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# Expression of Budding Uninhibited by Benzimidazole Related-1 and Telomerase in Perilesional Area of Oral Squamous Cell Carcinoma

Dena Nadhim Mohammad

Department of Oral Diagnosis, College of Dentistry, University of Sulaimani, Iraq

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**Corresponding Author:**
**Name: Name:** Dena N. Mohammad
**E-mail:**
dena.mohammad@univsul.edu.iq
**Tel:** 009647701485881
**Affiliation:**
Assist. prof., Oral Diagnosis Department, College of Dentistry, University of Sulaimani, Sulaimani, Kurdistan, Iraq.

**Abstract**

Objective: The premalignant lesions are regarded as intermediate conditions for subsequent cancer development. Even clinically, normal-appearing mucosa could harbor early precancerous genetic mutations. This study determines the immunohistochemical expression of Budding uninhibited by benzimidazole related-1 (BubR1) and Telomerase in the perilesional area of oral squamous cell carcinoma. Materials and methods: The study involved thirty-three formalin-fixed paraffin embedded blocks of the perilesional area of oral cancer. The stain distribution, subcellular localization, and intensity of these two markers in response to their cellular localization were determined and then related to Byrne's grading system. Fisher's exact test and spearman's rho test were applied to analyze the data. $P \leq 0.05$ is considered significant. Results: BubR1 showed prominent, continuous distribution in the full thickness of perilesional samples (54.5%). The subcellular localization of BubR1 was significantly related to Byrne's grading system ($P \leq 0.001$). High discontinuous distribution of telomerase throughout the epithelia was detected (63.6%). Byrne's grading system showed high significant relations to both stain distribution and subcellular localization of telomerase, as P- values were .001 and .002, respectively. Both markers showed high significant full thickness localization of buccal mucosa. Conclusions: Both makers overexpression through multiple layers of oral epithelia in the perilesional area might determine their valuable role in the early detection of oral cancer. Furthermore, the significant mixed subcellular localization of these markers in high-grade cancer could delineate their combined role in developing the second primary tumor.

## Introduction:

Oral squamous cell carcinoma (OSCC) is a commonly detected head and neck cancer. It is most commonly seen in certain parts of the world, with a high mortality rate. Early detection of oral cancer is an essential factor for a good prognosis and increased survival rate. Even though the oral cavity can be easily examined clinically, most OSCC patients cannot be identified early. This could be attributed to that patients do not seek dental care regularly, and most lesions are passed undetectable in their early stage [(1)]. The premalignant lesions and conditions are considered intermediate lesions for subsequent cancer development. Even clinically, normal-appearing mucosa could harbor early precancerous genetic mutations [(2).] The cells in the field of cancerization have gained some of the phenotypic alterations required for malignancy that are associated with underlying mutations of the related genes. These changes could include an increased growth rate, decreased death rate, or increased immune evasion., then passing through multiple phenotype states will lead to oral cancer [(3).] Substantial genetic or epigenetic changes in epithelial cells of the field of cancer might lead to cancer. Such changes can occur without any associated histological abnormalities and at a substantial distance (hundreds/thousands of cells) from the original tumor [(4).] Telomerase has two different subunits; a functional catalytic protein subunit of human telomerase reverse transcriptase (hTERT) encoded by the TERT gene, located at chromosome 5p15.33; and an RNA component known as human telomerase RNA component (hTERC or hTR), encoded by the TERC gene on chromosomal region 3q26 [(5,6).] OSCC showed increased telomerase activity compared to the oral potentially malignant lesions [(7)]. An essential factor in the spindle assembly checkpoint function is BUbR1. This checkpoint protein directs proper attachment of microtubules to kinetochores and regulates chromosome-spindle attachment checkpoint signaling [(8,9).] The hBUB1B gene, which encodes BUbR1, is located on human chromosome 15q14-21, which has a high rate of loss of heterozygosis and is related to many tumors, which are colon, urinary bladder, breast, lung, and head, and neck carcinomas [(10).] The BUbR1 showed high expression in the superficial invasive islands than the deep ones and indicated that this marker overexpression is associated with an early invasion of OSCC [(11).] Loss of heterozygosity at the chromosomes 3p and 9p detected in keratinocytes of premalignant lesions of the upper aerodigestive tract. That is associated with a 3.8-fold risk of malignant transformation. Later on, additional subsequent losses at any of the chromosomes 4q, 8p, 11q, and 17p had a 33-fold increased risk of carcinomatous change [(12).] Previous studies evaluated the immunohistochemical expression of adhesion markers (P-cadherin and B-catenin) in normal-appearing epithelium adjacent to OSCC [( 13,14).] This is the first study that determines the role of regulatory checkpoint protein of mitotic spindle apparatus (BUbR1) and telomerase activity in phenotypic normal apparent epithelial cells adjacent to OSCC to identify the early molecular alteration in the cancerized field.

## Materials and methods

### Study sample

**Thirty-three** formalin-fixed archived paraffin-embedded blocks of histologically normal epithelia of the perilesional area from different oral sites (buccal mucosa, tongue, lip, and floor of mouth) of OSCC graded by Byrne's grading system as (15 WDSCC,15 MDSCC,3 PDSCC). The samples were obtained from the histopathological lab of the College of Dentistry, University of Sulaimani**.** The present study was approved by the Committee of Ethics of the College of Dentistry and Medical Science, the University of Sulaimani, Sulaimani, Iraq (cod number: 153). This research was conducted between October 2019 to April 2020.

**Tissue preparation and evaluation**

Three serial 5µm tissue sections cut from each block. One was stained with Hematoxylin and Eosin to verify the perilesional area, and the other two sections were stained immunohistochemically with the two antibodies. Biotin free immunoenzymatic antigen detection system (expose mouse/rabbit specific HRP/DAB micro polymer detection (abcam®, UK) used. Sections were deparaffinized and rehydrated. Antigens retrieval was achieved by boiling the sections in citrate buffer for 15 min (pH-6), then allowed to cool at room temperature. Endogenous peroxidase blocking was accomplished by hydrogen peroxidase for 10 min, and then protein block was added to the sections (10 min). Sections incubated with mouse monoclonal BubR1 and rabbit polyclonal anti-Telomerase reverse transcriptase-(dilution 1:200, Abcam; UK) for 45 min and then washed with PBS four times. Complement was applied to the sections and incubated for 10 min. The secondary antibody conjugate was used for 15 min and washed. Sections were stained by DAB in the dark for 5min. Lastly, hematoxylin was used as a counterstain for 25 sec. Then sections were dehydrated, cleared, and mounted with DPX and evaluated. Normal human tonsil was used as a positive control for telomerase, while normal spleen tissue for BubR1 according to manufacturer protocol. The negative controls for both antibodies were done by omitting the primary antibody and applying antibody diluents alone. The evaluation of staining was done according to the following parameters ( Pyo et al. 2007) [15]:

1. Localization of staining (basal and parabasal layer) or (full thickness) expression with (continuous or discontinuous) distribution throughout the epithelia of the perilesional area.
2. Sub-cellular localization (nuclear, cytoplasmic, mixed).
3. Satin intensity (faint, moderate, strong).

**Statistical analysis**

The data were analyzed by the SPSS version 16.0 software program. Fisher's exact test and spearman's rho test were used to analyze, compare, and correlate the data. $P \leq 0.05$ considered being significant.

## Results:

The demographic data of archived cases were shown in Table (1)**.** All the studied samples of the perilesional area revealed positive expression for both BubR1 and telomerase. BubR1 showed continuous full-thickness localization in 18 cases (54 .5%) of the sample, while 6 cases (18.1%) had discontinuous basal and parabasal localization Table (2). A significant difference was reported between the stain distribution and localization in different layers (P=.005). Cytoplasmic subcellular localization within full thickness was predominant (36.3%) Fig. (1,A), followed by mixed localization (27.2%) Fig. (1,B). On the other hand, in basal and parabasal localization, three cases of both cytoplasmic and mixed subcellular localization were seen Fig.(1 C,D). Still, no significant differences were detected regarding both subcellular localization and intensity in response to localization of staining, as shown in Table (2). Byrne's grading system illustrated a high significant difference in the subcellular localization of BubR1 ($P \leq 0.001$) Table (3). Telomerase showed a discontinuous distribution of staining in both basal parabasal and full-thickness localizations, which were 9.09% and 63.6%, respectively, as seen in Table (4). The basal and parabasal layers revealed nuclear subcellular localization in three cases Table (4), Fig.(1,E). While full thickness showed high mixed subcellular localization 54.5% Fig.(1,F). There was a high significant relation of subcellular localization to staining localization of telomerase in the perilesional area ($P \leq 0.001$) Table (4). Finally, both staining distribution and subcellular localization of telomerase showed high significant relations to Byrne's grading system, as P-

values were .001 and .002, respectively, as shown in Table (5).
BubR1 and telomerase expressed prominent full-thickness localizations in buccal mucosa and tongue perilesional areas, with significant relations as P-values were .01 and .007, respectively. Furthermore, the buccal mucosa revealed a significant continuous distribution of BubR1 (36.3%), while the discontinuous distribution of telomerase in tongue and buccal mucosa (27.2%) Table (6). Lastly, both markers (BubR1and telomerase) showed statistical significance regarding their localization of staining in different layers of epithelia (P≤0.001 ).

## Discussion:

Alterations of biomarkers in the genetically transformed with histologically normal cells of the peritumor field could aid in the early detection of cancer. Furthermore, these markers give way to monitoring the adjacent tumor progression and or preventing the transformation of premalignant lesions into invasive cancers [16]. In head and neck tumors, the field of cancerization is initiated either by molecular events affecting many cells from different sites simultaneously or by a single clonal progenitor that is associated with widespread clonal expansion or lateral spread [17]. The markers used in the evaluation of the field of cancerization should have the ability to be detected within the early stage of precancerous lesions and conserved during the progression of these lesions. The genetic mutations that appeared in the field of cancerization are; loss of heterozygosity (LOH), microsatellite instability, chromosomal alterations, p53 gene mutation, which are identified by a polymerase chain reaction, Immunohistochemistry, and in situ hybridization [18,19]. BubR1 showed expression in all studied perilesional samples, with prominent cytoplasmic subcellular localization. Lira et al., in their study found high cytoplasmic expression of BubR1 in invasive OSCC (70%), with low expression in nonmalignant oral samples [20]. Other study showed a significant increase in BubR1 expression with the advanced grade of both oral dysplastic and cancerous lesions [9]. The high expression of BubR1 in the present study indicated the role of this marker in the identification of early chromosomal instability in altered epithelia adjacent to OSCC. Furthermore, the significant continuous distribution of this marker in the full thickness of the perilesional area demarcated that the patches or a small group of cells could share contiguous common genetic alterations at the same time; later on, subsequent genetic changes lead to clonal expansion [19]. All perilesional samples had telomerase expression, with predominant full-thickness layer localization. While Raghunandan et al. 2016 found a lower expression of telomerase in full-thickness epithelia of their oral dysplastic samples (38.09%), with a dramatic increase in scoring mean of telomerase in advancing from normal oral mucosa to oral dysplasia and OSCC [21]. Overexpression of telomerase in OSCC was also detected by other studies, which were 89%,74%,75% [5,22,23]. Commonly telomerase activity was not observed in most adult tissues except the stem cells of proliferative tissues. While human malignancies revealed 80-90% of telomerase expression, so this high detection in the perilesional area of this study could be a useful diagnostic tool for early detection of carcinogenesis [22,23]. The present study illustrated significantly high mixed subcellular localization of telomerase in the full thickness of the epithelium. Raghunandan et al., 2016. in their research identified high nuclear localization in both oral dysplastic and OSCC samples [21].

In this study, the subcellular localization in the cytoplasm could represent phosphorylated and inactive forms of the protein, which later on transferred to the nucleus and promote the unlimited proliferation ability of suprabasal cell layers of the epithelium in the field of cancerization [24]. The subcellular localization of BubR1 and telomerase in the perilesional area were significantly related to the high grade of adjacent OSCC, which indicated that loss

of differentiation in the adjacent original tumor might give a chance for the development of a second field tumor that arises from the same field of the original tumor and share identical genetic mutations [25]. Full-thickness localizations of BubR1 and telomerase in buccal mucosa and tongue could be attributed that these sites were more commonly exposed to irritation factors that were associated with subsequent genetic alterations. Accordingly, the planning of site-specific strategies of prevention, diagnosis, and treatment are needed. Finally, the significant correlations between telomerase and BubR1 localization of staining within different layers of epithelia might suggest that multiple gene mutations are needed for initiation and then the further progression of the carcinogenesis in the perilesional area adjacent to OSCC. Some possible limitations may be highlighted. First, the selected archived samples underwent various fixation procedures affecting the antigen expression despite the retrieval step. Besides, these samples represent different intraoral locations that some of which may be more aggressive. Second, the literature of previous studies is limited and did not provide the foundations for comparison. Therefore, future studies are needed for confirming and identifying molecular alterations in the perilesional region of malignant and premalignant surgical samples.

## Conclusion

The expression of BubR1 and telomerase suprabasally in the perilesional area of OSCC determine their combined role in the early identification of oral cancer. The mixed subcellular localization of these two markers might clarify those multiple genetic aberrations are required in the field of cancerization for the development and progression of oral carcinogenesis.

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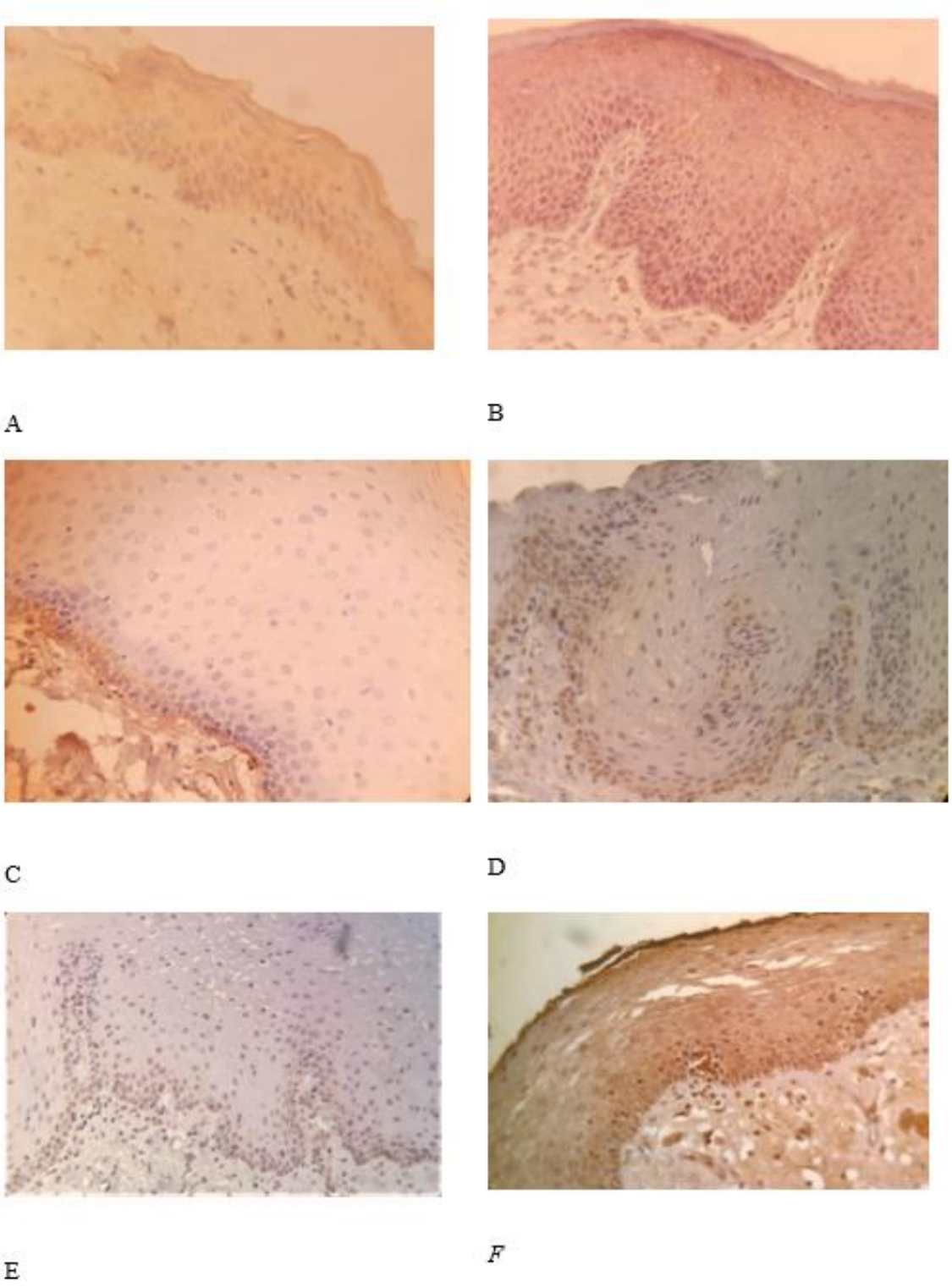


Fig.(1):- BubR1 and telomerase expression in perilesional area of OSCC. A-BubR1 cytoplasmic full thickness localization .B-BubR1 mixed full thickens localization. C-BubR1 cytoplasmic basal and parabasal localization D- BubR mixed basal and parabasl localization E-Telomerase nuclear basal and parabasal localization . F- Telomerase mixed full thickness localization.

Table (1): Demographic distribution of the sampl

| | | No | % |
|---|---|---|---|
| Sex | Male | 24 | 72.72 |
| | Female | 9 | 27.27 |
| | Total | 33 | 100 |
| Site of the lesion | Buccal mucosa | 15 | 45.45 |
| | Tongue | 9 | 27.27 |
| | lip | 6 | 18.18 |
| | Floor of the mouth | 3 | 9.09 |
| | Total | 33 | 100 |

Table (2): BubR 1 expression in perilesional area of 33 cases, regarding staining distribution, subcellular localization and intensity

| | | Cellular localization | | | | P-value |
|---|---|---|---|---|---|---|
| | | Basal and parabasal | | Full thickness | | |
| | | No | % | No | % | |
| Stain distribution | Continuous | 0 | 0 | 18 | 54.54 | .005 |
| | Discontinues | 6 | 18.18 | 9 | 27.27 | |
| Subcellular localization | Nuclear | 0 | 0 | 6 | 18.18 | .61 |
| | Cytoplasmic | 3 | 9.09 | 12 | 36.36 | |
| | Mixed | 3 | 9.09 | 9 | 27.27 | |
| Intensity | Faint | 3 | 9.09 | 9 | 27.27 | .13 |
| | Moderate | 3 | 9.09 | 6 | 18.18 | |
| | Strong | 0 | 0 | 12 | 36.36 | |

Table (3): BubR 1 cellular localization, stain distribution and subcellular localization in relations to Byrne's grading system.

| | | Well (15) | | Moderate (15) | | Poor (3) | | P-value |
|---|---|---|---|---|---|---|---|---|
| | | No | % | No | % | No | % | |
| Cellular localization | Basal and parabasl | 3 | 20 | 3 | 20 | 0 | 0 | 1.0 |
| | Full thickness | 12 | 80 | 12 | 80 | 3 | 100 | |
| Stain distribution | Continuous | 9 | 60 | 6 | 40 | 3 | 100 | .22 |
| | Discontinuous | 6 | 40 | 9 | 60 | 0 | 0 | |
| Sub-cellular localization | Nuclear | 3 | 20 | 3 | 20 | 0 | 0 | .000 |
| | Cytoplasmic | 12 | 80 | 3 | 20 | 0 | 0 | |
| | Mixed | 0 | 0 | 9 | 60 | 3 | 100 | |

Table (4): Telomerase expression in 33 perilesional regions of OSCC in response to stain distribution, subcellular localization and intensity.

| | | Cellular localization | | | | P-value |
|---|---|---|---|---|---|---|
| | | Basal and parabasal | | Full thickness | | |
| | | No | % | No | % | |
| Stain distribution | Continuous | 0 | 0 | 9 | 27.27 | .54 |
| | Discontinuous | 3 | 9.09 | 21 | 63.63 | |
| Subcellular localization | Nuclear | 3 | 9.09 | 0 | 0 | .000 |
| | Cytoplasmic | 0 | 0 | 12 | 36.36 | |
| | Mixed | 0 | 0 | 18 | 54.54 | |
| Intensity | Faint | 0 | 0 | 15 | 45.45 | .03 |
| | Moderate | 3 | 9.09 | 6 | 18.18 | |
| | Strong | 0 | 0 | 9 | 27.27 | |

Table (5): Telomerase relations of cellular localization, distribution and subcellular localization to Byrne's grade system

| | | Well (15) | | Moderate (15) | | Poor (3) | | P-value |
|---|---|---|---|---|---|---|---|---|
| | | No | % | No | % | No | % | |
| Cellular localization | Basal and parabasl | 3 | 20 | 0 | 0 | 0 | 0 | .29 |
| | Full thickness | 12 | 80 | 15 | 100 | 3 | 100 | |
| Stain distribution | Continuous | 0 | 0 | 6 | 40 | 3 | 100 | .001 |
| | Discontinuous | 15 | 100 | 9 | 60 | 0 | 0 | |
| Subcellular localization | Nuclear | 3 | 20 | 0 | 0 | 0 | 0 | .002 |
| | Cytoplasmic | 9 | 60 | 3 | 20 | 0 | 0 | |
| | Mixed | 3 | 20 | 12 | 80 | 3 | 100 | |

Table (6): BubR 1 and telomerase cellular localization and distribution in response to different oral sites of the sample

| | | | Oral sites | | | | | | | | P-value |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Tongue | | Buccal mucosa | | Floor of mouth | | Lip | | |
| | | | No | % | N | % | No | % | No | % | |
| BubR 1 | Cellular localization | Basal and parabasal | 3 | 9.09 | 0 | 0 | 0 | 0 | 3 | 9.09 | .01 |
| | | Full thickness | 6 | 18.18 | 15 | 45.45 | 3 | 9.09 | 3 | 9.09 | |
| | Cellular distribution | Continuous | 3 | 9.09 | 12 | 36.36 | 3 | 9.09 | 0 | 0 | .001 |
| | | Discontinuous | 6 | 18.18 | 3 | 9.09 | 0 | 0 | 6 | 18.18 | |
| Telomerase | Cellular localization | Basal and parabasal | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 9.09 | .007 |
| | | Full thickness | 9 | 27.27 | 15 | 45.45 | 3 | 9.09 | 3 | 9.09 | |
| | Cellular distribution | Continuous | 0 | 0 | 6 | 18.18 | 3 | 9.09 | 0 | 0 | .001 |
| | | Discontinuous | 9 | 27.27 | 9 | 27.27 | 0 | 0 | 6 | 18.18 | |

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