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Angiogenin and MMP-2 as potential biomarkers in the differential diagnosis of gestational trophoblastic diseases

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Abstract

Background: Gestational trophoblastic diseases (GTDs) are characterized by vascular abnormalities of the trophoblast, but their pathogenesis is unknown. Angiogenin (ANG) and matrix metalloproteinase (MMP)-2, which are molecules implicated in the angiogenic process, may play some role in this process.

Material and methods: We determined ANG and MMP-2 in the placental tissues of 26 patients who had a benign mole (BM), 12 patients with gestational trophoblast neoplasia (GTN) (1 invasive hydatidiform mole, 10 choriocarcinomas, and 1 placental-site trophoblastic tumor), and 28 normal chorionic villi (NCV) subjects using immunohistochemistry staining. We obtained the serum samples from 20 patients with GTDs and 20 early pregnant women and evaluated them by the enzyme linked immunosorbent assay.

Results: ANG expression in GTN (66.7%) and BM (100%) samples were both significantly higher (strong/intermediate staining) than in NCV (60.7%) samples ($P < .001$). Similarly, the immunoreactivities of MMP-2 in the GTN (66.7%) and BM (80.8%) samples were significantly elevated compared to that of the NCV (57.1%) samples ($P < .001$). The levels of ANG and MMP-2 in the maternal serum of the GTN group were both significantly higher than those of the control group ($P < .001$). ANG and MMP-2 expressions were associated with gestation age, clinical stage, and FIGO stage. A positive correlation between ANG and MMP-2 expression was observed ($rs = 0.725$; $P < .01$).

Conclusion: ANG and MMP-2 levels were significantly elevated in the placental tissues and maternal serum from patients with GTDs. Further studies with more patients may clarify the vascular abnormalities in GTDs and determine potential biomarkers in the differential diagnosis of GTDs.

Abbreviations: ANG = angiogenin, BM = benign mole, ELISA = the enzyme linked immunosorbent assay, EVT FIGO = International Federation of Gynecology and Obstetrics, GTDs = gestational trophoblastic diseasesdingshi, GTN = gestational trophoblast neoplasia, hCG = human chorionic gonadotropin, IHC = immunohistochemical analysis, MMP-2 = matrix metalloproteinase-2, MUC15 = Mucin 15, NCV = normal chorionic villi, PLGF = placental growth factor, VEGF = vascular endothelial growth factor.

Keywords: ANG, ELISA, gestational trophoblastic diseases, immunochemical staining, MMP-2

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All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Tangdu Hospital, Air Force Military Medical University, Xi'an) (No.TDLL-20140225).and with the Helsinki Declaration of 1964 and later versions.

The informed consent for participation was obtained from all subjects.

The datasets used and analyzed in the present research are available from the corresponding author on reasonable request.

The authors have no conflicts of interests to disclose.

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

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1. Introduction

Gestational trophoblastic diseases (GTDs) consist of a heterogeneous group of neoplastic disorders arising from placental tissues, including benign moles (BM), invasive hydatidiform moles, placental site trophoblastic tumors, and choriocarcinoma.[1] GTDs are characterized by an abnormal proliferation of the trophoblasts, including cytotrophoblasts, syncytiotrophoblasts, and extravillous implantation site trophoblasts, and are either nonneoplastic (hydatidiform moles) or true neoplastic (gestational trophoblast neoplasia [GTN]).[2] BM refers to placental villus trophoblast hyperplasia and edema after pregnancy, forming blisters with a "grape-like" appearance of different sizes. It is also known as the vesicular fetal block. Most patients who have BM can be cured after treatment. Approximately 15% to 20% of patients will undergo a malignant transformation into GTN; however, risk factors for progression are unclear.

GTN may develop following any gestational event, but commonly occurs after a molar pregnancy, which adds an approximate 1% to 2% risk of becoming an invasive hydatidiform mole.[3] There are 3 well-defined subtypes of GTN, with distinct pathobiology and different clinical management, including gestational choriocarcinoma, placental site trophoblastic tumors, and epithelioid trophoblastic tumors.[4] The broad differential diagnoses and diversity of their precursor lesions result in diagnostic uncertainty.[5] Moreover, 85% of patients with a benign hydatidiform mole achieve spontaneous remission, whereas 15% develop into GTN and require chemotherapy.

Several studies have applied a series of indicators to distinguish patients who are at risk for subsequent post-molar persistent diseases, such as a large uterine size at the time of examination, high levels of serum human chorionic gonadotropin (hCG), and a large ovarian theca lutein cyst.[6–8] The malignant potential of GTN can be evaluated by the regression curve of hyperglycosylated-hCG in collaboration with clinical characteristics.[9] However, the heterophilic antibodies in the serum samples can cause false-positive hCG results. There are no clinical biomarkers to predict post-molar GTN before the initiation of hCG surveillance.[10]

In obstetrics, the new formation of blood vessels is significant for blastocyst implantation and the development of the placenta and GTN.[11] In endometrial cancer, vascular density can be used as a prognostic indicator of metastatic potential.[12] Shaarawy and Sharkawy reported that circulating levels of angiogenic factors correlate well with both tumor burden and tumor stage in endometrial cancer.[13] Angiogenin (ANG) is a potent inducer of neovascularization.[14] In many types of tumors, the worse the differentiation of the tumors, the stronger the ANG expression.[15–17] Serum ANG levels in choriocarcinoma cases were significantly higher than in healthy pregnant women.[18] However, its expression in GTN tissues and mechanism remain unknown, as cases are rare.[19]

Human placentation processes require a balance between the degradation of the maternal decidual vasculature and the concurrent angiogenesis directed by the developing embryo.[20] MMPs are extracellular matrix remodeling proteases that act as critical mediators of the tumor microenvironment and play a key role in tumor angiogenesis, invasiveness, and metastatic potential.[21] MMP expression is altered in the placentas of patients with GTD compared to normal placentas.[22–24]

ANG is implicated in the angiogenic process. The molecule binds to actin, which is followed by the dissociation of the actin-ANG complex from the cell surface. Subsequently, the tissue plasminogen activator is activated, generating plasmin and degrading the basement membrane matrix.[25,26] The destruction of the existing basement membrane may be a prerequisite for endothelial cell migration during de novo vascularization.[27,28] However, there has been no link between ANG/MMPs and GTD diseases established. We assume that ANG and MMP-2 may play some roles in the pathogenesis of vascular changes seen in GTDs. To prove our hypothesis, we examined ANG levels in the placental tissue and maternal serum of patients with various GTDs.

2. Materials and methods

2.1. Patients and specimens

The present study was performed per the ethical standards in the 1964 Declaration of Helsinki. The study was approved by the ethics committee of the participating hospitals. All patients or their family members provided written informed consent. The clinical and laboratory data reported in this study were obtained at the time of tissue or serum sampling.

We included 38 patients with GTDs from Xi-Jing Hospital between January of 2006 to July of 2014. The paraffin blocks of placental villi tissues were obtained from the Department of Pathology. The diagnosis was confirmed by 2 experienced pathological doctors. The GTD clinical stages were defined according to the guidelines of the International Federation of Gynecology and Obstetrics (FIGO). Tissues from 28 gestational age-matched controls were sampled from patients undergoing elective termination of pregnancy at 7 to 12 weeks gestation in Tangdu Hospital between January and December of 2014. None of these patients received radiotherapy or chemotherapy before surgery.

The chorionic villi were dissected free from fetal membranes within 15 minutes after delivery. The specimens were fixed in 4% buffered formaldehyde for at least 24 hours, dehydrated, and embedded in paraffin. The paraffin-embedded tissue blocks were sectioned into a 6-μm thickness, dewaxed in xylene, and hydrated in an ethanol gradient. Serum samples were obtained from the peripheral blood of 20 pregnant women who had normal delivery within 10-weeks of gestation and 20 patients with GTDs in Tangdu Hospital between January and July of 2014. The histomorphology was confirmed by the Department of Pathology in Tangdu Hospital.

2.2. Immunohistochemical analysis and scoring

We incubated the slides with 3% hydrogen peroxide for 30 minutes to block the endogenous peroxidase activity and washed them with the washing buffer (0.5% albumin from bovine serum (BSA) and 0.025% Tween-20 in PBS, pH 7.4) 3 times. We conducted antigen retrieval by heating the slides at 100°C for 30 minutes in an ethylene diamine tetraacetic acid (EDTA) solution. We incubated the slides with 10% normal horse serum at room temperature for 30 minutes and then incubated them with the primary antibody diluted in the antibody buffer (1% BSA and 0.1% Tween-20 in PBS, pH 7.4), including rabbit anti-human ANG (1:80; sc-1408, Santa Cruz Biotech, Santa Cruz, CA) and MMP-2 (1:80; sc-8835, Santa Cruz Biotech, Santa Cruz, CA), in

Table 1

Associations of ANG and MMP-2 expressions with the clinicopathological characteristics of patients with GTD.

Characteristics	N	ANG			*P* value*	MMP-2			*P* value*
		−	+	++		−	+	++	
Age (year)									
<40	30	3	16	11	<.001†	12	7	11	<.001†
>-40	8	2	6	0		5	2	1	
Sample type									
NCV	28	11	13	4	<.001‡	12	12	4	<.001‡
BM	26	0	19	7		5	11	10	
GTT	12	4	7	1		4	6	2	
TNM stage									
I	4	1	2	1	.042‡	1	2	1	.042‡
II	1	0	1	0		0	0	1	
III	2	1	1	0		1	1	0	
IV	7	0	6	1		0	5	2	
FIGO stage									
poor	6	1	5	0	.031†	2	2	2	.014†
high	8	2	4	2		1	4	3	

BM = benign mole, GTT = gestational trophoblast tumor, NCV = normal chorionic villi.

* $P < .05$, significant different.

† Date are analyzed with Mann–Whitney test.

‡ Date are analyzed with Kruskal–Wallis test.

a moist chamber at 4°C for 16 hours. We performed subsequent procedures using a Vector Universal Elite Kit according to the manufacturer's instructions (Vector Laboratories, Inc., Burlingame, CA).

The specimens were quantified using the Image-Pro Plus 6.0 and immunoreactivity score (IRS) system[22] based on the percentage (0: <5%, 1: 6%–25%, 2: 26%–50%, 3: 51%–75%, and 4: >75%) and intensity (0: negative, 1: week, 2: moderate, and 3: intense) of positive cells stained with the respective antibodies. The analysis (n = 4) was done in triplicate. The final score was obtained by multiplying the score of the percentage and the intensity, and was stratified as negative staining (−: 0) or positive staining (+: 1-4, and ++: > 5). The specimens were scored separately by 2 pathologists, who were not informed of their clinical or clinicopathological status. Specimens were restored if the difference in score from the 2 pathologists was more than 3.

2.3. The enzyme-linked immunosorbent assay (ELISA)

The concentrations of ANG and MMP-2 in the serum of patients with GTDs and healthy pregnant women were evaluated by human ANG and MMP-2 ELISA kits (DAN00 and MMP200, R&D Systems Inc., Minneapolis, MN) per the manufacturer's instructions. The absorbance was measured at 450 nm using a 96-well automatic spectrophotometer (Bio-Tek Instruments, Inc. Winooski, VT).

2.4. Statistical analysis

We used SPSS software (version 19.0; SPSS Inc, Chicago, Ill.) for statistical analysis. For the immunohistochemical analysis (IHC) results, the Mann–Whitney *U* test and the Kruskal–Wallis H test were applied to analyze the association of ANG and MMP-2 expression with the clinicopathological characteristics of patients with GTD. The ELISA data were assessed using the Student *t* test and were reported as mean ± SEM. Pearson (rs) rank correlation and linear regression analysis were applied to analyze the relationship between the expressions of ANG and MMP-2 in different groups. Differences with a $P < .05$ were considered statistically significant.

3. Results

The immunostaining patterns of ANG and MMP-2 in the samples of normal chorionic villi (NCV), benign mole (BM), and GTN are summarized in Table 1. The IRS scores of ANG and MMP staining were significantly associated with the gestation age, clinical stage, and FIGO stage of GTD patients ($P < .05$). Positive staining of ANG and MMP-2 was more frequently detected in GTD patients with a higher clinical stage (III and IV) and advanced FIGO stage, indicating that the levels of ANG and MMP-2 were associated with GTD progression.

As shown in Figure 1, the ANG proteins were positively stained into a brownish-yellow granular, which was mainly located in the trophoblast cytoplasm and cell membrane, with a few located in the nucleus. Most of the MMP-2 proteins were located in the decidual cells and extravilli trophoblast cells (EVT). The ANG and MMP-2 immunoreactivity were intense in the cytoplasm of over 90% of the GTN cells. The semi-quantitative analysis revealed that the ANG expression in GTN (66.7%) and BM (100%) samples were significantly higher (strong/intermediate staining) than in the NCV (60.7%) samples ($P < .001$). Similarly, the immunoreactivities of MMP-2 in the GTN (66.7%) and BM (80.8%) samples were significantly elevated compared to that of NCV (57.1%) samples ($P < .001$). ANG expression in GTN samples was positively correlated with MMP-2 expression (Table 2).

To further elucidate the correlation between MMP-2 and ANG in GTD, we evaluated the maternal serum of patients with GTD and healthy pregnant women by ELISA. The results showed that the levels of ANG and MMP-2 in the GTD group were both significantly higher than that in the control group ($P < .001$) (Fig. 2A and B). The extent of change in ANG was much greater

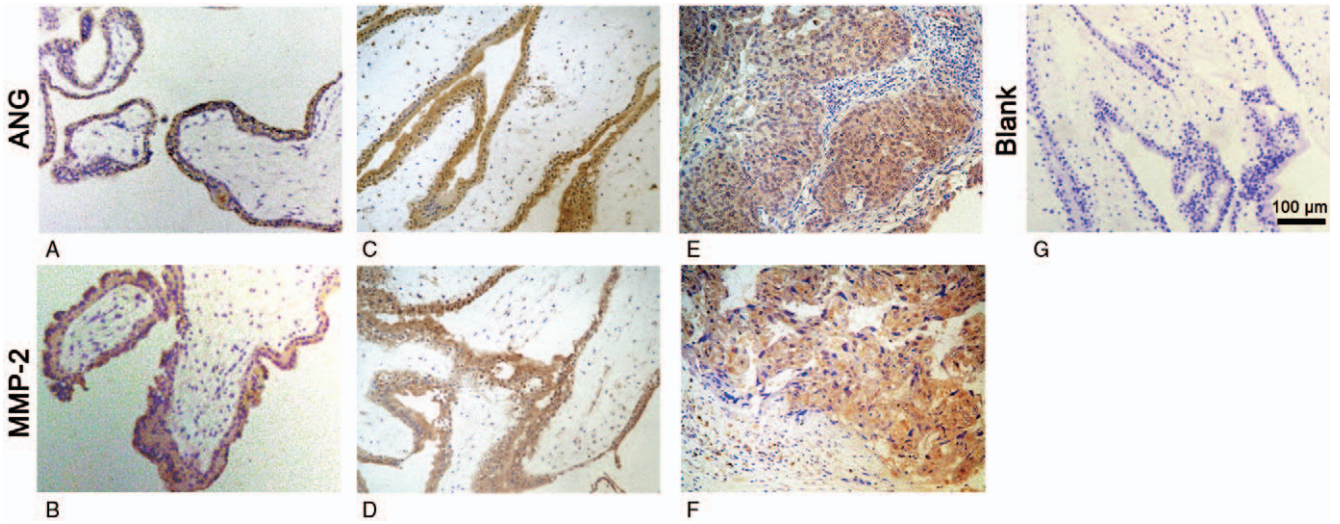

Figure 1. The immunoreactivity of ANG and MMP-2 in normal chorionic villi (A and B, ×100), benign mole (C and D), and gestational trophoblast tumor (E and F, × 200) samples and blank control (G, ×200).

Table 2

The relationship between the expressions of ANG and MMP-2 in NCV, BM, and GTT samples.

	MMP-2											
	NCV				BM				GTT			
ANG	–	+	++	P/r_s	–	+	++	P/r_s	–	+	++	P/r_s
–	10	0	1	0.000/0.725**	0	0	0	0.035/0.401*	2	1	0	0.013/0.606*
+	2	11	0		7	6	3		3	7	1	
++	0	2	2		2	3	7		0	0	2	

**Correlation is significant at the 0.01 level. (2-tailed).

*Correlation is significant at the 0.05 level. (2-tailed)

BM = benign mole, GTT = gestational trophoblast tumor, NCV = normal chorionic villi.

*$P < .05$, significant different.

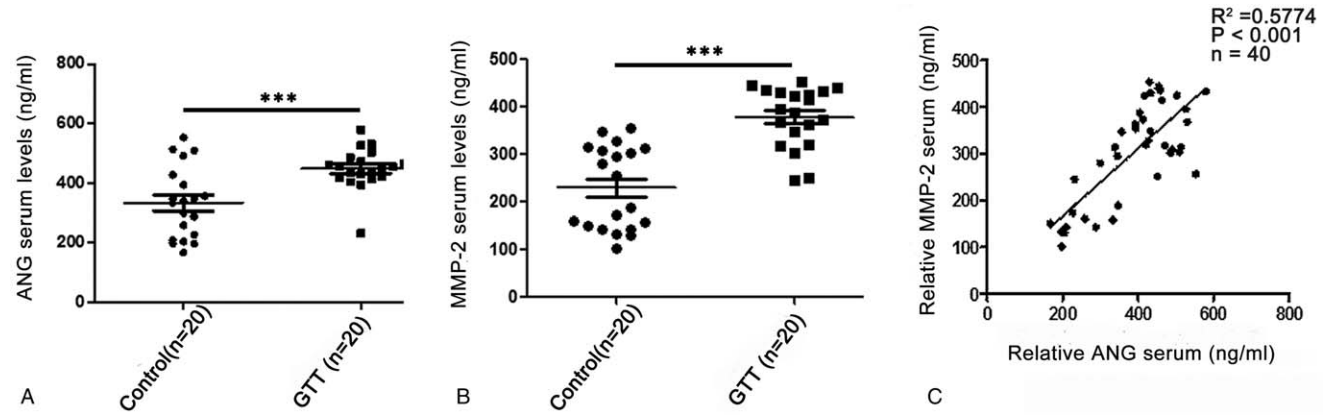

Figure 2. The relationship between the levels of ANG and MMP-2 in the serum of GTD and normal controls. (A and B) The levels of ANG and MMP-2 in the serum of GTD and normal controls were detected by ELISA. All results are representative of 3 independent experiments. The data were reported as the mean ± SEM, n = 3. ***Significant difference, $P < .001$, Student *t* test. (C) The correlation between ANG and MMP-2 levels in the serum from patients with GTD was analyzed using the Pearson correlation and linear regression analysis.

Table 3

The levels of ANG and MMP-2 in the maternal serum of patients with GTD and healthy pregnant women.

Group	Age (year)	Angiogenin (ng/mL)	*P* value	MMP-2 (ng/mL)	*P* value
Control (n = 20)	26.72 ± 0.07	372.65 ± 1.42	<.001	260.60 ± 1.73	<.001
GTD (n = 20)	33.05 ± 0.09	457.88 ± 0.64		388.98 ± 0.66	

than that in MMP-2 (Table 3). Moreover, there was a significant positive correlation between the ANG and MMP-2 levels in the serum of patients with GTD (Fig. 2C).

4. Discussion

Compared with other types of malignant tumors, GTN is biologically and immunologically unique.[29] Altered expression of several growth regulatory factors and oncogenes have been identified to investigate the relationship between the normal placenta and GTN tissues.[30] However, the rarity of GTDs renders incompleteness in the global scan, even with advancements in molecular and pathological research. In this study, we collected the tissue and serum samples of 38 and 20 patients with GTD from Xi-Jing Hospital and Tangdu Hospital, respectively, and analyzed the expression of ANG and MMP-2.

We demonstrated three novel findings in this study. First, there was a significant difference in the ANG and MMP-2 levels of the placenta tissues and maternal serum among normal subjects and patients with GTD diseases. Second, the levels of ANG and MMP-2 were both significantly up-regulated in those with GTD. Lastly, there are various identified GTD risk factors, such as age, prior history of GTD, prior abortion, maternal blood group, and familial history. However, none of these have been experimentally proven due to the condition's rarity.[31] As shown in Table 1, ANG and MMP-2 expression was statistically associated with gestation age, clinical stage, and FIGO stage ($P < .05$). Positive staining of ANG and MMP-2 was more frequently detected in samples of GTD patients with a higher clinical stage (III or IV) as well as advanced FIGO stage, indicating that the expressions of ANG and MMP-2 should be associated with GTD progression of GTD.

ANG is a member of the ribonuclease (RNase) superfamily, which can induce tumor angiogenesis and promote tumor cell survival, growth, and metastasis.[32,33] In a similar previous study, a gestational-dependent increase in ANG expression was observed with the term placenta secreting significantly higher amounts when compared to first-trimester villi.[34] Despite the well-recognized requirement for vascular recruitment and permeability during placentation, little is known about ANG production by trophoblasts and the molecular regulation of its expression in pathological pregnancies. ANG commonly circulates in human serum without a proliferative impact. Using the IHC and ELISA assay, we demonstrated an over-expression of ANG in maternal serum and placental tissues of GTN patients for the first time. Moreover, we demonstrated that ANG localized to the villous syncytiotrophoblast cells in GTD placenta tissues and maternal serum similar to that in normal patients by using IHC. Shaarawy et al found that the serum ANG concentration showed diagnostic and prognostic values for the differential diagnosis of GTD,[18] which was consistent with our results.

In addition to ANG, several angiogenic factors have been reported in the placenta that show differential expression in the GTD placenta, such as vascular endothelial growth factor (VEGF),[35] placental growth factor (PLGF),[36] Mucin 15 (MUC15),[37] insulin-like growth factor-1(IGF-1),[38] and epidermal growth factor receptor (EGFR).[39] Moreover, there is a significant positive correlation between angiogenic factors. For instance, a correlation has been found between VEGF and ANG levels during fetal development and maternal diabetes.[40,41] Based on this premise, ANG could function as a potent vasculogenic inducer in the pregnant uterus, facilitating the dynamic developmental events in this highly specialized organ.

Trophoblasts are invasive because of their ability to secrete MMPs, including MMP-2 (gelatinase A, 72 kDa) and MMP-9 (gelatinase B, 92 kDa), to degrade the decidual extracellular matrix (ECM) and basement membrane components. Adequate invasion of the placental trophoblast cells during placentation ensures a successful pregnancy, while both excessive and inadequate invasion lead to pregnancy complications, such as choriocarcinoma and preeclampsia.[42] Our results showed that MMP-2 was significantly over-expressed in the placental tissues and maternal serum of patients with GTD compared to those of women with normal pregnancies. As reported in our previous study, MMP-2 expression could be regulated by miR-519d-3p, suppressing trophoblast cell invasion and angiogenesis and enhancing cell apoptosis.[43] Here we found that ANG expression was positively correlated with MMP-2 in vivo (Fig. 2C), indicating that ANG may participate in the pathological processes underlying GTD. In bladder cancer, ANG expression resulted in the hypomethylated state of the *MMP-2* gene, which improved *MMP-2* gene expression. ANG and MMP-2 overexpression was correlated with a reduction in disease-free survival.[44] Thus, we surmised that ANG might promote trophoblast cell invasion and migration by regulating MMP-2.

Therefore, our study provides the first evidence for significantly elevated levels of ANG and MMP-2 in the placental tissues and maternal serum of patients with GTD compared with healthy pregnant women. Although we did not find significant differences between these groups due to the limited number of subjects, our results suggest that ANG and MMP-2 might work in coordination in GTD angiogenesis. Further studies with more patients would clarify the relationship between ANG and MMP-2 in the placenta tissues and maternal serum of patients with GTDs.

5. Conclusion

In this study, we identified elevated expressions of ANG and MMP-2 in the placenta tissues and serum of GTD patients, consistent with the higher risk factor levels of GTD, such as age, prior history of GTD, prior abortion, maternal blood group, and familial history. Considering that ANG and MMP-2 overexpression could confirm the existence of vascular reactivity and endothelial disturbance in GTDs, ANG, and MMP-2 in tissues and maternal serum could be used as biomarkers of GTDs. Further studies with more GTDs patients could elucidate the relationship between these vascular abnormalities and GTDs.

Author contributions

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