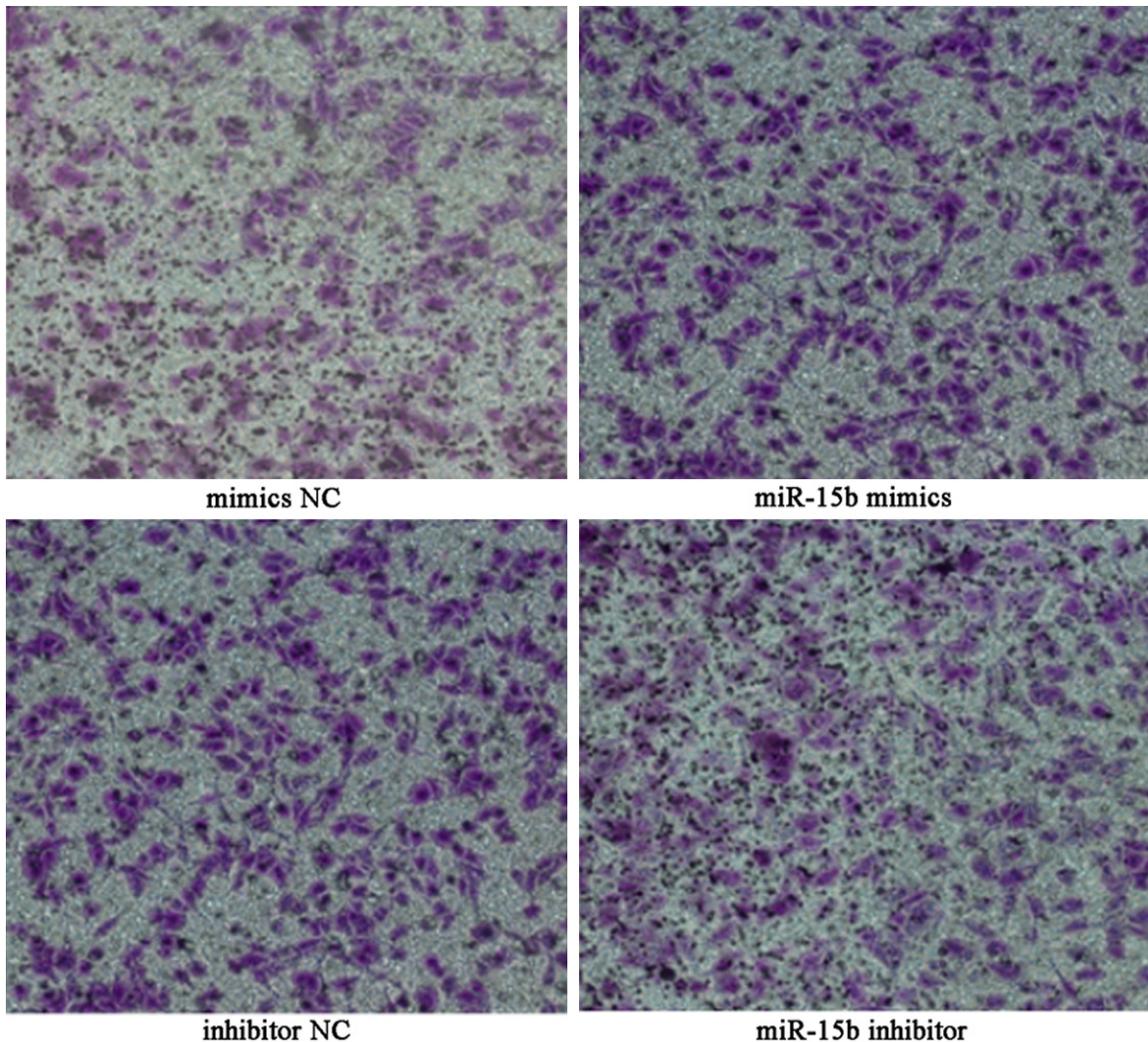


Figure 3. MiR-15b impact on CNE2 cells invasion.

MiR-15b impact on CNE2 cell proliferation

MTT assay was performed to detect miR-15b impact on CNE2 cell proliferation. MiR-15b up-regulation significantly inhibit CNE2 cell proliferation ($P < 0.05$), whereas miR-15b inhibition markedly promoted CNE2 cell proliferation ($P < 0.05$) (**Figure 2**). It indicated that miR-15b over-expression in CNE2 cells was facilitate to suppress NPC cell abnormal proliferation.

MiR-15b impact on CNE2 cells invasion

Transwell assay was adopted to determine miR-15b impact on CNE2 cell invasion. The results revealed that miR-15b upregulation failed to affect CNE2 cell invasion significantly ($P > 0.05$), whereas miR-15b inhibition also showed

no obvious effect on CNE2 cell invasion ($P > 0.05$) (**Figures 3 and 4**). It suggested that miR-15b in CNE2 cells did not affect cell invasive ability.

MiR-15b impact on Bcl-2 mRNA expression

Real time PCR was adopted to detect miR-15b impact on Bcl-2 mRNA level in CNE2 cells. Over-expressed miR-15b obviously declined Bcl-2 mRNA expression ($P < 0.05$). On the contrary, miR-15b inhibitor markedly upregulated Bcl-2 mRNA level ($P < 0.05$) (**Figure 5**).

MiR-15b impact on Bcl-2 protein expression

Western blot was used to test miR-15b impact on Bcl-2 protein expression. It demonstrated

MiR-15b suppress NPC proliferation

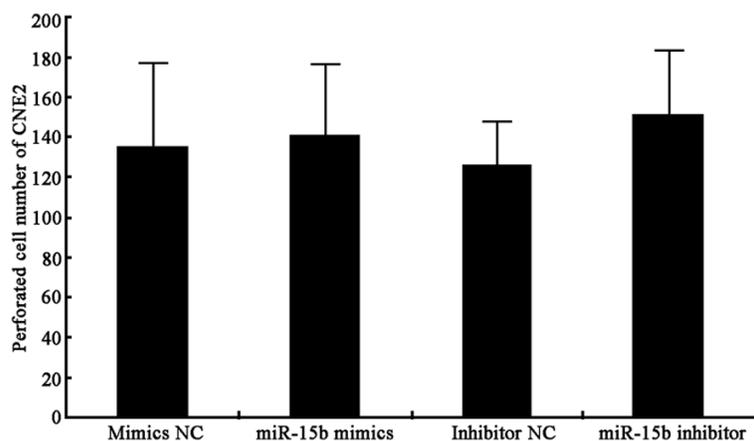


Figure 4. MiR-15b impact on CNE2 cells invasion analysis.

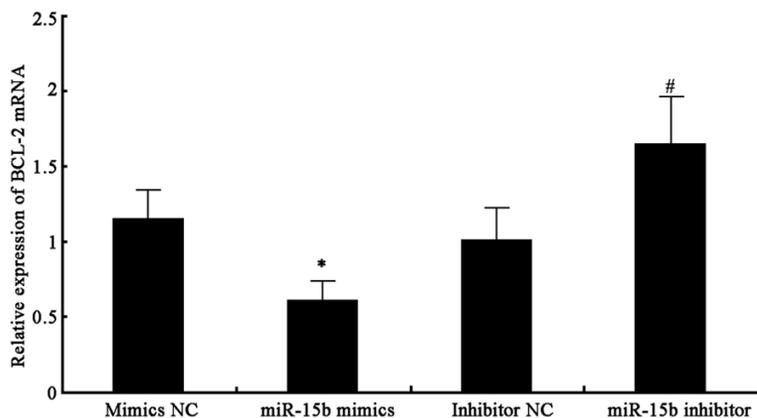


Figure 5. MiR-15b impact on Bcl-2 mRNA expression. * $P < 0.05$, compared with mimics NC group; # $P < 0.05$, compared with inhibitor NC group.

that similar with mRNA results, miR-15b mimics transfection significantly reduced Bcl-2 protein expression ($P < 0.05$). In contrast, miR-15b inhibitor transfection obviously enhanced Bcl-2 protein level ($P < 0.05$) (Figures 6 and 7). It suggested that miR-15b overexpression suppressed CNE2 proliferation may through regulating Bcl-2 gene and protein expression.

Discussion

Most NPC patients have been in advanced stage when diagnosed because of its late clinical symptoms. NPC belongs to proliferative tumor with rapid progress speed [16]. The incidence area of NPC is concealed, leading to difficult complete resection. Though radiotherapy is an important therapeutic approach for NPC, local recurrence and distant metastasis

may result in treatment failure [17]. Therefore, effective molecular marker can assist to evaluate NPC treatment strategy, curative effect, and prognosis. It is helpful for individualized treatment and best strategy choice that may provide new hope for NPC clinical diagnosis and treatment [18].

Study found that miRNAs expression in plants and animals may affect cell growth, proliferation, differentiation, apoptosis, and other processes [19]. Transcription factors, cell growth factors, and cell death genes can be regulated by miRNAs at transcriptional level, which directly affects body's metabolism and growth [20]. MiR-15b is widely distributed in a variety of organs, including heart, liver, and kidney. MiR-15b is closely related to body development and growth without tissue specific expression [21]. This study confirmed that miR-15b mimics transfection promoted its overexpression and inhibited NPC cell proliferation, while miR-15b inhibitor transfection obviously reduced miR-15b

level and promoted cell proliferation. However, no matter miR-15b overexpression or downregulation failed to affect NPC cell invasion. We further analyzed the related mechanism of miR-15b impact on NPC cells. Anti-apoptosis gene Bcl-2 is one of the target genes of miR-15 family. MiRNAs usually can regulate multiple downstream target genes mRNA or protein expression, so as to activate different signaling pathways that affected tumor cell biological behavior [22, 23]. Whether miR-15b may affect NPC proliferation through Bcl-2 is not confirmed. Our results verified that miR-15b mimics transfection obviously downregulated Bcl-2 mRNA and protein levels. On the contrary, miR-15b inhibitor transfection markedly promoted Bcl-2 mRNA and protein expression. It indicated that miR-15b may affect NPC progress by regulating Bcl-2 expression.

MiR-15b suppress NPC proliferation

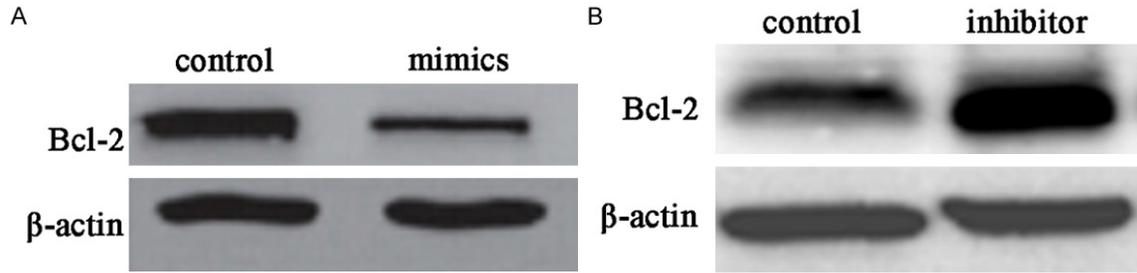


Figure 6. MiR-15b impact on Bcl-2 protein expression. A. miR-15b mimics transfection; B. miR-15b inhibitor transfection.

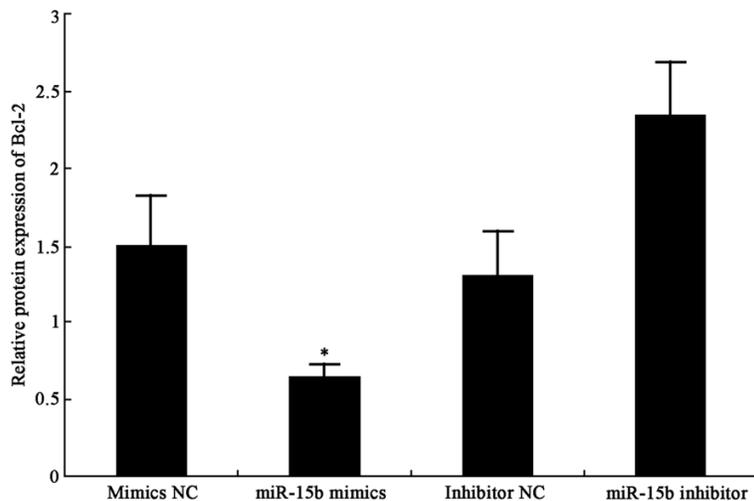


Figure 7. MiR-15b impact on Bcl-2 protein expression analysis. * $P < 0.05$, compared with mimics NC group; # $P < 0.05$, compared with inhibitor NC group.

In conclusion, miR-15b can suppress NPC cell proliferation and promote tumor cell apoptosis by downregulating Bcl-2 protein expression. It provides new theoretical evidence for molecular target selection for NPC clinical diagnosis and treatment.

Disclosure of conflict of interest

None.

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