RESEARCH ARTICLE

Roles of GST-π and polβ Genes in Chemoresistance of Esophageal Carcinoma Cells

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Abstract

The main aim of this study was to investigate the roles of GST-π and polβ genes in the chemoresistance of esophageal carcinoma cells. Eukaryotic expression vectors containing each gene were constructed and transfected into EC9706 cells, and the biological effects of the two genes assessed based on a resistance index. We additionally investigated the *in vitro* and *in vivo* anti-resistance effects of GST-π and polβ genes using recombinant lentiviruses carrying siRNAs against the two genes. Our results showed that upregulation of GST-π and polβ genes suppresses chemosensitivity of esophageal carcinoma cells to cisplatin, while downregulation of these two genes with RNAi technology reverses this chemoresistance. Multi-site injection of recombinant lentivirus targeting the GST-π gene into transplanted cDDP tumors effectively reversed their chemoresistant phenotype. However, the same treatment against the polβ gene did not lead to significant efficacy against chemoresistance.

Keywords: GST-π - polβ - drug resistance - esophageal carcinoma - RNA interference

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Introduction

Increasing rates of morbidity and mortality from malignant tumors have been reported in recent years. Esophageal cancer is the most common gastrointestinal malignancy, with chemotherapy as the mainstay for both unoperated and operated patients (Jemal et al., 2011; Bouwman and Jonkers, 2012; Moroney et al., 2012). However, multidrug resistance (MDR) in tumor cells often occurs due to several reasons, such as suboptimal drug use, unstable genome, heterogeneous expression, and frequent mutation (Liang et al., 2010; Bock and Lengauer, 2012). MDR is a defense mechanism that facilitates the maintenance of self-stability, but also constitutes the prominent reason for chemotherapeutic failure and tumor relapse (Larsen, 2000). Therefore, chemoresistance has become a major obstacle of tumor chemotherapy. Cisplatin is an important tumors resistant to chemotherapeutic drugs. Studying drug resistance mechanism of esophageal squamous carcinoma and finding meaningful molecular targets of the drug resistance is currently of intense interest. We (Li et al., 2006; Li et al., 2007) induced treated the human esophageal squamous cancer cell line, EC9706, with cis-diamminedichloroplatinum (cDDP), and established a chemoresistant cell line, EC9706/cDDP. Then we found expression levels of GST-π and polβ genes were significantly increased in the EC9706/cDDP cell line, compared with the parental cell line, EC9706, suggesting that the two genes are involved in the mechanism of drug-resistant to cDDP of esophageal cancer. Here we further investigated the effects of GST-π and polβ on esophageal cancer chemoresistance with experiments in which expression levels of the genes were regulated using lentiviruses harboring specific siRNAs.

Materials and Methods

Cell lines and cell culture

The well-differentiated human esophageal squamous cancer cell line, EC9706, was a kind gift from the Cancer Research Institute (Cancer Hospital) of the Chinese Academy of Medical Sciences & Peking Union Medical College. The chemoresistant cell line, EC9706/cDDP, was generated previously by our group. Cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 μg/μl streptomycin, and 100 μg/μl penicillin in a humidified incubator containing 5% CO2 at 37°C.

Construction of eukaryotic vectors containing GST-π and polβ genes

The GST-π mRNA sequence was obtained from GenBank (BT019950). Primers with BamHI and XhoI restriction sites were designed for amplification of the complete GST-π cDNA sequence. The sequence of the upstream primer was: 5-' AATGGATCC TCCACCATGCCGCCCTACACCGTGGT -3', and downstream primer: 5'- GACCTCGAGCTACTGTTTCCC GTTGCCAT -3'.

The polβ mRAN sequence was obtained from GenBank (M13140). Primers with BamHI and Pst I restriction sites were designed for amplification of complete polβ cDNA as follows: 5'-TACT GCAG GCCA CCAT GAGC AAAC

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Table 1. Single-strand DNA Template Sequence of the Hairpin siRNA of Targeted GST-π

GST1: 5'GATCCGAGGCAAGACCTTCATTGTTTCAAGAGAACAATGAAGGTCTTGCCTCTTTTTTC 3'
GST2: 3'G CTCCGTTCTGGAAGTACAAAGTTCTCTTGTTACTTCCAGAACGGAGAAAAAAGAGCT 5'
GSTc1: 5' GATCCTACGCTGACTTGATTGTTCTTCAAGAGAGAACAATCAAGTCAGCGTATTTTTTC 3'
GSTc2: 3'GATGCGACTGAACTAACAAGAAGTTCTCTCTTGTTAGTTCAGTCGCATAAAAAAGAGCT 5

Table 2. Single-strand DNA Template Sequence of Hairpin siRNA of Targeted polβ

pol1 5' GATCCGCGTGAGCCAAGCTATCCACTTCAAGAGAGTGGATAGCTTGGCTCACGTTTTTTC 3'
pol2 3' GCGCACTCGGTTCGATAGGTGAAGTTCTCTCACCTATCGAACCGAGTGCAAAAAAGAGCT 5'
polc1 5' GATCCGTCAATGGTCGTGTAGAGTTTCAAGAGAACTCTACACGACCATTGACTTTTTTC 3'
polc2 3' GCCAGTTACCAGCACATCTCAAAGTTCTCTTGAGATGTGCTGGTACATGAAAAAAGAGCT 5'

GGAA GGCG CCGC AGG-3' (upstream sequence), and 5'-CTGG ATCC TTCG CTCC GGTC CTTG GGTT C-3' (downstream sequence).

PCR products were retrieved and purified with β-actin as an internal reference. Target gene sequences were ligated into the pIRES2-AcGFP1 blank vector, and transformed into Escherichia coli DH5α.

Transfections

Cells (1×10^6) were transfected with 2 μg eukaryotic expression vector containing the GST-π (pIRES2-AcGFPI-GST-π) or polβ (pIRES2-AcGFPI-polβ) gene in 4 μl of Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's instructions. Blank plasmids were used as controls.

Cells transfected with pIRES2-AcGFP1-GST-π and pIRES2-AcGFP1 blank plasmid were designated 'EC9706-GST' and 'EC9706-KC', respectively, while those transfected with pIRES2-AcGFP1-polβ were denoted 'EC9706-polβ' cells.

Construction of lentivirus vectors expressing of siRNA targeting GST-π and polβ

The mRNA sequences of GST-π (GenBank BT019950) and polβ (GenBank M13140) were sent to the Dharmacon siDESIGN Center (http://www.dharmacon.com/sidesign/) for siRNA design. Two siRNA sequences confirmed as effective targets from a preliminary experiment by our group was selected for study (Tables 1, 2). One negative control siRNA containing a scrambled sequence with the same nucleotide composition was additionally selected. A 9-nucleotide loop (TTCAAGAGA) was selected for all shRNAs. The shRNA-annealed oligonucleotides were ligated into a lentiviral vector (pRNAT-U6.2/Lenti). A lentiviral vector containing siRNA targeting the GST-π gene and the corresponding control vector were designated pRNAT-U6.2/Lenti-GST and pRNAT-U6.2/Lenti-GSTC, respectively. The lentiviral vector containing siRNA targeting the polβ gene and the corresponding control vector were designated pRNAT-U6.2/Lenti-polβ and pRNAT-U6.2/Lenti- polβC, respectively.

Packaging of the lentivirus

Infectious viral supernatant fractions (in DMEM with 1% FBS) were derived via transient co-transfection of 293FT (6×10^6) cells using Lipofectamine 2000TM (Invitrogen, USA). In total, 12 μg of plasmid (8 μg of lentiviral vector carrying shRNA to 4 μg of packaging plasmid ViraPower™ Packaging Mix) was used, and the viral supernatant collected at 24, 48 and 72 h post-transfection. 293FT cells were inoculated in six-well plates at a density of 2×10^5/well for 24 h and infected with virus. The culture medium was diluted from $1:10^3$ to $1:10^{10}$. Diluted virus solution was added to the cell culture plate at a concentration of 400 μl/well for 4 to 6 h at 37°C. Following replenishing of the medium, cells were cultured for a further 24 h, and green fluorescent protein (GFP)-positive cells counted under a fluorescence microscope. The virus titer was calculated with the following formula: virus titer (GFU/ml) = number of GFP-positive cells × virus dilution factor/volume of inoculum.

pRNAT-U6.2/Lenti-GST and pRNAT-U6.2/Lenti-GSTC viruses were abbreviated to GSTsi and GSTsiC respectively, and pRNAT-U6.2/Lenti-pol and pRNAT-U6.2/Lenti-polC viruses to polsi and polsiC, respectively.

Virus infection

EC9706/cDDP cells were inoculated in 6-well plates at a density of 2.5×10^5/well for 24 h in RPMI 1640 containing 10% fetal bovine serum. Diluted virus was added at a cell-virus ratio of 1:10. To confirm virus infection, untreated EC9706/cDDP cells were added to the culture medium of infected cells for 48 h. Fluorescence was evident in cases where the infection was successful (data not show).

Semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

The relative levels of GST-π and polβ mRNA were determined using RT-PCR. Total RNA was extracted with the RNA Extraction Kit (Qiagen) and cDNA obtained with the miScript Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. Optical density values of the target gene and β-actin gene bands were analyzed using the Gel-Pro Analyzer image analysis system.

Western blotting

Cells were washed with PBS at 4°C three times, lysed for 30 min on ice, centrifuged at 12000 g at 4°C for 15 min, and the supernatant fraction collected. Protein was separated using SDS-PAGE (50 μg per well) and transferred to PVDF membranes. The membrane was blocked with 5% bovine serum albumin (BSA) and the corresponding primary (dilution 1:200) and secondary antibodies added (goat anti-rabbit polyclonal antibody, dilution 1:1500). The membrane was washed and incubated with enhanced chemiluminescence substrate.

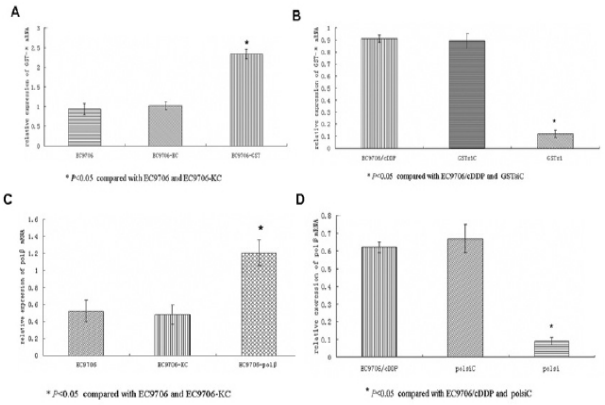

Figure 1. Relative fold GST-π and polβ mRNA levels in different cell lines. (A) Relative expression of GST-π mRNA in EC9706, EC9706-KC and EC9706-GST cell lines. Expression levels of GST-π mRNA in EC9706-GST cells were significantly higher than those in EC9706 or EC9706-KC cells. (B) Relative expression of GST-π mRNA in EC9706/cDDP, GSTsiC and GSTsi cell lines. The GST-π mRNA level in siRNA-treated cells was significantly lower than that in the other two groups after transfection for 48 h (P <0.05). Transfection with GST-π-specific siRNA suppressed the GST-π levels. (C) Relative expression of polβ mRNA in EC9706, EC9706-KC and EC9706-polβ cell lines. Expression levels of polβ mRNA in EC9706-polβ cells were significantly higher than those in EC9706 or EC9706-KC cells. (D) Relative expression of polβ mRNA in EC9706/cDDP, polβsiC and polβsi cell lines. The polβ mRNA level in siRNA-treated cells was significantly lower than that in the other two groups after transfection for 48 h (P <0.05). Transfection with polβ-π-specific siRNA suppressed polβ levels

Protein bands were exposed to X-ray film and analyzed.

Drug sensitivity assays

Cells in the exponential phase were inoculated in 96-well plates at 200 μl cells per well (3×10^4/ml) for 24 h. Various concentrations of cDDP were used to treat cells for 24 h, using three wells for each concentration. The blank control group was left untreated. MTT (5 mg/ml) was added at a concentration of 20 μl/well for 4 h. Dimethyl sulfoxide (DMSO) was added at a concentration of 200 μl/well and mixed thoroughly. The optical densities of wells were determined with a microplate reader at 550 nm. The relative inhibition rate (%) was taken as: (1-OD value of the treated well/OD value of the control well) × 100%, and the IC_{50} value calculated. Resistance index (RI) was assessed as IC_{50} of resistant cells/parental cells.

Animal experiments

Nude mice (BALB/C-nu/nu, 4-6 weeks) were provided by the Institute of Laboratory Animal Sciences of the Chinese Academy of Medical Sciences and Peking Union Medical College. Animals were maintained in specific pathogen-free conditions, and provided sterilized water, food and materials.

Twenty nude mice were randomly inoculated with either EC9706 (n=4) or EC9706/cDDP cells (n=16). Cells were digested with 0.25% trypsin, washed twice with PBS, and resuspended in PBS at a density of 1×10^7/ml. Mice were subcutaneously injected with 0.2 ml cell suspension in the armpit of the left forearm, and growth of the inoculated tumor was recorded.

Treatment was initiated when the transplanted tumor reached 5 mm in diameter. Mice with EC9706 tumors were treated with a multi-site injection of 0.1 ml cDDP (200 μg/ml, 0.85 μg/g body weight) into the tumor once every 3 days four times. Mice inoculated with EC9706/cDDP were randomly divided into four groups, specifically, PBS, cDDP (200 μg/ml), cDDP+GSTsi (200 μg/ml cDDP and GSTsi mixed in equal volumes) and cDDP+polsi (200 μg/ml cDDP and polsi mixed in equal volumes) groups. Treatment agents were injected into multiple sites of the tumor at a volume of 0.1 ml per site once every 3 days four times, and the tumor sizes monitored. After completion of therapy, all mice were sacrificed. Tumor weights of all mice were measured, and tumor volumes calculated according to the formula: V (cm^3)=W-10 .This study was approved by the Human Research Ethics Committee of Zhengzhou University.

Statistical analysis

Results were analyzed using SPSS software 17.0, and compared using one-way analysis of variance (ANOVA). Data were presented as means ± standard deviation (SD) of three independent experiments. P<0.05 was considered statistically significant.

Results

GST-π mRNA levels detected using RT-PCR

The relative expression levels of GST-π mRNA normalized to β-actin in the EC9706, EC9706-KC, and EC9706-GST groups were 0.94±0.14, 1.02±0.10, and 2.34±0.12, respectively (Figure 1A). Expression of GST-π mRNA in EC9706-GST cells was 2.49-fold higher than that in EC9706 cells (P<0.05).

The relative expression levels of GST-π mRNA normalized to β-actin in cells treated with GSTsi and GSTsiC and untreated EC9706/cDDP cells were 0.27±0.03, 0.89±0.06, and 0.91±0.03, respectively (Figure 1B). Expression of GST-π in GSTsi-infected cells was significantly decreased by 86.8%, compared with that in GSTsiC-infected and EC9706/cDDP cells (P<0.05).

polβ mRNA levels detected using RT-PCR

The relative expression levels of polβ mRNA normalized to β-actin in EC9706, EC9706-KC, and EC9706-GST cell groups were 0.52±0.13, 0.48±0.11, and 1.21±0.15, respectively (Figure 1C). Expression of polβ mRNA in EC9706-polβ cells was 2.33-fold higher than that in EC9706 cells (P<0.05).

The relative expression levels of polβ mRNA normalized to β-actin in untreated EC9706/cDDP and cells treated with polsiC and polsi were 0.62±0.03, 0.28±0.05, and 0.09±0.02, respectively (Figure 1D). Expression of polβ mRNA in polsi-infected cells was significantly decreased by 90.2%, compared with that in polsiC-infected and EC9706/cDDP cells (P<0.05).

GST-π protein levels detected via Western blotting

GST-π protein expression was observed in untreated EC9706, EC9706-KC, and EC9706-GST cells (Figure 2A). Expression of GST-π in EC9706-GST cells was

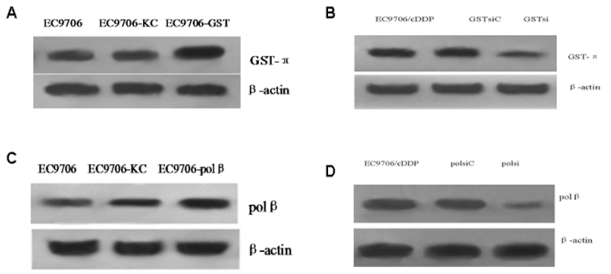

Figure 2. Protein levels of GST-π and polβ in different cell lines. (A) Western blot analysis of GST-π protein in EC9706, EC9706-KC and EC9706-GST cell lines. Expression levels of GST-π protein in EC9706-GST cells were significantly higher than those in EC9706 or EC9706-KC cells. (B) Western blot analysis of GST-π protein in EC9706/cDDP, GSTsiC and GSTsi cell lines. The GST-π protein level in siRNA-treated cells was significantly lower than that of the other two groups after transfection for 48 h (P <0.05). Transfection with GST-π-specific siRNA suppressed GST-π levels. (C) Western blot analysis of polβ protein in EC9706, EC9706-KC and EC9706- polβ cell lines. Expression levels of polβ protein in EC9706-polβ cells were significantly higher than those in EC9706 or EC9706-KC cells**.** (D) Western blot analysis of polβ protein in EC9706/cDDP, polβsiC and polβsi cell lines.The level of polβ protein in siRNA-treated cells was significantly lower than that in the other two groups after transfection for 48 h (P <0.05). Transfection with polβ-specific siRNA suppressed polβ levels

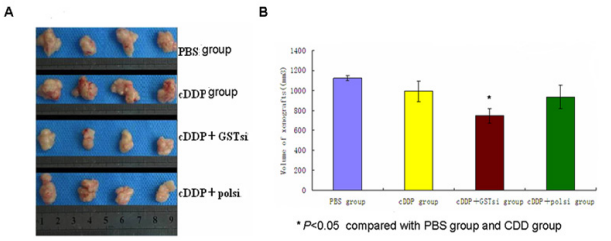

Figure 3. (A) Different Xenografts after Therapy with Different Methods. Mice inoculated with EC9706/cDDP were randomly divided into four groups: PBS, cDDP, cDDP+GSTsi, and cDDP+polsi. Treatment agents were injected into multiple sites of the tumor at a volume of 0.1 ml for each site once every 3 days four times. (B) Comparison of Volumes of Xenografts (after Therapy with Different Methods). The mean tumor size in the PBS and cDDP groups grew continuously during treatment and reached 1124.00±25.13 mm^3 and 990.00±99.33 mm^3 after treatment (P>0.05). The cDD+GSTsi group showed decreased tumor growth and markedly reduced mean tumor size after treatment (747.50±72.30 mm^3), compared with both PBS and cDDP groups (P<0.05). However, no significant differences were evident among the cDDP+polsi (937.50±118.70 mm^3), PBS and cDDP groups (P>0.05)

significantly increased, compared with that in EC9706 and EC9706-KC cells. As expected, infection with lentivirus GSTsi led to a significant decrease in GST-π protein expression (Figure 2B).

Polβ protein levels detected via Western blotting

Polβ protein expression in untreated EC9706, EC9706-KC, and EC9706-polβ cells is presented in Figure 2C. Expression of polβ in EC9706-polβ cells was significantly increased, compared with that in EC9706 and EC9706-KC cells. Additionally, infection with lentivirus polsi suppressed polβ protein expression to a significant extent (Figure 2D).

MTT assay findings

Data from the MTT assay showed that IC_{50} values for cDDP in EC9706-GST, EC9706-polβ, EC9706-KC and untreated EC9706 cells were 9.48, 7.16, 4.37 and 4.02 μg/ml, respectively. RI values for EC9706-GST and EC9706-polβ cells were 2.36 and 1.78, respectively.

IC_{50} values in cells infected with GSTsi, GSTsiC and untreated EC9706/cDDP were 38.56, 61.24, 63.11 μg/ml, with RI of 9.59, 15.23, 15.7, respectively. GSTsi infection led to a significant increase in sensitivity to cDDP (P<0.05), suggesting that suppression of GST-π expression attenuates chemoresistance.

IC_{50} values in cells infected with polsi, polsiC and untreated EC9706/cDDP were 55.71, 65.41, 63.11 μg/ml, with RI values of 13.9, 15.5, 15.7, respectively. Polsi infection significantly enhanced sensitivity to cDDP (P<0.05), again suggesting that suppression of polβ expression attenuates chemoresistance.

Effects of recombinant lentivirus on tumor growth in nude mice

Both EC9706 and EC9706/cDDP cells generated subcutaneous tumors in nude mice on day 8 with a success rate of 100%. Tumors had grown to 4 to 5 mm in diameter on day 12. No significant differences in mean time to tumorigenesis were evident between EC9706 (7.75±0.96d) and EC9706/cDDP cells (8.00±0.82d) (P>0.05).

The mean tumor size in the PBS and cDDP groups grew continuously during treatment and reached 1124.00±25.13 mm^3 and 990.00±99.33 mm^3 after treatment (P>0.05). The cDDP+GSTsi group showed decreased tumor growth and significantly reduced mean tumor sizes after treatment (747.50±72.30 mm^3), compared with both PBS and cDDP groups (P<0.05) (Figure 3A, B). In contrast, no significant differences were evident among the cDDP+polsi (937.50±118.70 mm^3), PBS, and cDDP groups (P>0.05) (Figure 3A, B). No remote metastasis was observed in internal organs, including lung, liver and kidney, in all groups.

Discussion

After nearly 40 years of research, the complex mechanisms underlining MDR of tumors remain unclear. It is suggested that different tumor cell types have their own unique mechanisms leading to MDR (Gillet and Gottesman, 2010; Al-Lazikani et al., 2012). The association between GST and tumors was initially reported by Wang (Wang and Few, 1985). Accumulating evidence has shown that GST is upregulated in many tumors and reduces the cytotoxicity of anti-cancer drugs, leading to chemoresistance in tumor cells (Burg et al., 2002; Yu et al., 2006; Tang et al., 2013). The housekeeping gene, polβ, is universally expressed in mammalian cells and participates in DNA repair. Recent research has revealed the involvement of polβ in chemoresistance in tumor cells. For example, upregulated polβ expression is associated with resistance to cisplatin in tumor cells (Li et al., 2007; Illuzzi and Wilson, 2012). Previous research by our group further disclosed that GST-π and polβ genes are likely to

play an important role in function in MDR in EC9706/cDDP cells.

Specific siRNAs targeting chemoresistant genes designed using RNAi technology have shown considerable potential in reversing MDR (Maddalena et al., 2011; Li et al., 2012; Zhao et al., 2012; Yin et al., 2012). In the current study, we constructed recombinant lentivirus containing siRNA targeting GST-π and polβ genes. Infection of the human esophageal carcinoma cell line, EC9706/cDDP that is resistant to cisplatin, with recombinant virus led to significantly decreased GST-π and polβ mRNA and protein levels and increased sensitivity to cDDP. Our results confirmed that GST-π and polβ genes function in resistance to cisplatin in esophageal cells, and may therefore be potential targets for effective chemotherapy.

Silencing GST-π and polβ genes attenuated chemoresistance in EC9706/cDDP cells, but did not decrease the IC_{50} to the levels of sensitive cells. Accordingly, we propose that an additional mechanism may be involved in chemoresistance of EC9706/cDDP cells that does not involve GST-π and polβ genes.

Nude mice preserve the morphology and genetics of primary tumors and are thus commonly used to study tumor MDR (Rygaard, Povlsen, 1969; Shen et al., 2005; Li et al., 2005). In this study, combined treatment with cDDP and GSTsi significantly restored the sensitivity of transplanted tumor cells to cDDP, suggesting that recombinant lentivirus targeting the GST-π gene can successfully reverse the chemoresistance of esophageal cancer cells. In contrast, combined treatment with cDDP and polsi failed to show significant efficacy in terms of reducing tumor size and promoting chemosensitivity, compared with the cDDP and PBS groups, implying that targeting the polβ gene is not an effective strategy to reverse tumor cell chemoresistance. Although recombinant lentivirus targeting the polβ gene induced successful downregulation of gene expression and decrease in RI, MDR of transplanted tumors was not significantly altered. This finding may be attributable to the low titer and uneven distribution of the virus *in vitro*, leading to failure to block the target gene.

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