

Comparison of the clinicopathologic features of solitary and multifocal papillary thyroid carcinoma and the utility of testing an additional tumor nodule for BRAF^{V600E} in multifocal cases

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Abstract

A retrospective review of the tumor focality and BRAF^{V600E} mutational status of all thyroidectomy cases diagnosed with papillary thyroid carcinoma (PTC) between 1/2010 and 7/2013 was performed. A total of 122 cases were included in this study consisting of 65 solitary cases and 57 multifocal cases with a total of 209 tumor nodules. In the multifocal tumors with absent BRAF^{V600E} mutation in the dominant nodule, an additional tumor focus was submitted for testing. The clinicopathologic characteristics of the cases were then compared and analyzed. Significant association with BRAF^{V600E} mutation was driven by previously established unfavorable histologic features such as usual variant, capsular invasion, lymphovascular invasion, extrathyroidal extension, and lymph node metastasis. We did not establish this association with tumor multifocality in this study. Although more cases of mPTC (multifocal papillary thyroid carcinoma) had BRAF^{V600E} mutation in comparison with the solitary cases (56 vs 44%), we did not find a significant difference between the two groups ($p = 0.0689$). Furthermore, at least one of the unfavorable histologic features was also noted in three out of the five multifocal cases with discordant mutations in the two largest nodules. The heterogeneity of BRAF^{V600E} mutational status in mPTC is evident in this study and although it seems prudent to further test additional foci, it appears that in most cases there are already histologic features that support a more aggressive behavior. Selective testing based on histomorphology may be a more practical approach in these cases.

Keywords: papillary thyroid carcinoma; microcarcinoma; solitary; multifocal; BRAF^{V600E}; mutation; dominant nodule; heterogeneity

Introduction

Papillary thyroid carcinoma (PTC) is the most common cancer of the thyroid accounting for more than 80% of all cases among all races. The incidence has been steadily increasing through the years with currently estimated new diagnosis of 60,220 and 1,850 deaths in the United States in 2013 [1]. PTC is often initially diagnosed with the use of fine needle aspiration (FNA) of thyroid nodules. It is also a common incidental finding in many patients undergoing thyroidectomy for non-malignant causes such as multinodular goiter and chronic thyroiditis [2, 3]. The steady increase in the number of cases has also been attributed to the incidental finding of papillary thyroid microcarcinomas (PTMC) in up to 79.3% of cases [3-6]. About 20% are categorized as multifocal PTC (mPTC) with a dominant nodule (> 1 cm) and/or multiple microcarcinomas (≤ 1 cm) [7-9].

Concurrent with these reports is the refinement of the approach to diagnosis and prognosis of PTC with the use of molecular techniques both at the FNA cytology and surgical histology levels. Several genetic alterations have been reported in PTC including RET, NTRK1, RAS and BRAF [10, 11]. BRAF^{V600E} mutation is reported to be the most

prevalent and specific genetic alteration in PTC and has been utilized for both diagnosis and prognosis. When this mutation is present, it has a strong diagnostic value for PTC when cytology yields an indeterminate result [12]. This mutation is particularly associated with more aggressive subtypes especially the tall cell variant. It is also reported to be present in anaplastic carcinoma especially those that arose from PTC. The presence of this mutation is associated with a more aggressive behavior including extrathyroidal

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extension, lymph node metastasis, advance stage, tumor recurrence and reduced sensitivity to radioiodine therapy [13, 14].

At our institution, testing for BRAF^{V600E} mutation is also utilized post-surgically after a definite diagnosis of PTC for the management of patients. We noticed a few cases of mPTC's with aggressive pathologic features such as capsular invasion, lymphovascular invasion, extrathyroidal extension and lymph node metastasis with absent BRAF^{V600E} mutation in the tested dominant nodule. This study therefore aims to determine the rate of BRAF^{V600E} mutation in cases of PTC with multifocal nodules when this mutation is absent in the dominant nodule. The clinicopathological characteristics of the cases are compared with the solitary tumors with and without mutation.

Materials and methods

733 patients underwent thyroid lobectomy, subtotal to total thyroidectomy at Boston Medical Center from 1/2010 to 7/2013. 277 cases were diagnosed with PTC. BRAF^{V600E} assay was performed on 128 cases, 122 of which were included in this study. Six cases were excluded due to insufficient diagnostic material for testing. Results of the testing were abstracted from the records and the corresponding clinicopathologic features recorded. This was done in accordance with the Boston Medical Center Internal Review Board (H-32597). The cases were segregated according to tumor focality and their BRAF^{V600E} mutation status. The number and size of tumor nodules were also recorded. All multifocal tumors with absent mutation in the dominant nodule were reviewed and an additional tumor focus was submitted for BRAF^{V600E} assay if not done previously. Features of aggressiveness including capsular and lymphovascular invasion, distant metastasis and number of regional lymph nodes were then recorded and compared. Fisher's exact test was used to examine the association between the different variables.

Tissue preparation

The tissue was fixed in 10% formalin and paraffin-embedded and thereafter sectioned. Sections were stained for hematoxylin & eosin and submitted for microscopic examination. The diagnosis of PTC was rendered based on the characteristic nuclear features of overlapping, ground-glass nuclei, nuclear pseudoinclusions and nuclear grooves. The cases were further classified according to the diagnosed histologic variant. For our study, cases were grouped into usual, follicular, microcarcinoma and other histologic variants. Tumor nodules ≤ 1 cm were classified as microcarcinoma.

BRAF^{V600E} assay

The block containing the lesion was submitted for BRAF^{V600E} assay. The H&E stained sections were reviewed by the submitting pathologist and the area of the lesion is identified. In specimens with multifocal tumors, the block with the largest nodule was marked and submitted for the assay. Multifocal tumors with absent BRAF^{V600E} mutation in the dominant nodule were reviewed and the second largest tumor focus was submitted for BRAF assay. DNA was extracted by proteinase K digestion and boiling method. DNA was quantified with spectrophotometer and further diluted to different working concentrations for PCR assay.

Allele-specific PCR (AS-PCR) was performed in duplicates with DNA concentrations ranging from 25 to 100 ng per PCR reaction. PCR products were run on a 3% agarose gel and mutation status was compared and assayed with a positive and negative control.

Results

A total of 122 cases of PTC were included in this study (Table 1). The median age was 47 years, range 22-79 and included 97 females and 25 males with females predominating at a ratio of about 4:1. The cases consisted of 65 solitary tumors and 57 mPTC's. The solitary and dominant tumor nodules were classified histologically at 29% (36) usual variant, 31% (37) follicular variant, 38% (47) microcarcinomas and two others consisting of Warthin-like and mixed pattern. The rate of BRAF^{V600E} mutation among all cases was 47% (57). Capsular invasion was identified in 23% (28), lymphovascular invasion in 9% (11), extrathyroidal extension in 14% (17) and lymph node metastasis in 16% (19) cases.

Table 1 Clinicopathological features of cases included in the study, n = 122.

	Number (%)
Age year, median (range)	47 (22-79)
Gender	
Female	97 (80%)
Male	25 (20%)
Tumor focality	
Solitary	65 (53%)
Multifocal	57 (47%)
PTC Variant	
Usual	36 (29%)
Follicular	37 (31%)
Microcarcinoma	47 (38%)
Other	2 (2%)
BRAF ^{V600E} mutation status	
Mutation present	57 (47%)
Mutation absent	65 (53%)
Capsular invasion	28 (23%)
Lymphovascular invasion	11 (9%)
Extrathyroidal extension	17 (14%)
Lymph node metastasis	19 (16%)

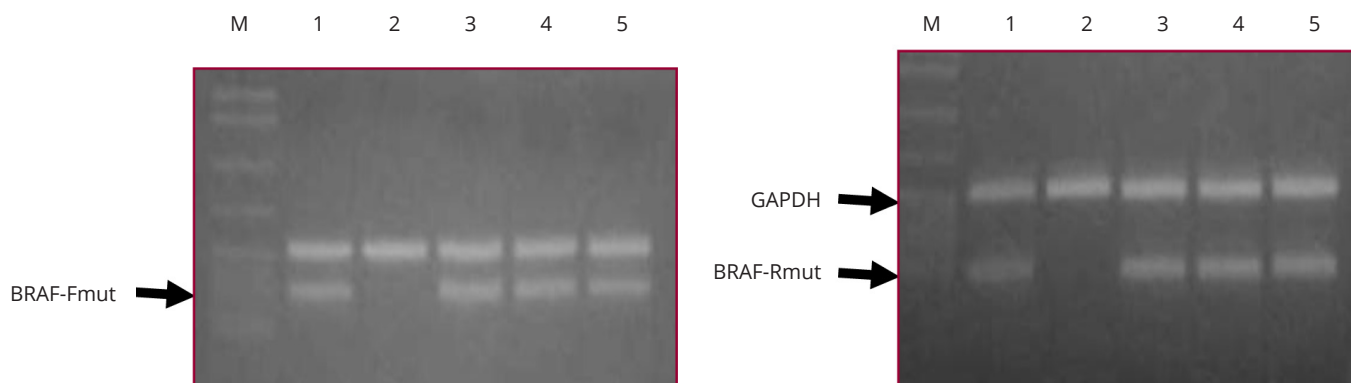
The clinicopathological characteristics between the solitary and multifocal tumors are compared in Table 2. There was no age or gender difference between solitary vs multifocal tumors ($p = 0.2762$ and 0.1780 respectively). Furthermore, there were no significant differences between these two groups based on aggressive histological features such as capsular invasion, lymphovascular invasion, and lymph node metastasis, however extrathyroidal extension was noted to be higher in multifocal tumors ($p = 0.0391$).

Table 2 Comparison of the clinicopathological features between solitary and multifocal PTC cases and tumor nodules.

	Solitary, 65 (53%)	Multifocal, 57 (47%)	p
Age year			
<45	29 (45%)	32 (56%)	0.2762
>45	36 (55%)	25 (44%)	
Gender			
Female	55 (85%)	42 (74%)	0.178
Male	10 (15%)	15 (26%)	
Capsular invasion	13 (20%)	15 (26%)	0.5181
Lymphovascular invasion	3 (5%)	8 (14%)	0.1112
Extrathyroidal extension	5 (8%)	12 (21%)	0.0391
Lymph node metastasis	7 (11%)	12 (21%)	0.1385
Tumor Nodules	Solitary, n = 65	Multifocal, n = 144	
PTC Variant			
Usual	14 (21%)	27 (19%)	0.7073
Follicular	18 (28%)	21 (14%)	0.0341
Microcarcinoma	31 (48%)	96 (67%)	0.014
Other	2 (3%)	0 (0%)	N/A
Nodule size (cm)			
≤ 2	51 (78%)	126 (88%)	0.1008
2.1 - 4	8 (12%)	12 (8%)	0.014
≥ 4.1	6 (10%)	6 (4%)	0.0121

A total of 209 nodules were identified from all cases consisting of 65 solitary nodules and 144 nodules from the cases with mPTC's (Figure 1). They are compared further down in Table 2. The follicular histologic variant of PTC comprised a significantly higher proportion of the solitary cases ($p = 0.0341$). On the other hand, microcarcinomas were identified at a significantly higher rate in multifocal tumors ($p = 0.0140$). Of note was the usual variant which showed no difference in distribution between the two groups ($p = 0.7073$). When they were compared by tumor size driven by AJCC cancer staging (pT1: tumor size 2 cm or less; pT2: tumor more than 2 cm but not more than 4 cm and pT3: tumor more than 4 cm, all limited to the thyroid) [15], a difference was noted only with nodules larger than 2 cm (pT2, $p = 0.0140$) and more than 4 cm (pT3, $p = 0.0121$). Tumor nodules measuring 2 cm or larger were found more frequently in solitary PTC's.

Table 3 compares the cases according to their BRAF^{V600E} mutation status. There was no difference in the rate of BRAF^{V600E} mutation between solitary and multifocal tumors ($p = 0.0689$). Capsular invasion, lymphovascular invasion, extrathyroidal extension and lymph node metastasis were significantly more frequent in cases with BRAF^{V600E} mutation ($p = 0.0045, 0.0234, 0.0029, 0.0004$ respectively). There were no differences noted between the cases with 1, 2 or more than 3 tumor nodules. Among the 209 nodules identified from all cases consisting of 65 solitary nodules and 144 nodules from the cases with mPTC's, a total of 152 tumor nodules were tested for BRAF^{V600E} mutation. When the tumor nodules that were tested were compared according to histologic variant, there was a significant association noted between the usual variant and the

**Figure 1** BRAF^{V600E} AS-PCR result: Left panel, Forward strand; Right panel, Reverse strand. M, DNA size standard marker; lane 1, positive control; lane 2, negative control; lanes 3,4,5, cases with BRAF^{V600E} mutation positive for both.

presence of BRAF^{V600E} mutation ($p = 0.0001$). An inverse relationship was noted with the follicular variant. There was no association noted when the tumor nodules were stratified according to size per pathologic T classification (pT1 vs pT2 vs pT3).

Five out of the 32 multifocal cases were found to have a heterogenous BRAF^{V600E} mutational status after testing the second largest tumor nodule in the cases that showed no mutation in the dominant nodule. Table 4 shows a tabulation of the histopathologic features of these cases. The number of tumor foci ranged from 2 to 5, with four cases arising from the same lobe. The nodule size

tested for BRAF^{V600E} mutation ranged from 0.5 to 3 cm. Three of the five nodules with heterogenous BRAF^{V600E} mutation already displayed at least 2 of the following aggressive histopathologic features: capsular invasion, lymphovascular invasion, extrathyroidal extension and lymph node metastasis. Two cases did not show any of these histopathologic features but the second nodule tested positive for BRAF^{V600E} mutation (Figure 2).

Discussion

BRAF^{V600E} is the most common genetic mutation in PTC and many studies have found association with adverse prognostic features including more aggressive subtypes,

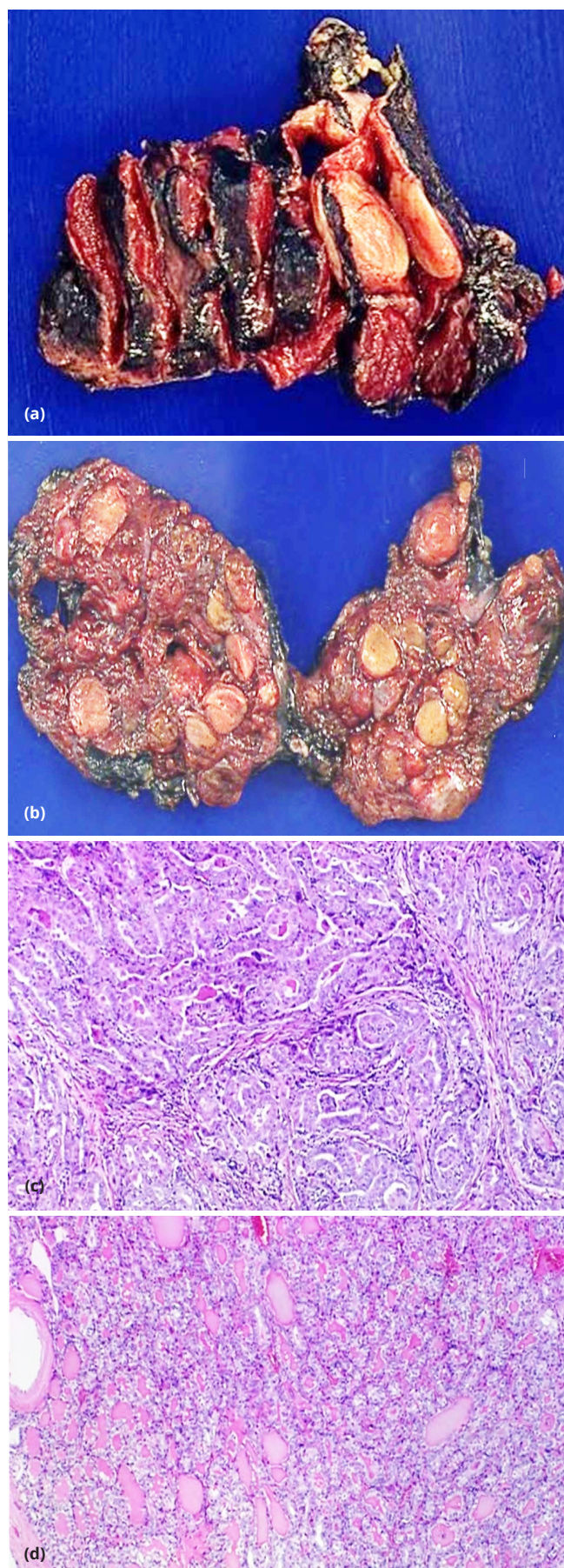
Table 3 Comparison of the histopathologic features of the PTC cases and tumor nodules according to BRAF^{V600E} mutation status.

	Mutation present, n = 57	Mutation absent, n = 65	p
Unifocal (65)	25 (38%)	40 (62%)	0.0689
Multifocal (57)	32 (56%)	25 (44%)	
Capsular invasion	20 (35%)	8 (12%)	0.0045
Lymphovascular invasion	9 (16%)	2 (3%)	0.0234
Extrathyroidal extension	14 (25%)	3 (5%)	0.0029
Lymph node metastasis	16 (28%)	3 (5%)	0.0004
Number of nodules			
1	25 (44%)	40 (62%)	0.0689
2	18 (32%)	17 (26%)	0.5514
≥ 3	14 (24%)	8 (12%)	0.0999
Tumor nodules, n = 152	n = 57	n = 95	
PTC variant			
usual	27 (47%)	11 (12%)	0.0001
follicular	5 (9%)	35 (37%)	0.0001
micro carcinoma	24 (42%)	48 (50%)	0.4017
other	1 (2%)	1 (1%)	N/A
Nodule size (cm)			
≤ 2	49 (86%)	70 (74%)	0.1035
2.1-4	5 (9%)	15 (16%)	0.3216
≥ 4.1	3 (5%)	10 (10%)	0.3726

Table 4 Histopathologic features of the tumor foci with heterogeneous BRAF^{V600E} mutation in multifocal PTC.

Case	#1	#2	#3	#4	#5
Number of tumor foci	5	4	2	3	2
Location	Bilat- eral	Uni- lateral	Uni- lateral	Uni- lateral	Uni- lateral
*Histologic variant	Usual	Usual	Fol- licular	Usual	Micro
Size of the tumor nodule tested (cm)					
Dominant nodule (BRAF ^{V600E} wild type)	3	2.3	1.4	1.5	0.9
Non-dominant nodule (BRAF ^{V600E} mutant)	1.4	0.8	0.5	1.2	0.5
Histopathology					
Capsular invasion	(+)	(-)	(-)	(+)	(-)
Lymphovascular invasion	(-)	(-)	(-)	(+)	(-)
Extrathyroidal extension	(-)	(+)	(-)	(+)	(-)
Lymph node metastasis	(+)	(+)	(-)	(+)	(-)

Note: *Histologic variant of nodules > 1 cm, otherwise classified as microcarcinoma.

**Figure 2** Case 1 showing a solitary nodule in right thyroid lobe (a) with classic PTC (b). Case 2 showing multiple nodules in left thyroid lobe (c) with a follicular variant PTC in the dominant nodule and microcarcinomas in 3 other nodules (d).

extrathyroidal extension, lymph node and distant metastasis, as well as increased recurrence and mortality [16, 17]. Melck and colleagues also demonstrated that cases of PTC with BRAF^{V600E} mutation were more likely to require cervical reoperation compared to those without mutation [18]. The overall rate of BRAF mutation in PTC has been reported up to 83% [19, 20]. The presence of this mutation has also been reported to have a higher incidence in mPTC [21]. Given a significant number of cases has this mutation and the association with adverse features, the possibility of missing the presence of this mutation when testing only the dominant nodule in mPTC's may affect the overall prognosis for these patients.

Most studies report a rate of mPTC's in the range of 20-27% but has been reported to be present in up to 78.1% of patients, usually with a dominant tumor nodule and multiple microcarcinomas [7, 8, 16, 22, 23]. The number of tumor nodules usually ranges from two to six with a mean size of about 1 cm [16, 24]. This study reports a rate of 47% for multifocal cases. And consistent with other reports, majority of the mPTC cases consisted of multiple microcarcinomas and were classified as pT1. The only histologic feature we found to be significantly associated with multifocality was extrathyroidal extension ($p = 0.0391$), however the other features such as capsular invasion, lymphovascular invasion, and lymph node metastasis appeared to be more frequent in mPTC. The increased proportion of follicular variant PTC in the solitary cases in our study may explain why these aggressive features were less frequent in this group. These findings allude to previous reports correlating multifocality with a more aggressive behavior [7, 9, 22, 25]. Various endeavors to elucidate the origin and behavior of mPTC include both independent clonal origin of these tumors as well as intraglandular spread and subsequent clonal evolution to acquire a more aggressive phenotype [4, 11, 24, 26]. Demonstration of the heterogeneity of BRAF^{V600E} mutation in mPTC's also further supports the independent clonal origin of these tumors [18, 26]. These varying propositions on the origin of these tumors can affect our practice of tumor nodule selection for ancillary studies which include BRAF^{V600E} mutation. Would testing the dominant tumor nodule be sufficient in cases of mPTC?

In this study, we report a rate of 56% for BRAF^{V600E} mutation in mPTC. This rate included five cases (9%) with discordant mutational status when the second largest nodule was tested. We note that our rate is lower than reported in other studies which found discordance in up to 40% of the cases [26, 27]. Although more cases of mPTC had BRAF^{V600E} mutation in comparison with the solitary cases (56 vs 44%), we did not find a significant difference between the two groups ($p = 0.0689$). We also did not find a significant association with the number or size of the nodules. Our findings however, are concordant with other studies in showing the landmark histologic features associated with BRAF^{V600E} mutation such as the usual histologic variant, capsular invasion, lymphovascular invasion, extrathyroidal extension, and lymph node metastasis. Furthermore, at least one of these features was also noted in three out of the five multifocal cases with discordant mutations in the two largest nodules. The cases which did not show any of these histologic features were subtyped as follicular

variant and microcarcinomas. On further review of the microcarcinomas, they were determined to demonstrate follicular subtypes.

PTC generally has a good prognosis with a 10-year survival rate of over 90% after appropriate treatment; however, recurrence rate is reported in up to 32% of cases [1, 28]. Disease progression and recurrence have been associated with unfavorable pathologic features such as extrathyroidal extension, lymph node and distant metastasis, advanced pathologic stage (Stage III/IV), as well as the presence of BRAF^{V600E} mutation [29, 30]. There are also reports associating multifocality with a more aggressive behavior [9, 25].

Limitations

Our limitations include those characteristic of a retrospective study. We also did not test all the nodules in the mPTC cohort which probably explains our lower rate compared to other studies. Except for those with discordant mutational status, the microcarcinomas were not further subtyped. Given there are reports associating multifocality with adverse prognosis [9, 25], we recommend continued efforts towards elucidating the adverse features specific to multifocal cases, at both the histologic and molecular levels. Of note, all the cases of follicular variant papillary thyroid carcinoma were classified under the old nomenclature as reported in the original diagnosis. Although, subclassification of encapsulated and non-encapsulated variants was not done, the low incidence of BRAF^{V600E} mutation in this group appears reflective of the new classification and nomenclature.

Conclusions

We showed in this study that the mutational status in the dominant nodule in mPTC's does not necessarily reflect the mutational status of all the tumors in the thyroid including microcarcinomas. It is probably prudent to test the second dominant tumor nodule in mPTC's for BRAF^{V600E} mutation; however the decision must be done in conjunction with other histologic features which are already likely to be present and are predictive of a more aggressive behavior. Selective testing may be a more practical approach in these cases.

Conflicts of interest

The authors declare no conflicts of interest.

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