Abstract. The carboxyl terminus of heat shock cognate 70 interacting protein (CHIP) is an E3 ubiquitin ligase, which can promote ubiquitylation and degradation of many tumor-related proteins. However, the expression of CHIP in human gastric cancer has not been investigated. In this study, the mRNA and protein levels of CHIP expression in 53 cases of gastric cancer and matched normal tissues were determined by quantitative real-time PCR, western blotting and immunohistochemistry. We showed that CHIP was registered from basal to middle portions of normal gastric mucosa. CHIP expression was notably decreased or lost in human gastric cancer samples compared with the matched normal non-cancer samples. The correlations between CHIP downregulation and the clinicopathological characteristics were also evaluated. The expression of CHIP was significantly lower in the gastric cancer samples compared with the matched normal non-cancer samples at both mRNA and protein levels (P<0.05 and P<0.05, respectively). More importantly, the downregulation of CHIP was correlated with TNM stage (P=0.048) and lymph node metastasis (P=0.010) at the mRNA levels. In addition, the downregulation of CHIP was correlated with lymph node metastasis (P=0.021) and tumor differentiation (P=0.009) at the protein levels. Taken together, at both mRNA and protein levels, the decreased expression of CHIP was correlated with lymph node metastasis. Furthermore, our study suggests that a negative correlation exists between CHIP expression and tumor malignancy in human gastric cancer.

Introduction

Gastric cancer (GC), is the fourth most common type of malignancy and the second most common cause of cancer death in the world (1), over 70% of the gastric cancer cases occur in the developing countries, and half of the total cases occur in Eastern Asia (mainly in China) (2). Gastric cancer is a biologically and genetically heterogeneous carcinoma (3), and accumulating evidence has suggested that various genetic and epigenetic alterations are related to human gastric cancer (4), including overexpression of oncogenes such as c-met and c-erbB2 (5-7), inactivation of tumor suppressor genes such as p53, β-catenin and PTEN (8-10), as well as alterations of cell cycle regulators, cell adhesion molecules and DNA repair genes (4). Sakata et al have reported that methylation of HACE1 (HECT domain and ankyrin repeat containing E3 ubiquitin-protein ligase 1) and downregulation of EGFL8 (epidermal growth factor-like domain 8) were intimately related to gastric cancer (11,12). The majority of gastric cancer cases are diagnosed at advanced stages which are generally resistant to chemotherapy or radiotherapy, and the current 5-year survival rate of gastric cancer is <20% (13,14). Nevertheless, if gastric cancer could be diagnosed at an early stage, it is a curative disease. Therefore, it is crucial to identify clinically useful biomarkers that can diagnose gastric cancer at an early stage (15). Thus, further investigations to identify genetic changes as new parameters for assessing the progression of gastric cancer are necessary.

Carboxyl terminus of heat shock cognate 70 interacting protein (CHIP) is a cytoplasmic protein containing a 34-amino-acid tetratricopeptide repeat (TPR) domain (16), which is referred to in protein-protein interactions (17), an intervening charged domain and a ‘U-box’ domain (18). The U-box domain contains an E3 ubiquitin ligase activity and can induce ubiquitylation and subsequent proteasome-dependent degradation of tumor-related proteins (19,20). Therefore, many studies have focused on the relationship between CHIP and carcinomas. For instance, CHIP acts as an upstream regulator of oncogenic pathways and inhibits cell growth and metastatic potential by degrading oncogenic proteins including SRC-3 in breast cancer (21). And a recent report demonstrated CHIP...
contributes to the oncogenesis of glioma (22). Moreover, a present study found that CHIP interacts with endogenous Met in lung cancer cells (H358 cells) via inducing the ubiquitination and degradation of Met receptor and CHIP inhibits the tumor growth by decreasing Met in vivo (23). However, the expression of CHIP in human gastric cancer remains unknown. Therefore, the current study was carried out to evaluate the expression of CHIP in gastric cancer and to explore the correlations between CHIP expression and clinicopathological characteristics of gastric cancer. In our present study, we found the decreased expression of CHIP is associated with the clinically aggressive phenotype in gastric cancer.

Materials and methods

Clinical patient samples. Fifty-three patients (median age, 56.0 years; range, 16 - 77 years; 32 males, 21 females) with primary gastric cancer were included in this study. A total of 53 paired cancerous samples and matched normal mucosa located at least 6 cm away from the tumor site were collected from patients who underwent initial surgical resection at Tongji Hospital, Tongji Medical College (Wuhan, China) between April 2011 and January 2012. The non-cancerous samples were confirmed to be without any tumor cell infiltration by histological examination. All patients were pathologically diagnosed as stomach carcinoma, without any metastatic diseases or any other tumors. Informed written consent was obtained from all the patients and the study was approved by the local ethics committee. For each sample, a portion of the lesion was frozen in liquid nitrogen immediately after surgical resection and then stored at -80˚C, while another portion was fixed in 10% formalin-buffered and paraffin-embedded.

Total RNA extraction and first strand cDNA synthesis. RNAiso Plus extraction of total RNA was carried out essentially according to the manufacturer’s instructions (Takara, Dalian, China). The RNA pellets were dissolved in 40 µl of RNase-free water and stored at -80˚C. RNA integrity was assessed prior to cDNA synthesis. The concentration of total RNA was measured by UNICO UV-2800 spectrophotometric readings (Shanghai, China) and the OD260/OD280 ratio of all RNA samples were up to 2.0. The first strand cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, MBI, Lithuania) according to the manufacturer’s protocol.

Polymerase chain reaction and quantitative real-time PCR. Polymerase chain reactions (PCR) were performed in a total volume of 20 µl, containing 10 µl 2X Taq PCR MasterMix, 0.5 µl of each primer (10 pM each), 1 µl cDNA template and 8 µl sterile water. The amplification protocol consisted of an initial denaturation at 94˚C for 5 min, followed by 35 cycles of denaturation for 30 sec at 94˚C, annealing for 45 sec at 64˚C and extension for 30 sec at 72˚C, followed by a final extension at 72˚C for 10 min. The PCR products were verified by 1.5% agarose gel electrophoresis and analyzed using the Gel Doc™ XR Imaging System (Bio-Rad, Foster City, CA, USA). The PCR and real-time PCR primers for CHIP (151 bp): forward, 5’-GGAGGCAGGCTACGAAGCAGTAC-3’; reverse, 5’-TGATGCCACTGGGCGTGATGC-3’. The primers of CHIP and GAPDH genes were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA). Quantitative real-time PCR was performed with a continuous fluorescence detector - StepOne machine (Applied Biosystems, Forster City, CA, USA). Quantitative real-time PCR reaction was carried out using SuperReal PreMix SYBR-Green kit (Tiangen Biotech Co., Ltd., Beijing, China). The cycling parameters were: initial denaturation at 95˚C for 15 min, followed by 40 cycles at 95˚C for 10 sec, 64˚C for 30 sec and 72˚C for 30 sec. The cycling was followed by melting curve analysis to distinguish specificity of the PCR products. CHIP expression was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control in the same sample. Each sample was run three times. No template controls (no cDNA in PCR reaction) were run to detect unspecific or genomic amplification and primer dimerization. The average threshold cycle (Ct) for three replicates per sample was used to calculate ΔCt. Relative quantification of CHIP expression was calculated with the 2^(-ΔΔCt) method.

Tissue immunohistochemistry and immunoblotting. Tissue immunohistochemistry (IHC) was performed using a standard peroxidase-based staining method. Tissue sections (4 µm) were dewaxed in xylene, hydrated with graded ethanol. Then antigen retrieval was performed by pretreatment of the slides in 0.01 M citrate buffer (pH 6.0) using a microwave oven. Subsequently, the sections were treated with 3% hydrogen peroxide (H2O2) for 10 min in order to block endogenous peroxidase. The sections were washed with 0.01 M phosphate-buffered saline (PBS) (pH 7.4), and were incubated with rabbit anti-CHIP antibody (dilution 1:250; Abcam Co., USA) overnight at 4˚C. The sections were then washed with 0.01 M PBS and incubated with biotinylated goat anti-rabbit IgG (SP9000, Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China). For each sample, the omission of primary antibody was used as a negative control. In addition, total protein was extracted only with a tissue lysis buffer containing protease and phosphatase inhibitors (50 mM Tris-base pH 7.4, 100 mM NaCl, 1% NP-40, 10 mM EDTA, 20 mM NaF, 1 mM PMSF, 3 mM Na4VO4, protease inhibitor mixture), the concentration of protein for each sample was determined using the Enhanced BCA Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Protein samples (20 µg) were separated by 10% SDS-polyacrylamide gel electrophoresis and then transferred to nitrocellulose membranes (transfer buffer: 25 mM Tris, 190 mM glycine, 20% methanol, 0.5% sodium dodecyl sulfate). The membranes were washed in Tris-buffered saline (TBS) (20 mM Tris-HCl, pH 7.6, 140 mM NaCl) and blocked with 5% bovine serum albumin (BSA) in TBS containing 0.5% Tween-20 (TBS-T). The membranes were incubated overnight at 4˚C with the primary antibody rabbit anti-CHIP (dilution 1:1000; Cell Signaling Technology, Inc., USA). Membranes were washed with TBS-T solution, incubated for 60 min with horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG (dilution 1:3000; Upstate Biotechnology, Lake Placid, NY), washed with TBS-T, rinsed with double deionized water and immersed in enhanced chemiluminescence (ECL)-detecting substrate (SuperSignalWest Pico; Pierce Chemical Co., Rockford, IL, USA). Images were captured with Micro
Chemi (DNR Bio-Imaging Systems, Israel), the pictures were scanned and the optical density of the bands was determined using NIH ImageJ software (National Institutes of Health, Bethesda, MD) and was standardized to GAPDH detected using mouse anti-GAPDH monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Each case of gastric cancer and the matched normal mucosa was repeated at least 3 times.

Table I. Association between the mRNA expression of CHIP with histopathological features of gastric cancer patients.

<table>
<thead>
<tr>
<th>CHIP mRNA levels</th>
<th>Total (n=53)</th>
<th>Not decreased&lt;sup&gt;a&lt;/sup&gt; (n=16)</th>
<th>Decreased&lt;sup&gt;a&lt;/sup&gt; (n=37)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
<td>No. (%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>32</td>
<td>8</td>
<td>25</td>
<td>0.310</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>8</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤55</td>
<td>26</td>
<td>10</td>
<td>38</td>
<td>0.059</td>
</tr>
<tr>
<td>&gt;55</td>
<td>27</td>
<td>6</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>TNM stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1+T2</td>
<td>11</td>
<td>6</td>
<td>55</td>
<td>0.048</td>
</tr>
<tr>
<td>T3+T4</td>
<td>42</td>
<td>10</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>5</td>
<td>71</td>
<td>0.010</td>
</tr>
<tr>
<td>Positive</td>
<td>46</td>
<td>11</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>45</td>
<td>12</td>
<td>27</td>
<td>0.185</td>
</tr>
<tr>
<td>Well and moderated</td>
<td>8</td>
<td>4</td>
<td>50</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>The decreased folds of >2 were defined as decreased and the decreased folds of ≤2 were specified as not decreased.

Table II. Association between the protein levels of CHIP with clinicopathological data in gastric cancer patients.

<table>
<thead>
<tr>
<th>CHIP expression</th>
<th>Total (n=53)</th>
<th>Not decreased&lt;sup&gt;a&lt;/sup&gt; (n=24)</th>
<th>Decreased&lt;sup&gt;a&lt;/sup&gt; (n=29)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. (%)</td>
<td>No. (%)</td>
<td>No. (%)</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
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<tr>
<td>Male</td>
<td>32</td>
<td>15</td>
<td>47</td>
<td>0.774</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
<td>9</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤55</td>
<td>26</td>
<td>15</td>
<td>58</td>
<td>0.075</td>
</tr>
<tr>
<td>&gt;55</td>
<td>27</td>
<td>9</td>
<td>33</td>
<td></td>
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<td>TNM stages</td>
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<td></td>
</tr>
<tr>
<td>T1+T2</td>
<td>11</td>
<td>7</td>
<td>64</td>
<td>0.170</td>
</tr>
<tr>
<td>T3+T4</td>
<td>42</td>
<td>17</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>7</td>
<td>6</td>
<td>86</td>
<td>0.021</td>
</tr>
<tr>
<td>Positive</td>
<td>46</td>
<td>18</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>45</td>
<td>17</td>
<td>38</td>
<td>0.009</td>
</tr>
<tr>
<td>Well and moderated</td>
<td>8</td>
<td>7</td>
<td>87.5</td>
<td></td>
</tr>
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</table>
Statistical analysis. The non-parametric Mann-Whitney U-test was used to analyze the mRNA expression levels of CHIP in the gastric cancerous samples and the matched normal samples of human gastric cancers. The significance of correlations between CHIP expression and clinicopathological characteristics was analyzed by Student's t-test and Pearson's $\chi^2$ test (Tables I and II). The continuous data were expressed as mean ± SEM. All statistical analyses were two-sided and performed by the SPSS 13.0 software package (SPSS Inc., Chicago, IL, USA). The level of statistical significance was set at P<0.05.

Results

Fifty-three patients suffered from gastric cancer were involved in this research. The gastric cancerous tissues and the matched normal non-cancerous tissues from each patient were detected to determine the expression of CHIP at both mRNA and protein levels. The clinicopathological characteristics including gender, age, TNM stage, lymph node metastasis and tumor differentiation of each patient were evaluated in this study.

CHIP mRNA levels were decreased in gastric cancer and the relationship with histopathologic features. The matched normal mucosa and cancerous tissue samples which were normalized to GAPDH levels were detected from each GC patients (n=2) by PCR (Fig. 1). The results showed mRNA levels of CHIP in cancerous tissues were decreased compared with normal tissues. Furthermore, the expression of CHIP mRNA was detected in 53 gastric cancer samples and the corresponding normal samples by real-time PCR analysis. The relative mRNA expression of CHIP in the gastric cancer samples was significantly lower than that in the corresponding normal samples (3.44±1.33 vs. 11.40±2.87, 2$^{-\Delta\Delta Ct}$, P=0.022, paired t-test). As shown in Table I, the downregulation of CHIP expression occurred in 70% (37 of 53) of gastric cancer patients. Furthermore, the clinical significance of decreased CHIP expression correlated with the clinicopathological data was also explored. There were remarkable differences in CHIP mRNA expression in pT1/T2 stage tumors vs. pT3/T4 stage tumors (P=0.048, $\chi^2$ test), and lymph node non-invasive tumors vs. lymph node invasive tumors (P=0.01, $\chi^2$ test) (Table I). CHIP mRNA was reduced 2.68±1.14-fold in 11 pT1/T2 stage tumors and 8.67±3.42-fold in 42 pT3/T4 stage tumors (P=0.048, Z=-1.974, Mann-Whitney U-test), respectively. In addition, CHIP mRNA was decreased 0.97±0.48-fold in 7 lymph node non-invasive cancers and 8.41±3.13-fold in 46 lymph node invasive cancers (P=0.008, Z=-2.67, Mann-Whitney U-test) (Fig. 2).

Protein expression of CHIP was downregulated in gastric cancers and the correlation with clinicopathological parameters. In this study, the protein levels of CHIP were also examined using western blot analysis. The presence of CHIP in the normal gastric mucosa was confirmed (Fig. 3). However, CHIP protein expression was notably reduced in cancerous samples compared with the matched normal mucosa in 4 cases of gastric cancer (Fig. 3). An immunohistochemical assay was used to estimate the endosomatic status of CHIP expression.
in normal stomach. High levels of CHIP expression occurred in non-cancerous gastric epithelial cells but not in adjacent stromal or inflammatory cells (Fig. 4A). The immunohistochemical staining was not observed in the superficial gastric foveolar cells, but was remarkable in the epithelium from the neck region to deeper glands (Fig. 4A). Therefore, CHIP was shown to align from the basal to middle portions of the gastric mucosa. CHIP staining was diffuse throughout the cytoplasm of the gastric epithelial cells (Fig. 4A). In Fig. 4B, immunohistochemical staining was remarkably decreased at the protein level of CHIP expression in cancerous tissue compared with normal tissue. However, in the cancerous sample, CHIP expression was significantly reduced in well-/moderate-/poor-differentiated gastric cancer (Fig. 4C-E), and with the differentiation turning poor, the staining was gradually less strong. In addition, CHIP downregulation was found in 55% (29 of 53) of gastric cancer patients (Table II). We further assessed the correlations between downregulated CHIP expression and clinicopathological features (Table II). Statistical data showed that downregulated CHIP expression...
was associated with lymph node metastasis and tumor differenti- 
ation. CHIP expression was decreased in 14% (1 of 7) of 
lymph node non-invasive gastric cancer and in 61% (28 of 
46) of lymph node invasive gastric cancer (P=0.021, χ² test) 
(Table II). CHIP expression was decreased in 12.5% (1 of 8) 
of well-/moderated-differentiated gastric cancer and in 62% 
(28 of 45) of poor-differentiated gastric cancer (P=0.009, χ² test) (Table II).

Discussion

Increasing amounts of evidence strongly suggest that E3 ubiquitin ligases are involved in cancer proliferation and tumorigenesis. Furthermore, E3 ubiquitin ligases, such as murine double minute 2 (MDM2), S-phase-kinase-associated protein (Skp)-Cullin-F-Box (SCF), inhibitor of apoptosis protein have emerged as prognostic biomarkers and potential cancer drug targets (24). As a member of the E3 ubiquitin ligases, CHIP has been demonstrated to be involved in tumori- 
genesis, proliferation and invasion in several malignancies (21). 
CHIP is an E3 ubiquitin ligase that induces the ubiquitination and 
proteasomal degradation of its substrates. CHIP interacts 
with Hsp/Hsc70 and Hsp90 through its TPR domain and nega- 
tively regulates chaperone functions. The U-box domain at 
the carboxyl terminus of CHIP contains its E3 ubiquitin ligase 
activity, and was able to promote ubiquitylation and degrada-
tion of many tumor-related proteins, such as ErbB2 in breast 
cancer and ovarian cancer (25,26). ErbB2 overexpression 
contributes to the evolution of a substantial group of human 
cancers and signifies a poor clinical prognosis (25). Previous 
study suggests that ErbB2 is a target of CHIP and wild-type 
CHIP induces ErbB2 ubiquitination and downregulation in vitro 
(25). CHIP overexpression results in decreased levels 
of endogenous ERα in ERα-positive breast cancer MCF7 
cells (27). In addition, CHIP interacted with Met receptor 
leading to proteasomal degradation of the receptor in vitro and 
CHIP overexpression inhibited Med-mediated lung cancer cell 
growth and invasion (23). Other tumor-related proteins such as 
p53 (28,29), FOXO1 (30,31) and hypoxia-inducible factor 
(HIF)-1-α (32) can also be regulated by CHIP.

Because CHIP can regulate these tumor-related proteins 
through ubiquitylation and degradation, it might play an 
important role in cancers. Kajiro et al showed that CHIP 
suppresses tumor progression by inhibiting oncopgenic path-
ways in human breast cancer. Knockdown of CHIP (shCHIP) 
significantly enhanced the metastatic potential of the cancer 
cells due to increased expression of Bel2, Akt1, Smad and 
Twist. These observations demonstrated that CHIP inhibits 
anchorage-independent cell growth and metastatic potential 
by degrading oncopgenic proteins including SRC-3 (21). 
Interestingly, the roles of CHIP in gliomas were totally 
opposite to those in breast cancer. Xu et al showed that CHIP 
expressed stronger in high-grade gliomas than in low-grade 
gliomas. Glioma cells proliferation and colony formation were 
enhanced due to overexpression of CHIP, while knockdown of 
CHIP suppressed proliferation and colony formation. Notably, 
CHIP RNAi lentivirus significantly delayed tumor growth. In 
contrast, overexpression of CHIP resulted in enhanced tumor 
growth in a nude mouse xenograft model. This study demon-
strated that CHIP contributes to oncogenesis of glioma (22).

These results indicate that CHIP might play different roles 
in different human cancers. However, the role of CHIP in the 
progression of gastric cancer has not been investigated.

In the current study, we presented some primary data that 
CHIP was frequently downregulated in gastric cancer using 
RT-PCR, real-time PCR, western blot and immunohisto-
chemical assays. We showed that CHIP was expressed in the 
neck and deeper glands of gastric mucosa in normal tissues. 
However, CHIP expression was significantly decreased in the 
cancerous tissues. Notably, it was almost disappeared in some 
highly lymph node invasive gastric cancer patients. Meanwhile, 
the well-differentiated and moderate-differentiated samples 
showed higher expression of CHIP than the poorly-differen-
tiated gastric cancer samples. Therefore, it seems that a negative 
correlation exists between CHIP expression and tumor malign-
nancy in human gastric cancer.

Invasion and metastasis of tumor cells are major causes of 
mortality in cancer patients. In the present study, we found that 
CHIP expression was almost absent in the advanced gastric 
cancer, such as lymph node invasive gastric cancer and poorly-
differentiated gastric cancer. Therefore, CHIP may play a 
significant role in the progression of gastric cancer.

Thus, further investigation on the molecular mechanism 
between CHIP expression and lymph node metastasis would 
provide some useful insight into the understanding of carcino-
genesis of gastric cancer. However, our study only reported the 
primary data on the relationships between CHIP downregula-
tion and clinically aggressive phenotype of gastric cancer. We 
showed that the decreased CHIP expression was associated 
with lymph node metastasis, TNM stage and tumor differentia-
tion. Such information indicates that CHIP may be a potential 
diagnostic biomarker and therapeutic target for gastric cancer. 
However, our study only investigated the correlations between 
CHIP and clinicopathological characteristics of gastric cancer, 
and a further prospective analysis to elucidate the molecular 
mechanism of the downregulated CHIP in gastric cancer could 
be informative.

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