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RESEARCH REPORT

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Lack of association between *LGMN* and Alzheimer's disease in the Southern Han Chinese population

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Abstract

Recently, functional studies have demonstrated that legumain (LGMN) cleaves both amyloid β-protein precursor and tau, promoting senile plaques and formation of neurofibrillary tangles, which may play a crucial role in the pathogenesis of Alzheimer's disease (AD). However, the genetic role of *LGMN* in AD has not been clearly elucidated. Here, we used Sanger sequencing to investigate the single independent (single-variant association test) and cumulative (gene-based association test) effects of variants in the *LGMN* gene as potential susceptibility factors for AD, in a cohort comprising 676 AD cases and 365 elderly controls from the Han population of South China. In single-variant association analysis, none of the common variants in *LGMN* were statistically significant. In gene-based analysis, the *LGMN* gene also showed no association with AD. The results of our replication study in the Alzheimer's Disease Neuroimaging Initiative cohort also showed no association between *LGMN* and AD. These findings suggest that the *LGMN* gene may not be a critical factor for AD development.

KEYWORDS
genetics, risk factor, single nucleotide polymorphism, SKAT-O, δ-secretase

Abbreviations: AD, Alzheimer's disease; ADNI, Alzheimer's Disease Neuroimaging Initiative; APOE, apolipoprotein E; APP, amyloid β-protein precursor; Aβ, amyloid-β; CI, confidence interval; EDTA, ethylenediaminetetraacetic acid; EOAD, early-onset Alzheimer's disease; HWE, Hardy–Weinberg equilibrium; LGMN, legumain; LOAD, late-onset Alzheimer's disease; MAF, minor allele frequency; NCs, normal controls; NFTs, neurofibrillary tangles; OR, odds ratio; PCR, polymerase chain reaction; SKAT-O, sequence kernel association test-optimal; SNPs, single-nucleotide polymorphisms; WGS, whole-genome sequencing.

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1 | INTRODUCTION

As the most common neurodegenerative disorder, Alzheimer's disease (AD) has garnered increasing attention. It is characterized by memory loss and executive dysfunction and significantly interferes with daily life activities in the late stage (Scheltens et al., 2016). Amyloid plaques and neurofibrillary tangles (NFTs) are two major pathological features of AD (Kozlov, Afonin, Evsyukov, & Bondarenko, 2017). Amyloid plaques are principally composed of abnormally folded amyloid-β (Aβ) 40 and Aβ42, the most abundant forms of Aβ. Aβ is generated from amyloid-β protein precursor (APP) by β-secretase and then γ-secretase in the amyloidogenic pathway (Thinakaran & Koo, 2008; O'Brien & Wong, 2011; Tiwari, Atluri, Kaushik, Yndart, & Nair, 2019). Studies have shown that Aβ plays a critical role in the initiation of AD pathology (Hardy & Higgins, 1992; Selkoe & Hardy, 2016). In addition, Aβ oligomers also induce abnormal tau hyperphosphorylation, which promotes the formation of NFTs (Fan et al., 2019).

Although the etiology of late-onset sporadic AD remains unclear, a growing body of evidence suggests that a large proportion of these cases are significantly influenced by genetic factors. Genome-wide association studies and next-generation sequencing have identified many susceptibility loci associated with AD (Harold et al., 2009; Lambert et al., 2009, 2013; Seshadri et al., 2010; Hollingworth et al., 2011; Jonsson et al., 2013; Cruchaga et al., 2014; Vardarajan et al., 2015; Sassi et al., 2016). *LGMN* maps to chromosome 14q32.12 and encodes the age-dependent, pH-controlled lysosomal cysteine protease, legumain, also known as asparagine endopeptidase or δ-secretase (Zhang et al., 2015; Gao et al., 2018). *LGMN* was found to be casually associated with late-onset AD (LOAD) after adjusting for apolipoprotein E (*APOE*) in Caribbean Hispanic families (Vardarajan et al., 2018). In addition, cleavage of APP and tau by legumain has been shown to be involved in the pathological processes underlying AD. APP is cleaved by δ-secretase at two major sites, N373 and N585 residues; cleavage at N585 increases Aβ level by enhancing β-secretase 1-mediated APP processing. In addition, a study showed that knockout of δ-secretase in 5XFAD and APP/PS1 mouse models led to a reduction in senile plaque formation (Zhang et al., 2015). Moreover, it has also been reported that tau cleavage by δ-secretase inhibits its microtubule polymerization and promotes neurotoxicity (Zhang et al., 2014). Subsequently, it was shown that an oral small molecule inhibitor of δ-secretase identified through high-throughput screening abolished AD pathologies in P301S and 5XFAD transgenic mice, resulting in cognitive function improvement (Zhang et al., 2017).

To the best of our knowledge, no study has determined whether there is a genetic association between *LGMN* and AD in Chinese populations. Thus, we conducted a mutation screening study using Sanger sequencing to determine the correlation between *LGMN* and AD in southern Han Chinese. We also performed a replication study in the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort.

2 | MATERIALS AND METHODS

2.1 | Study subjects

A total of 676 Han Chinese AD patients (256 males, 420 females) were recruited from the Department of Neurology, Xiangya Hospital, Central South University (Hunan, China). All patients underwent a detailed neurologic examination by two experienced neurologists and met the National Institute of Neurological and Communicative Disorders and Stroke and Alzheimer's Disease and Related Disorders Association criteria (McKhann et al., 1984) for probable or definite AD. The patients had a mean age at onset of 66.07 ± 10.69 years (range, 33 to 91 years). Among the 676 AD patients, 88 (13.02%) had a positive family history and the remaining 588 (86.98%) were sporadic cases. In addition, 315 (46.60%) of the 676 patients were diagnosed with early-onset AD (EOAD, age ≤ 65) and 361 (53.40%) had LOAD (age > 65). No pathogenic mutation in *APP, presenilin 1,* or *presenilin 2* was reported in this cohort. In addition, 365 unaffected community-dwelling individuals without symptoms of AD were recruited as healthy controls and matched for ethnicity and area of residence (175 males and 190 females, mean age 70.65 ± 5.65 years, range 63–87 years). The study was approved by the Ethics Committee of Xiangya Hospital, Central South University (institutional review board equivalent). Written informed consent was obtained from all participants involved in the study. The demographic and clinical information of all subjects is presented in Table 1.

2.2 | Genetic testing

Blood samples (10 ml per subject) were obtained by venipuncture from each subject and transferred to ethylenediaminetetraacetic acid (EDTA) tubes. Genomic DNA was isolated from peripheral blood leukocytes using a standard protocol (Jiao *et al*., 2014; Jiao *et al*., 2015). All isolated DNA samples were measured for quality and quantity by a fluorometer and normalized to 50 ng/μL. Polymerase chain reaction (PCR) was performed on the exonic regions and exon-intron boundaries of *LGMN* (NM_005606). Each PCR fragment was sequenced from both directions on the ABI 3730xl Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). Primers for *LGMN* (exons 1–14) and *APOE* genotypes amplification were designed according to the GenBank entries (Table S1). Sequencher software was used

TABLE 1 Clinical and demographic data of 676 AD cases and 365 NCs

	AD cases ($n = 676$)	Control individuals ($n = 365$)	P
Age (year)	66.07 ± 10.69	70.65 ± 5.65	$p < 0.001$
Gender (% male)	37.87	47.95	$p = 0.002$
Education (year)	7.83 ± 4.29	10.02 ± 2.85	$p < 0.001$
Disease duration (y)	3.41 ± 2.61	-	
MMSE (score)	10.89 ± 7.19	27.35 ± 3.01	$p < 0.001$
MoCA (score)	7.3 ± 5.45	-	
CDR (score)	1.34 ± 0.72	-	
ADL (score)	35.21 ± 13.84	-	
NPI (score)	15.61 ± 16.38	-	
Hachinski (score)	2.07 ± 1.19	-	
HAMD (score)	5.44 ± 5.22	-	

Note: MMSE, Mini-Mental State Examination; MoCA, Montreal Cognitive Assessment; CDR, Clinical Dementia Rating; ADL, Activities of Daily Living; NPI, Neuropsychiatric Inventory; Hachinski, Hachinski Ischemic Scale; HAMD, Hamilton Depression Scale. All of them are neuropsychological assessment scales.

for DNA sequences analysis. Variants were checked against established databases (Genome Aggregation Database [gnomAD] and dbSNP v.150). The effects of missense variants on the function of proteins were predicted by SIFT (Ng & Henikoff, 2001), PolyPhen-2 (Adzhubei et al., 2010), and 12 other related silico tools.

2.3 | ADNI dataset

To perform the replication study in an independent cohort, we obtained whole-genome sequencing (WGS) data from the ADNI database (adni.loni.usc.edu). Participants enrolled in the ADNI were from North America. Our ADNI cohort consisted of 247 patients with AD and 243 normal controls (NCs). Variants with read depth ≥ 5 and allele depth ≥ 2 were retained for subsequent analysis. For the single-variant association test, only variants with an MAF ≥ 0.01 were considered, whereas variants with an MAF < 0.01 were used for the gene-based association test.

2.4 | Statistical analysis

Descriptive statistics are presented as the mean ± standard deviation. The Mann–Whitney U test was used to compare age, gender, education, and Mini-Mental State Examination between the AD group and the control group. Comparisons of allele and genotype frequency distributions of *APOE* between the two groups were performed with the chi-square test. Single-nucleotide polymorphisms (SNPs) with a P value less than 0.0001 were considered to be inconsistent with Hardy–Weinberg equilibrium (HWE). According to the minor allele frequency (MAF) in the controls, the variations were classified into common variations (0.01 ≤ MAF ≤0.5) and rare variations (0 < MAF <0.01). For common variants, allele frequencies were calculated in cases and controls, and the chi-square test on allelic association was performed. Logistic regression analysis corrected for sex, age, and *APOE ε4* status (*APOE ε4+, APOE ε4-*) was also undertaken. Regarding the rare variations, we collapsed all rare variants together, and the sequence kernel association test-optimal (SKAT-O) test (Lee, Wu, & Lin, 2012) was carried out with adjustment for three covariates (sex, age, and *APOE ε4* status) to study their cumulative effect on the AD trait. The analysis was further stratified based on age at onset (≤ 65 or > 65 years) and presence or absence of *APOE ε4* alleles. Bonferroni correction was performed to correct for multiple comparisons of our cohort and the ADNI cohort. All computations were performed in PLINK 1.9. $p < 0.05$ was considered statistically significant.

TABLE 2 The distribution of APOE alleles and genotypes in AD cases and NCs

APOE	Case ($n = 676$,%)	Control ($n = 365$,%)	P
ε2	54 (4.0)	64 (8.8)	<0.001
ε3	939 (69.5)	591 (81.0)	<0.001
ε4	359 (26.5)	75 (10.2)	<0.001
ε2/2	1 (0.1)	2 (0.5)	0.283
ε2/3	41 (6.1)	54 (14.8)	<0.001
ε2/4	11 (1.6)	6 (1.6)	0.984
ε3/3	339 (50.2)	237 (65.0)	<0.001
ε3/4	220 (32.5)	63 (17.3)	<0.001
ε4/4	64 (9.5)	3 (0.8)	<0.001

3 | RESULTS

3.1 | APOE allele frequencies and genotypes

The distribution of *APOE* allele frequencies and genotypes of all participants is presented in Table 2. Consistent with previously reported results, the frequencies of the *APOE* ε 4 allele were significantly higher in AD cases than in the NCs, and the frequencies of the ε2 allele and ε3 allele were significantly lower in AD cases than in the NCs. In terms of genotypes, the frequencies of *APOE* ε3/4 and ε4/4 genotypes

TABLE 3 Association analysis of common variants in *LGMN* in AD cases and NCs (0.01 ≤ MAF≤0.5)

Gene	Position	SNP	Nucleotide change	AA change	MAF		P_value	Corrected P	OR (95% CI)
					case	control			
LGMN	Chr14:93,170,741	rs2250672	g.93170741T > C [a]	-	0.460	0.455	0.7437	0.5102	1.031 (0.860–1.235)
	Chr14:93,170,901	rs2402189	g.93170901A > C,[a]	-	0.233	0.247	0.2974	0.2563	0.894 (0.724–1.104)
	Chr14:93,170,993	rs9791	c.1251G > A [b]	p.P417P	0.064	0.059	0.4950	0.4308	1.139 (0.783–1.658)
	Chr14:93,172,735	rs17128499	g.93172735C > A [a]	-	0.133	0.134	0.8979	0.8606	0.983 (0.754–1.281)
	Chr14:93,173,010	rs17128502	g.93173010C > T [a]	-	0.115	0.101	0.1580	0.2061	1.232 (0.922–1.647)
	Chr14:93,175,973	rs2273922	g.93175973G > A [a]	-	0.020	0.012	0.0595	0.0792	2.024 (0.958–4.275)
	Chr14:93,180,727	rs59223493	c.474T > C [b]	p.N158N	0.153	0.170	0.1212	0.2038	0.824 (0.644–1.653)
	Chr14:93,182,466	rs1242119	g.93182466G > A [a]	-	0.085	0.077	0.3458	0.3374	1.172 (0.842–1.632)
	Chr14:93,183,718	rs2295986	g.93183718C > T [a]	-	0.133	0.134	0.9056	0.8932	0.984 (0.755–1.282)
	Chr14:93,185,129	rs118128989	c.199G > A [b]	p.V67I	0.034	0.034	0.9787	0.8563	0.993 (0.605–1.630)
	Chr14:93,198,972	rs12885208	g.93198972A > T [a]	-	0.031	0.036	0.4536	0.6279	0.826 (0.500–1.364)
	Chr14:93,199,080	rs2236264	c.52G > A [b]	p.V18I	0.233	0.233	0.9954	0.8664	1.001 (0.809–1.238)
	Chr14:93,199,203	rs1613441	g.93199203T > A [a]	-	0.108	0.100	0.4116	0.5425	1.132 (0.842–1.520)
	Chr14:93,207,541	rs2025058	g.93207541A > G [a]	-	0.017	0.018	0.8749	0.7642	0.945 (0.471–1.900)

Note: MAF, minor allele frequency; OR, odds ratio; Corrected P, adjustment for age and gender and *APOE ε4*.

[a]NC_000014.8 version.

[b]NM_005606 version.

TABLE 4 Rare variants in the *LGMN* gene in the Chinese AD cohort

Gene	Position	cDNA change	AA change	Mutation type	MAF gnomAD	dbSNP	Functional predictions: pathogenic (total)	AD cases (*n*)	Controls (*n*)
LGMN	Chr14:93,170,693	c.1273A > G	p.M425V	Missense	0.0000735949	rs189997608	5(14)	1	0
	Chr14:93,171,007	c.1237G > C	p.E413Q	Missense	-	-	11(14)	0	1
	Chr14:93,171,049	c.1195G > A	p.E399K	Missense	0.0000121824	rs767989757	13(14)	1	0
	Chr14:93,172,859	c.1160G > A	p.R387Q	Missense	0.0000124149	rs376306388	8(14)	0	1
	Chr14:93,172,904	c.1115C > T	p.P372L	Missense	0.0000366635	rs765350803	2(14)	1	0
	Chr14:93,176,028	c.1009C > T	p.R337W	Missense	0.000170815	rs570992743	6(14)	1	0
	Chr14:93,176,135	c.902A > T	p.H301L	Missense	0.0000934906	rs200248140	4(14)	3	1
	Chr14:93,176,168	c.869C > G	p.A290G	Missense	0.00011426	rs151054975	6(14)	0	1
	Chr14:93,180,185	c.526C > A	p.H176N	Missense	0.0000162433	rs775800345	4(14)	1	0
	Chr14:93,182,560	c.325A > G	p.T109A	Missense	0.0000771617	rs779985081	13(14)	1	0
	Chr14:93,182,563	c.322G > A	p.V108I	Missense	-	-	12(14)	2	0
	Chr14:93,185,190	c.139−2_139−1insA	-	Splicing	-	-	-	0	1
	Chr14:93,199,100	c.32T > C	p.V11A	Missense	-	-	2(14)	1	0
	Chr14:93,199,103	c.29G > A	p.S10N	Missense	-	-	4(14)	2	0
Carriers (*n*)								14	5
Frequency (%)								2.07% (14/676)	1.37% (5/365)
SKAT-O test		$p = 0.49$ (adjusted by age, gender and *APOE ε4*)							

Note: Transcript NM_005606 has been used for *LGMN* variants nomenclature; cDNA, complementary deoxyribonucleic acid; gnomAD, genome aggregation database; SKAT-O, Sequence Kernel Association Test-Optimal test. The silico tools for predicting variants were (1) SIFT (http://sift.jcvi.org), (2) PolyPhen2-HDIV (http://genetics.bwh.harvard.edu/pph2), (3) PolyPhen2-HVAR (http://genetics.bwh.harvard.edu/pph), (4) LRT (http://www.genetics.wustl.edu/jflab/lrt_query.html), (5) MutationTaster (http://www.mutationtaster.org), (6) MutationAssessor (http://mutationassessor.org), (7) FATHMM (http://fathmm.biocompute.org.uk), (8)PROVEAN(http://provean.jcvi.org/), (9) M-CAP (http://bejerano.stanford.edu/MCAP), (10) CADD(http://cadd.gs.washington.edu/), (11) DANN (https://cbcl.ics.uci.edu/public_data/DANN/), (12) Eigen (http://www.columbia.edu/~ii2135/eigen.html), (13) GenoCanyon (http://genocanyon.med.yale.du/), (14) fitCons (http://compgen.cshl.edu/fitCons/).

were significantly higher in AD cases than in the NCs ($p < 0.001$), and the frequencies of ε2/3 and ε3/3 genotypes were lower in the AD group than in the NCs ($p < 0.001$; Table 2).

3.2 | Single common variant association test

We identified 14 common variants in total, all of which conformed to HWE. Before adjustment, none of the common variants detected in *LGMN* reached statistical significance (uncorrected $p > 0.05$). Moreover, after correcting covariates for sex, age, and *APOE ε4* status, no statistically significant difference was found between the two groups (corrected $p > 0.05$; Table 3). In *APOE ε4* status-stratified analysis, two SNPs (rs17128499, rs2295986) showed statistical significance after adjustment for age and sex (corrected $p = 0.0274$, 0.0173) between the *APOE ε4* carrier AD group and the *APOE ε4* carrier control group. However, neither of the two SNPs reached statistical significance after Bonferroni correction ($p > 0.05$; Table S2). In age at onset-stratified analysis, none of the common variants detected reached statistical significance after adjustment for sex and *APOE ε4* status in both the age at onset > 65 years group and ≤ 65 years group (corrected $p > 0.05$; Table S3).

3.3 | Gene-based rare variants association test

As shown in Table 4, 14 rare variants (0 < MAF <0.01) were identified in our cohort, and none of them significantly deviated from HWE. Nine of these variants were seen in patients only. One missense variant (H301L) was noted in patients and NCs. We also identified fewer variants (3 missense variants and 1 splicing variant) only in the NCs. In our data, 2.07% of AD cases (14/676) and 1.37% of NCs (5/365) carried these rare variants. The cumulative effect on AD of all 14 variants was analyzed by the SKAT-O test. We failed to identify the significant enrichment of rare variants across *LGMN* in AD patients compared with NCs (SKAT-O p corrected = 0.49) (Table 4). In further stratified analysis based on age at onset and *APOE ε4* status, the SKAT-O test results showed no association between *LGMN* and AD (SKAT-O p corrected > 0.05; Table S4).

3.4 | ADNI cohort results

To replicate our findings in other population, variants located in both the coding region and non-coding region of the *LGMN* gene were extracted from the WGS data of the ADNI database. In total, 109 common variants and 6 rare variants were identified. Between the AD group and NC group, three SNPs reached nominally statistical significance: rs1242100 (uncorrected $p = 0.0094$, odds ratio [OR] =0.613, 95% confidence interval [CI] =0.422–0.889), rs1242098 (uncorrected $p = 0.0121$, OR = 0.622, 95% CI = 0.428–0.903), and rs3783933 (uncorrected $p = 0.0497$, OR = 0.732, 95% CI = 0.536–1.000). No SNPs reached statistical significance after Bonferroni correction ($p > 0.05$; Table S5). A total of six non-synonymous SNPs were included to study their cumulative effects on the AD trait. SKAT-O test results showed no association between *LGMN* and AD (SKAT-O p corrected = 0.536; Table S6).

4 | DISCUSSION

In this study, the screening of variants in *LGMN* was first conducted in a Chinese cohort. Mammalian legumain (δ-secretase) encoded by the *LGMN* gene was cloned by Chen *et al*. in humans in 1997, which belongs to peptidase family C13 (Chen et al., 1997). Structurally, human legumain consists of a signal peptide composed of the first 17 residues, an N-terminal propeptide composed of 8 residues, a cysteine protease domain with 298 residues, and a C-terminal prodomain consisting of the remaining 110 residues (Chen et al., 1997). Recently, it has been reported that the C-terminal domain of legumain plays a key role in legumain activation and stability (Dall & Brandstetter, 2013). Functional studies have shown that δ-secretase cleaves not only APP but also tau, enhancing the formation of both Aβ and NFTs. In AD mouse models, deletion of δ-secretase can reduce the pathological changes of AD and restore cognitive function (Wang, Liu, Chen, & Ye, 2018). According to our results, we found that several variants identified in AD patients were located in the essential C-terminal domain, including four missense rare variants (p.M425V, p.E399K, p.P372L, and p.R337W). Due to the special domain in which these variants located, we cannot rule out their effects on the autoproteolytic activation of legumain, which may further affect the legumain cleavage of APP and tau.

A WGS study was previously performed in Caribbean Hispanic families, and the gene-based association analysis results showed a modest association between *LGMN* and LOAD after adjusting for *APOE* dosage ($p = 0.033$) (Vardarajan et al., 2018). In this study, after adjusting for sex, age, and *APOE ε4*, we did not find any association between *LGMN* and AD in the Chinese population. Moreover, in age at onset- and *APOE ε4* status- stratified analysis, no statistical difference was observed after Bonferroni correction in our cohort. Even so, it is still notable that the rare variants were observed at a higher frequency in AD cases compared with NCs. In addition, our replication study in the ADNI cohort also indicated no association between *LGMN* and AD.

Several possible reasons could account for these results. First, the number of subjects included in our study was relatively small, which was a limitation that contributed to the negative findings to some extent. Second, the inconsistency with previous results was due in part to the fact that the previous genetic study of *LGMN* was conducted in a western population, which have different genetic backgrounds compared to the Chinese population. Finally, the AD cases included in the study by Vardarajan et al. (2018) were LOAD and the gene-based association analysis indicated that *LGMN* was associated with LOAD. By contrast, the onset age of AD patients in our study ranged from 33 to 91 years, and in addition to LOAD patients, EOAD patients accounted for 46.60% of our AD cohort, which may have weakened the modest association shown in the previous study. Similarly, the association of the risk variants with AD varied across age groups, which has been shown in previous studies (Vardarajan et al., 2015; Verheijen et al., 2016). Therefore, when genetic association studies with AD are conducted, various factors should be taken into consideration such as the characteristics of the study population, genetic heterogeneity of the phenotype, and the sample size.

In conclusion, this is the first study to assess the genetic association between *LGMN* and AD in a Chinese cohort. Our results suggest that variants in *LGMN* may not be associated with AD in ethnic Han Chinese population. However, due to the limited sample size in the present study, replication studies should be conducted to further evaluate the potential association between *LGMN* and AD in samples with different ethnic or geographic origins.

DATA ACCESSIBILITY STATEMENT

All data can be accessed by contacting the corresponding author at xinliao@csu.edu.cn.

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CONFLICT OF INTEREST STATEMENT.

The authors have no conflicts of interest to report.

AUTHOR CONTRIBUTIONS.

XZ conceived the article and wrote the manuscript. LW, YZ, LG, XW, LZ, XL, XX, HL, XZ, CL, YZ, QY, ZL, YJ, YW, and HZ collected the data. LS, XXL, and BJ reviewed and edited the manuscript. All authors reviewed the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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