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CDH1 methylation in peritoneal washes as a prognostic factor for gastric cancer

Metilação de *CDH1* em lavados peritoneais como fator prognóstico para câncer gástrico

Metilación de *CDH1* en lavados peritoneales como factor pronóstico de cáncer gástrico

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Ana Carolina Favacho Miranda de Oliveira
ORCID: https://orcid.org/0000-0003-4985-2354
Universidade Federal do Pará, Brasil
E-mail: carolina_mirandaa@hotmail.com
Antônio André Conde Modesto
ORCID: https://orcid.org/0000-0003-1854-0358
Universidade Federal do Pará, Brasil
E-mail: antoniocmodesto@gmail.com
Fabiano Cordeiro Moreira
ORCID: https://orcid.org/0000-0002-2799-3546
Universidade Federal do Pará, Brasil
E-mail: fabiano.ufpa@gmail.com
Taíssa Maíra Thomaz Araújo
ORCID: https://orcid.org/0000-0002-1716-4445
Universidade Federal do Pará, Brasil
E-mail: taissa_araujo@hotmail.com
Marcelli Geise de Oliveira Prata da Silva
ORCID: https://orcid.org/0000-0002-3999-5565
Universidade Federal do Pará, Brasil
E-mail: m.prataa@outlook.com
Paulo Pimentel Assumpção
ORCID: https://orcid.org/0000-0003-3846-8445
Universidade Federal do Pará, Brasil
E-mail: assumpcaopp@gmail.com
Geraldo Ishak
ORCID: https://orcid.org/0000-0002-0837-4803
Universidade Federal do Pará, Brasil
E-mail: ishakgeraldo@gmail.com
Bárbara do Nascimento Borges
ORCID: https://orcid.org/0000-0002-5989-8287
Universidade Federal do Pará, Brasil
E-mail: pithecia@gmail.com
André Salim Khayat
ORCID: https://orcid.org/0000-0002-3451-6369
Universidade Federal do Pará, Brasil
E-mail: khayatas@gmail.com

Abstract
Gastric cancer is a major public health problem, considered the fifth most frequently diagnosed cancer worldwide. Peritoneal metastasis is the main causes of mortality related to gastric cancer. The hypermethylation of tumor suppressors genes is a promising factor for the development of predictive biomarkers. In gastric tumors, reduced expression of *CDH1* suppressor gene contributes to tumor invasion and triggering metastasis. We aim to evaluate the promoter region methylation profile of the *CDH1* gene as a peritoneal metastasis indicator in patients with gastric cancer. *CDH1* methylation profile were evaluate in 47 samples of peritoneal washes from patients with gastric cancer by bisulfite sequencing PCR (BSP). Thirty-three CpGs sites in the *CDH1* promoter region were individually evaluated. Samples with ≥ 10% of methylated CpGs sites were considered hypermethylated. Statistical analysis was performed by Fisher's Exact test using R-Studio program ($p \leq 0.05$). Twenty-nine (62%) samples showed hypermethylation in the promoter region of *CDH1*, with preferential methylation at the CpGs sites located in the region 5' upstream from gene. Statistical analysis showed that *CDH1* hypermethylation was significantly different in patients with advanced stages of tumor depth of invasion ($p=0.03$). Our results suggest that *CDH1* gene methylation analysis is a useful tool in predicting tumor progression and invasiveness in gastric cancer patients and could have a major impact for the field of precision cancer medicine.
Keywords: Gastric cancer; Peritoneal metastasis; Methylation; *CDH1*; Tumor progression.

Resumo
O câncer gástrico é um importante problema de saúde pública, considerado o quinto câncer mais diagnosticado no mundo. A metástase peritoneal é uma das principais causas de mortalidade relacionadas ao câncer gástrico. A

hipermetilação de genes supressores de tumor é um fator promissor para o desenvolvimento de biomarcadores preditivos. Em tumores gástricos, a expressão reduzida do gene supressor *CDH1* contribui para a invasão tumoral e desencadeamento de metástases. Nosso objetivo foi avaliar o perfil de metilação da região promotora do gene *CDH1* como indicador de metástase peritoneal em pacientes com câncer gástrico. O perfil de metilação de *CDH1* foi avaliado em 47 amostras de lavados peritoneais de pacientes com câncer gástrico por PCR de sequenciamento de bissulfito (BSP). Trinta e três sítios CpGs na região do promotor *CDH1* foram avaliados individualmente. Amostras com ≥ 10% de sítios CpGs metilados foram consideradas hipermetiladas. A análise estatística foi realizada pelo teste Exato de Fisher, utilizando o programa R-Studio (p≤0,05). Vinte e nove (62%) amostras apresentaram hipermetilação na região promotora de *CDH1*, com metilação preferencial nos sítios CpGs localizados na região 5' a montante do gene. A análise estatística mostrou que a hipermetilação de *CDH1* foi significativamente diferente em pacientes com estágios avançados de profundidade de invasão do tumor (p=0,03). Nossos resultados sugerem que a análise de metilação do gene *CDH1* é uma ferramenta útil na predição de progressão e invasividade tumoral em pacientes com câncer gástrico e pode ter um grande impacto para o campo da medicina de precisão do câncer.
Palavras-chave: Câncer gástrico; Metástase peritoneal; Metilação; *CDH1*; Progressão tumoral.

Resumen

El cáncer gástrico es un importante problema de salud pública, considerado el quinto cáncer más diagnosticado en el mundo. La metástasis peritoneal es una de las principales causas de mortalidad relacionada con el cáncer gástrico. La hipermetilación de los genes supresores de tumores es un factor prometedor para el desarrollo de biomarcadores predictivos. En los tumores gástricos, la expresión reducida del gen supresor *CDH1* contribuye a la invasión tumoral y al desencadenamiento de metástasis. Nuestro objetivo fue evaluar el perfil de metilación de la región promotora del gen *CDH1* como indicador de metástasis peritoneal en pacientes con cáncer gástrico. El perfil de metilación de *CDH1* se evaluó en 47 muestras de lavado peritoneal de pacientes con cáncer gástrico mediante PCR de secuenciación con bisulfito (BSP). Treinta y tres sitios CpG en la región promotora de *CDH1* se evaluaron individualmente. Las muestras con ≥ 10 % de sitios CpG metilados se consideraron hipermetiladas. El análisis estadístico se realizó mediante la prueba exacta de Fisher, utilizando el programa R-Studio (p≤0.05). Veintinueve (62%) muestras mostraron hipermetilación en la región promotora de *CDH1*, con metilación preferencial en los sitios CpG ubicados en la región 5' aguas arriba del gen. El análisis estadístico mostró que la hipermetilación de *CDH1* fue significativamente diferente en pacientes con estadios avanzados de profundidad de invasión tumoral (p = 0,03). Nuestros resultados sugieren que el análisis de la metilación del gen *CDH1* es una herramienta útil para predecir la progresión tumoral y la invasividad en pacientes con cáncer gástrico y podría tener un gran impacto en el campo de la medicina oncológica de precisión.
Palabras clave: Cáncer gástrico; Metástasis peritoneal; Metilación; *CDH1*; Progresión tumoral.

1. Introduction

Gastric cancer (GC) is an important public health problem, considered the fifth most frequently diagnosed cancer and the third leading cause of cancer death worldwide. The 5-year overall survival rate for these patients is low (10-20%) and the poor prognosis associated with GC is mainly due to late-stage tumor detection (Bray et al., 2018; Khan & Shukla, 2006). The peritoneum is one of the most common metastatic sites among patients with GC. Peritoneal metastasis can lead to intestinal obstruction and formation of large amounts of malignant ascites, being considered a major cause of gastric cancer-related mortality (Thomassen et al., 2014).

Although surgical resection is the main management modality, it does not prevent progression to peritoneal metastasis, especially in advanced stages (Yoo, Noh, Shin, Choi & Min, 2000). The distinct prognosis in patients with GC of the same clinical stage highlights that it does not reflect the biological potential of the tumor and new biomarkers are needed to complement the clinical parameters for a more accurate therapeutic decision (Sawada et al., 2015; Soleyman-Jahi et al., 2015; Soleyman-Jahi et al., 2015; Soleyman-Jahi et al. al., 2015).

Evidence suggests that analysis of the methylation profile of the promoter region of genes, especially tumor suppressors, is a promising factor for the development of predictive cancer biomarkers, tumor prognosis, and treatment response prediction (Koch et al., 2018). Furthermore, epigenetic changes in peritoneal washes samples have been suggested as potential non-invasive circulating biomarkers for early detection of gastric cancer (Dumitrescu, 2018).

CDH1 is a tumor suppressor gene that transcribes a transmembrane epithelial glycoprotein (E-cadherin), responsible for calcium-dependent cell adhesion to form organized tissues (Shenoy, 2019). The loss of these epithelial markers is related to

the epithelial mesenchymal transition (EMT), which the cell acquires a malignant phenotype (Prieto-García, Díaz-García, García-Ruiz & Agulló-Ortuño, 2017). In gastric tumors, reduced *CDH1* expression is often associated with promoter region hypermethylation, contributing to cellular phenotypes with greater tumor invasiveness and metastasis development (Tamura et al., 2000; Katoh, 2005; Li & Guo, 2019).

Given the central role of the *CDH1* gene in tumor progression, the relationship between the decrease or loss of E-cadherin and the acquisition of invasive and infiltrative cell phenotypes (Bruner & Derksen, 2018), this study aimed to investigate the methylation profile of the *CDH1* gene promoter region in peritoneal washes samples from patients with gastric cancer and its relationship with clinicopathological data.

2. Methodology

Patient selection

This research was approved by the Research Ethics Committee of the João de Barros Barreto University Hospital (Protocol nº 2.367.053). An informed consent form was obtained from each patient. Thirty-seven patients diagnosed with gastric adenocarcinoma under follow-up at University Hospital João de Barros Barreto (Belém, Pará, Brazil) participated in this study. For some patients, samples were collected in two different stages (laparoscopy and gastrectomy).

Sample collection

Forty-seven (47) peritoneal washes samples were collected (100 ml). These samples were centrifuged for 10 minutes at 1500 rpm. The pelleted cells were transferred to a 2 ml microtube and stored at -80°C.

DNA Methylation Assay

Genomic DNA was extracted using QIAamp DNA Mini Kit (QIAGEN). DNA treatment with bisulfite was done using the Epitec Bisulfite Kit (QIAGEN). The genomic fragments were amplified using a nested PCR strategy with specific primers previously described (Nojima et al., 2001). Nested PCR reactions were performed in 12.5 µl, containing 2 µl of bisulfite-modified DNA, 6.25 µl of 2x GoTaq® Colorless Master Mix (Promega Corporation), 1 µl of forward primer, 1 µl of reverse primer, and 4.25 µl of nuclease-free water. The reaction settings were 94°C for 2 min and 40 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min and 72°C for 10 min. The positive (methylated DNA) and negative (unmethylated DNA) controls used were EpiTect Control (QIAGEN) DNAs.

The methylation profile of *CDH1* CpG sites was analyzed by bisulfite sequencing (BSP) PCR using BigDye™ Terminator v3.1 Cycle Sequencing (ThermoFisher Scientific), following the guidelines described by the manufacturer, containing 0.5 µl of BigDye, 3 µl buffer, 1 µl primer (forward or reverse), 2 µl PCR product and 2 µl nuclease-free water. The thermocycling settings were 94°C for 2 min and 40 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1 min. The products of these reactions were purified by precipitation with Sephadex dextran gel (Sigma-Aldrich's). The purified pellets were suspended in 10 µl of formamide (Applied Biosystem) and denatured at 95°C for 3 min. Subsequently, they were added to the ABI 3500 automatic sequencer (Applied Biosystems). Samples with methylation ≥ 10% of their CpG sites were classified as hypermethylated (Bergman & Cedar, 2013).

Statistical data analysis

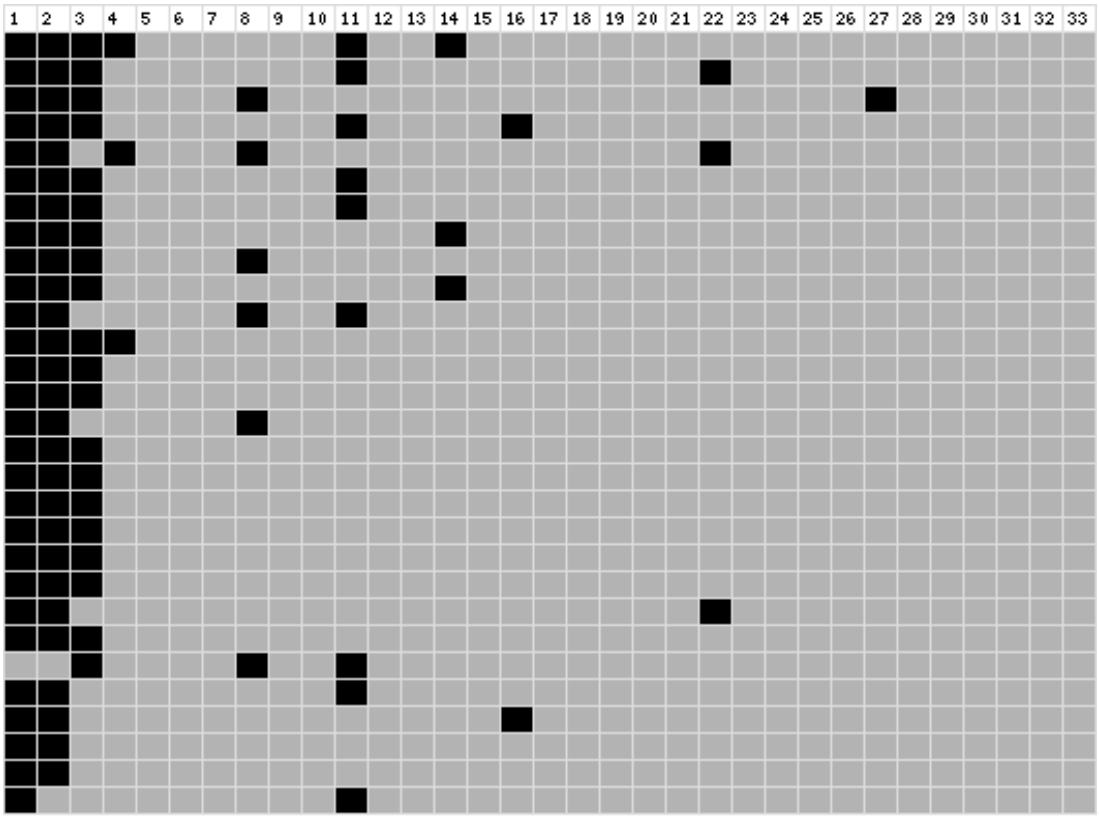
The electropherograms obtained from the sequencing were analyzed using the Chromas program version 2.6.6 (Technelysium). The BDPC program (Bisulfite sequencing Data Presentation and Compilation) was used to illustrate the methylation pattern of the fragments and prepare the methylation map (Rohde et al., 2008). All data analysis was performed

using the RStudio program (RStudio, Inc). Fisher's exact test was used to compare the different groups of variables and $p \leq 0.05$ was considered statistically significant.

3. Results

Among the 47 samples, 29 (62%) showed hypermethylation in the *CDH1* promoter region. Of the 33 CpG sites analyzed (fragment of 368 base pairs), we observed preferential methylation at the CpG sites located in the 5' region of the fragment (Figure 1). *CDH1* hypermethylation was found in 4 (33%) samples with initial tumor invasion depth (T1/T2) and in 18 (72%) samples with advanced tumor invasion depth (T3/T4) with statistical significance of p = 0.03 (OR = 0.204) (95% CI = 0.03310664-1.05552434). All associations between *CDH1* methylation profile and clinical features are described in table 1.

Figure 1: Methylation of 33 CpG sites of the analyzed fragment in the 5'→3' ascending direction. The lines represent the 29 samples hypermethylated in the promoter region of the *CDH1* gene; columns represent each CpG in the analyzed region; black: methylated CpG; gray: unmethylated CpG.



Source: Authors; BDPC program.

Table 1: Association of *CDH1* methylation profile in peritoneal washes with variables in patients with gastric cancer. M: methylated; UN: unmethylated.

	CDH1		
	M (%)	**U (%)**	***p-value***
Total	29 (62)	18 (38)	-
PLC			
Positive	4 (67)	2 (33)	1
Negative	25 (61)	16 (39)	
Procedure			
Laparoscopy	12 (57)	9 (43)	0.76
Gastrectomy	17 (65)	9 (35)	
Gender			
Male	20 (65)	11 (35)	0.75
Female	9 (56)	7 (44)	
Age			
≤ 60	17 (61)	11 (39)	1
> 60	12 (63)	7 (37)	
H. pylori			
(+)	6 (67)	3 (33)	1
(-)	23 (61)	15 (39)	
Tumor location			
Cardia	2 (50)	2 (50)	0.65
No cardia	27 (63)	16 (37)	
Clinical staging			
I/II	9 (47)	10 (53)	0.29
III/IV	14 (74)	5 (26)	
Depth of invasion			
T1/T2	4 (33)	8 (67)	0.03
T3/T4	18 (72)	7 (28)	
Lymph node metastasis			
N0	9 (53)	8 (47)	0.86
N1-N3	13 (65)	7 (35)	
Distant metastasis			
M0	17 (57)	13 (43)	0.67
M1	5 (71)	2 (29)	

Source: Authors.

4. Discussion

The main recurrence sites for gastric cancer patients include locoregional, distant or hematogenous metastases and in the peritoneum (Liu et al., 2016). Among these, peritoneal dissemination is the most frequent after curative resection (Zhu et al., 2019). Furthermore, it has been reported that the chances of peritoneal recurrences increase with the depth of invasion of the primary tumor (Fujiwara, 2007).

The Japanese Gastric Cancer Association (JGCA) recommends peritoneal lavage cytology (PLC) to detect free tumor cells within the peritoneal cavity, considered the gold standard technique for predicting peritoneal spread (JGCA, 2017;

Bentrem et al., 2005). Patients with positive PLC are classified as stage IV according to the JGCA and the Union for International Cancer Control (UICC) (Brierley, Gospodarowicz, and Wittekind, 2016). However, PLC has questionable sensitivity and many patients with negative PLC develop some recurrence (Liu et al., 2016).

Of the 47 peritoneal washes samples in this study, only 6 (13%) were PLC positive and 41 (87%) PLC negative. The positive PLC samples, 4 (67%) showed *CDH1* hypermethylation and the negative PLC samples, 25 (61%) showed *CDH1* hypermethylation. Although there is no statistical significance between PLC status and *CDH1* gene hypermethylation, these results reveal the low sensitivity of cytological tests in detecting free tumor cells compared to molecular tests, since hypermethylation was detected in most PLC negative. Kodera et al. (2002) previously demonstrated that molecular markers such as carcinoembryonic antigen (CEA) mRNA detected by PCR are more sensitive than PLC in gastric cancer. However, despite the recognized applicability of CEA, clinical tests with mRNA can be unstable due to the fragility of the molecules in relation to DNA.

Although the general level of methylation of CpGs in human genomic DNA is 70 to 80% (Jones, 2012), methylation levels in CpG islands are typically less than 10% for active genes (Bergman & Cedar, 2013). Hypermethylation of the promoter region of tumor suppressor genes has been proposed as one of the main mechanisms of gene silencing and research suggests their potential as biomarkers in carcinogenesis (Dumitrescu, 2018). In GC, gene inactivation caused by hypermethylation of the promoter region was found more frequently than by genetic mutations (Fu, 2015).

The spread of tumor cells in the peritoneal cavity from primary tumors is strongly associated with disease progression and a worse prognosis (Coccolini et al., 2013). Since the "seeds and soil" hypothesis, which proposes that the spread of tumor cells is guided by the interaction and cooperation between the tumor cells (seed) and the host organ (soil) (Paget, 1889), free tumor cells in peritoneal washings in patients with gastric cancer are considered important factors of peritoneal metastasis (Liu et al., 2016; Hoskovec et al., 2017; Lisiecki et al., 2017; Virgilio et al., 2018).

During cancer progression, downregulation of E-cadherin and upregulation of mesenchymal cadherins, such as N-cadherin, αvβ6 integrin, vimentin, and matrix metalloproteinase, allow epithelial neoplastic cells of the primary tumor to undergo morphological remodeling and functional (Prudkin et al., 2009). This process improves cell detachment and invasiveness, necessary for the initiation of metastasis, thus allowing tumor cells to invade, migrate and colonize distant sites (Paolillo & Schinelli, 2019).

Evidence suggests that epigenetic signatures may be one of the main ways of regulating the inhibition of intercellular adhesion and consequent penetration of tumor cells into the basement membrane in the tissues and vessels adjacent to the cancer, thus facilitating the spread of tumor cells (Peixoto et al., 2019). We found 62% of samples with *CDH1* promoter region hypermethylation and a significant association of *CDH1* hypermethylation between initial depth of tumor invasion (33%) and advanced depth of tumor invasion (72%) ($p=0.03$).

Our results were consistent with most of the literature, indicating that *CDH1* methylation, not only in tumor tissues but also in peritoneal washes, was significantly correlated with tumor progression (Hiraki et al; 2010; Hiraki et al., 2011). Yu et al. (2012) detected 48% of peritoneal washes from GC patients with hypermethylation of the *CDH1* promoter region correlated significantly with the depth of invasion ($p<0.05$), in addition to hypermethylation of the 5'CpG island of the *CDH1* promoter. However, as demonstrated in our results, no relationship was found with age, sex, location and H. pylori infection ($p>0.05$).

Previous studies have emphasized that *CDH1* hypermethylation is an important biomarker for the depth of T3/T4 tumor invasion and progression in esophageal cancer (Ling et al., 201; Zhi-Qiang et al., 2011), cervical cancer (Holubeková et al., 2016), melanoma (Venza et al., 2016) and gastric cancer (Li & Guo, 2019). The role of E-cadherin as an "invasion suppressor" (Vleminckx et al., 1991) has been well established in the context of gastric cancer, as its reestablishment

expression was accompanied by suppression of tumor invasion and metastasis (Ma, Siegal & Wei, 2016; Gao, Wang, Jing, Zhan & Wang, 2017).

Variations in the frequency of *CDH1* methylation can be found in different populations. Zeng et al. (2015) described that the frequency can range from 28.6% to 82.2% (average 61%) in cancerous tissues and from 0.00% to 54.5% (average 16%) in normal mucosa. Our analysis there was no statistical association between *CDH1* hypermethylation and lymph node metastasis (N) and distant metastasis (M), as previously described (Hiraki et al., 2010). These differences can be explained by the different tumor stages analyzed (Suzuki et al., 1999).

In our analyses *CDH1* hypermethylation was estimated quantitatively, using bisulfite sequencing, so that we could detect regions susceptible to hypermethylation. Borges et al. (2010) analyzed the same fragment of the *CDH1* promoter region from gastric tissues, using bisulfite sequencing, and a higher methylation rate was also found in the 5'CpG island. The authors highlighted the importance of the region as potential binding sites for transcription factors.

Aberrant methylation of the 5'CpG island of the *CDH1* gene has been reported in breast cancer (Caldeira et al., 2006), esophageal adenocarcinoma (Corne r al., 2001), colorectal cancer (Lu, Du, Zheng, Peng & Chen, 2014) and gastric cancer (Hiraki et al., 2011; Ma, Siegal & Wei, 2016). In acute leukemia, hypermethylation of the 5'CpG island of *CDH1* was associated with reduced or absent expression of E-cadherin and treatment with 5-aza-2'deoxycytidine, a drug that induces demethylation, was effective in restoring normal expression of transcript and e-cadherin protein. In this sense, the usefulness of *CDH1* hypermethylation as a molecular marker of tumor prognosis may also influence the choice of appropriate adjuvant therapy for patients (Corn et al., 2000).

5. Final considerations

The detection of *CDH1* methylation in peritoneal washes can be a very applicable guideline for the diagnosis and prediction of progression and invasion in gastric cancer, since the current available tools, such as PLC, do not provide reliable results. Our results also reinforce that hypermethylation of the 5'CpG island of the *CDH1* promoter is a frequent molecular event in gastric cancer, suggesting its important role in gene silencing.

From these initial analyzes of biomarkers for peritoneal recurrences in gastric cancer, it is suggested that the development of molecular biomarkers panels, to increase the accuracy of the results obtained, is a promising tool for precision medicine. In addition, after the validation of biomarkers, the application of more agile techniques, such as real-time PCR, can be applied in prognostic methods in research and diagnostic centers.

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