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Association of *CDKAL1*, *CDKN2A/B* & *HHEX* gene polymorphisms with type 2 diabetes mellitus in the population of Hyderabad, India

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***Background & objectives*: The genome-wide association studies (GWAS) have shown an association of type 2 diabetes mellitus (T2DM) with several novel genes. We report here the findings on the pattern of genetic association of three genes (*CDKAL1*, *CDKN2A/B* and *HHEX*) with T2DM in the population of Hyderabad, south India.**

***Methods*: A sample of 1379 individuals (758 T2DM cases and 621 controls) from Hyderabad, India, were genotyped for five single nucleotide polymorphisms (SNPs) of *CDKAL1* (rs7754840, rs7756992) *CDKN2A/B (*rs10811661) and *HHEX* (rs1111875, rs7923837) genes on Sequenom Mass Array platform.**

***Results*: The risk allele frequencies of the *CDKAL1* and *CDKN2A/B* SNPs were relatively higher in cases than in the controls and the logistic regression analysis yielded significant odds ratios suggesting that the variant alleles conferred risk for developing T2DM in this population. The *HHEX* gene did not show either allelic or genotypic association with T2DM. The multivariate logistic regression analysis with reference to both alleles and genotypes of *CDKAL1* SNPs showed significant association, suggesting an important role for this gene in the T2DM pathophysiology.**

***Interpretation & conclusions*: A significant association was seen of all the three SNPs of *CDKAL1* and *CDKN2A/B* genes with T2DM but none of the two SNPs of HHEX. Further studies are required to cross-validate our findings in a relatively larger sample. It is also necessary to explore other SNPs of *HHEX* gene to unequivocally establish the pattern of association of this gene with T2DM in this population.**

Key words Allele frequency - genetic association - *HHEX* gene - single nucleotide polymorphism (SNP) - type 2 diabetes mellitus (T2DM)

Type 2 diabetes mellitus (T2DM) is a complex disease with both genetic and environmental factors leading to various complications such as coronary heart disease, diabetic retinopathy, neuropathy and nephropathy. It has emerged as an epidemic affecting millions of people throughout the world. With rapid increase in urbanization coupled with sedentary occupations and lack of physical activity, there is an escalating prevalence of T2DM in the developing countries like India[1]. The typical characteristic

feature of 'Asian Indian Phenotype'[1], triggered by the environmental factors, makes Indians more predisposed to develop T2DM. On the other hand, India with its unique population structure and a number of endogamous caste and tribal populations, presents enormous ethnic, linguistic and genetic heterogeneity of its population in different geographic regions. Despite this heterogeneity and high prevalence of T2DM in India and given the large number of genetic variants that have been hitherto discovered through the candidate gene approach and GWAS (genome-wide association study), only a few validation studies have been carried out[2-6]. Many more populations from diverse regions and ethnic backgrounds need to be studied to depict representative susceptible/protective genetic profile associated with T2DM that might be different for this region when compared to the Caucasian/Western populations.

In India, Andhra Pradesh (AP) has been reported to have a relatively higher prevalence of T2DM than in other States in south India, Hyderabad with a prevalence of 16.6 per cent[1]. Inspite of this, except for a couple of our studies[7-9] on the association of *TCF7L2*, *IGF2BP2*, *SLC30A8, IRS-1, CAPN10* and *PPARG* gene polymorphisms with T2DM in the population of Hyderabad, there was no other genetic study pertaining to this problem. The GWAS studies have revealed association of a number of novel genes with T2DM[10-16]. The single nucleotide polymorphisms (SNPs) were subsequently replicated in different populations with strong association with T2DM and the meta-analysis conducted for each of these genes also confirmed the risk for developing T2DM[17-20]. Studies also showed a significant association of *CDKAL1* [cyclin-dependent kinase 5 (CDK5) regulatory subunit associated protein-1-like1] with small decreases in insulin response to a glucose load[12,14]. The *CDKN2A* and *CDKN2B* (cyclin dependent kinase 4 inhibitor 2A/B) are known as tumour suppressor genes and highly expressed in the pancreatic islets and play an important role in the beta cell function and regeneration. Graurp *et al*[21] showed that these genes confer impaired glucose homeostasis and pancreatic β-cell dysfunction. On the other hand, *HHEX* (haemopoetically expressed homeobox) encodes a transcription factor which is involved in Wnt signaling pathway, a fundamental pathway required for cell growth and development[10]. *HHEX* regulates cell proliferation and tissue specification underlying vascular and hepatic differentiation, while the gene *IDE* (insulin degrading enzyme) is involved in the function of pancreas. The SNPs in the *HHEX-IDE* locus were found to be associated with decreased pancreatic beta cell function[22]. We report here the results of analysis of five SNPs from the three genes: rs7754840 and rs7756992 (*CDKAL1*), rs10811661 (*CDKN2A/B*) and rs1111875 and rs7923837 (*HHEX*).

Material & Methods

A total of 758 T2DM cases (443 males and 315 females) and 621 controls (395 males and 226 females) were included during the year 2010-2011. The T2DM patients were recruited from J.P Endocrine Center, Hyderabad, based on their medical record and adhering to American Diabetes Association (ADA, 2010)[23] criteria that specifies fasting plasma glucose (FPG) ≥ 126 mg/dl or 2 h plasma glucose (PPG) ≥ 200 mg/dl or random plasma glucose (random blood sugar) (RBG) ≥ 200 mg/dl to be characterized as diabetic. The age of the patients ranged between 31-80 yr. We conducted free diabetes camps for the employees of different organizations in Hyderabad and individuals with ≥ 40 yr of age were tested for random blood sugar (where fasting and 2 h plasma glucose were not feasible) and those having blood sugar <140 mg/dl and with no positive family background for T2DM were recruited as controls. The background information and the anthropometric measurements collected on both cases and controls have been described elsewhere[7-9]. Given that the patients of the clinics as well as the employees of different organizations from where the controls were drawn constituted similar ethnic and linguistic backgrounds, broad matching for ethnicity of the cases and controls was ensured. Blood sample (5 ml) was drawn from all patients and controls after obtaining written informed consent. The study protocol was approved by the ethics committee of Indian Statistical Institute, Kolkata.

Genomic DNA was extracted using the phenol-chloroform method[24] and the isolated DNA was subsequently quantified using NanoDrop™ 2000 (Thermo Scientific, Wilmington, Delaware, USA). The SNPs of the respective genes *CDKAL1* (rs7754840, rs7756992), *CDKN2A/B* (rs10811661) and *HHEX* (rs1111875, rs7923837) were genotyped as part of a select panel of 15 SNPs of 9 prominent T2DM genes on Sequenom Massarray platform (Sequenom. Inc, San Diego, CA, USA) at The Centre for Genomic Application (TCGA), Delhi. Due to resource constraints, all GWAS identified SNPs could not be genotyped, and given the limited choice SNPs that were most significantly associated and more often replicated were genotyped. The assays were performed according

to the manufacture's specifications[25] and quality control (QC) procedure as described elsewhere[7].

Statistical analysis: Most of the statistical analyses were performed with the help of SPSS statistical software (version 18.0, IBM SPSS, Chicago, IL, USA). Data for continuous variables were expressed as mean±SD, median and range. Student's t test was used to determine the significance of difference in the means of the continuous variables between cases and controls. Chi-square test was used to assess the significance of heterogeneity of allele and the genotype frequencies between cases and controls. Logistic regression analysis was done under the assumption of general model, *i.e.* by taking the most frequent genotype as reference and body mass index (BMI) and waist-hip ratio (WHR) as covariates. The risk was estimated based on the significance of odds ratio (OR) with 95% C.I. obtained. Multivariate logistic regression was also carried out using R PROGRAM (version 2.15.2; R Foundation, *http://www.r-project.org/*). Post-hoc power of the study was estimated using G*Power (version 3.1) Universität Kiel, Keil Germany) and Quanto (version 1.2.4, Kiel University of South California, Los Angeles, CA, USA) softwares. The Hardy-Weinberg equilibrium was estimated by the $\chi 2$ test using Pypop (version 0.7.0, Thomson Laboratories, Department of Integrative Biology, University of California, Berkeley, CA, USA) and Haploview software (version 4.1, Broad Institute of MIT and Harvard, Cambridge, USA) was used to estimate linkage disequilibrium (LD).

Results

The details on the clinical profile of the subjects have been published elsewhere[7,8]. The patients showed significantly higher values for BMI, waist-hip ratio and random blood glucose (P<0.001) compared to controls.

Allele and genotypic association of different SNPs: The allele and genotype frequencies of the five SNPs are shown in Tables I and II. All these SNPs were found to be in Hardy-Weinberg equilibrium (Table I). The frequency of the risk alleles of *CDKAL1* SNPs, rs7754840 (C) and rs7756992 (G) and T allele of rs10811661 of *CDKN2A/B* SNP were relatively higher in T2DM patients than in the controls and the logistic regression analysis yielded significant odds ratios suggesting that the variant alleles conferred risk for developing T2DM. The frequencies of the heterozygotes (GC, AG) and homozygote genotypes (CC, GG) of the two SNPs of *CDKAL1* gene were found to be higher in patients than in the controls (P<0.003), suggesting risk confering nature. Although a similar pattern of association was observed in the homozygote genotype frequency (TT) of *CDKN2A/B* gene, the heterozygote (CT) was found to be higher in frequency in the controls. In the logistic regression analysis of the genotypes the heterozygote (GC) and homozygote (CC) genotypes of *CDKAL1* SNP showed significant odds ratio (P<0.01) and the observed association remained significant even after adjusting for covariates. While the genotype frequency of *CDKN2A/B* gene showed a marginal significance, it was not significant in the logistic regression analysis, with or without covariates. On the other hand, the two SNPs of *HHEX* gene (rs1111875, rs7923837) showed similar allele as well as the genotype frequencies among the patients and controls, suggesting lack of evidence for its association with T2DM.

Combined role of these genes: Results of multivariate logistic regression analysis: The linkage disequilibrium (LD) plot for different pairs of the five SNPs of the three genes considered in this study (Figure) suggested strong LD between the two SNPs (rs7754840, rs7756992) of *CDKAL1* and *HHEX* (rs1111875, rs7923837), respectively (*CDKAL1:* D'=0.90, r^2=0.73; *HHEX* : D'=0.87, r^2=0.70) and there was no LD between the other pairs of SNPs. Similar results were obtained when LD plots were constructed for cases and controls separately (results not presented). Therefore, one of the two SNPs with greater significance from

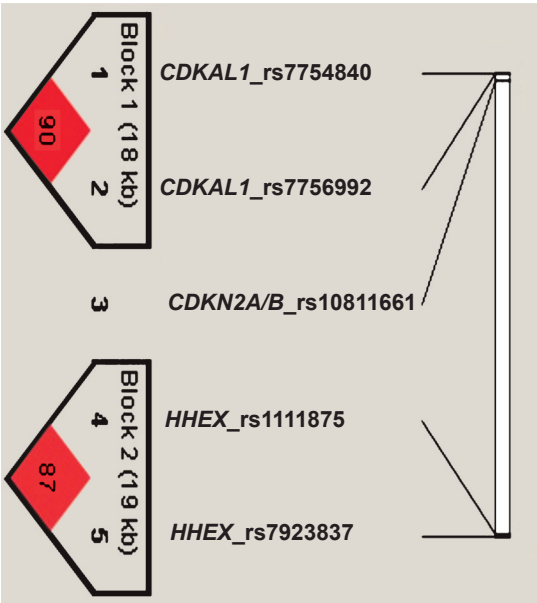

Figure. Linkage disequilibrium plot for the five SNPs of *HHEX*, *CDKN2A/B* and *CDKAL1* genes. D' values mentioned in the LD plot. LD is seen only between SNPs of the same gene, not across the genes.

Table I. Allele frequency distribution and odds ratio (OR) obtained from the logistic regression of *CDKAL1*, *CDKN2A/B* and *HHEX* genes in T2DM patients and controls

Genes	SNP	Patients / controls (2N)	Non-risk/Risk	T2DM patients	Controls	Allele	*P* value	OR	CI (95%)
CDKAL1	rs7754840	1344/1188	G	0.73	0.78	C vs G	0.001	1.36	1.14- 1.63
			C	0.27	0.22				
			#HWE (χ^2, *P* value)	(0.17, 0.68)	(0.19, 0.66)				
	rs7756992	1474/1188	A	0.70	0.76	G vs A	0.001	1.35	1.14 - 1.61
			G	0.30	0.24				
			HWE (χ^2, *P* value)	(1.56, 0.21)	(1.13, 0.29)				
CDKN2A/B	rs10811661	1466/1176	**T**	0.87	0.85	T Vs C	0.031	1.28	1.02 - 1.59
			C	0.13	0.15				
			HWE (χ^2, *P* value)	(0.03, 0.85)	(3.68, 0.06)				
HHEX	rs1111875	1458/1180	A	0.58	0.56	G Vs A	0.569	0.96	0.82 - 1.12
			G	0.42	0.44				
			HWE (χ^2, *P* value)	(0.06, 0.81)	(0,0.99)				
	rs7923837	1448/1178	A	0.55	0.54	G Vs A	0.707	0.97	0.84 - 1.13
			G	0.45	0.46				
			HWE (χ^2, *P* value)	(0.22, 0.64)	(0.07, 0.79)				

Risk allele in our study in bold
Risk allele based on previous study in bold and underlined (Ref. 4)
HWE, Hardy-Weinberg Equilibrium with 1 degrees of freedom
OR calculated with respect to risk allele

each of the two above genes were considered, along with rs1081661 of *CDKN2A/B* in the multivariate logistic regression. The allele and genotype data were analysed separately to investigate the combined role of these genes in the T2DM aetiology. The odds ratio was obtained using the most frequent genotype/allele as the reference. Allele based multivariate logistic regression suggested significant odds ratio for minor allele (G) of rs7756992 from *CDKAL1*, which was consistent with the individual SNP analysis. This SNP also showed similar pattern of association in genotypic multivariate logistic regression (*P*=0.009), which remained significant even after bonferroni correction for multiple testing, suggesting the independent risk conferring role of this gene in the manifestation of T2DM, after adjusting for confounding nature of effects of the other genes, if any (Table III). On the other hand, in allele based multivariate logistic regression analysis, rs10811661 of *CDKN2A/B* did not show significant association. However, in case of genotype wise multivariate logistic regression analysis, rs10811661 showed consistent results with individual SNP analysis. Although the minor allele G of rs1111875 (*HHEX* gene) also showed significant association with T2DM in the multivariate context (with protective role against developing T2DM), it was not significant after bonferroni correction for multiple testing. A similar, albeit non-significant pattern was observed in the genotype based logistic regression, both in the context of multivariate and individual SNP analyses.

Power of study: The post-hoc power of the study was calculated using G power for the SNPs of the *CDKAL1*, *CDKN2A/B* and *HHEX* genes. The three SNPs- rs7754840, rs7756992 and rs10811661 of the

Table II. Genotype frequency distribution and odds ratio from logistic regression of *CDKAL1*, *CDKN2A/B* and *HHEX* genes in T2DM patients and controls

Genes	SNP	Cases/ Controls (N)	Genotype	T2DM patients	Controls	*P* value	Genotype	Unadjusted for covariates			Adjusted for covariates		
								P value	OR	CI (95%)	P value	OR	CI (95%)
CDKAL1	rs7754840	672/549	GG	0.53	0.61	0.003	GC vs GG	0.009	1.36	1.08 - 1.71	0.017	1.34	1.05-1.70
			GC	0.40	0.35		CC vs GG	0.011	1.90	1.16 - 3.11	0.032	1.76	1.05 - 2.94
			CC	0.07	0.04								
	rs7756992	737/549	AA	0.48	0.57	0.002	AG vs AA	0.007	1.36	1.09 - 1.71	0.023	1.31	1.04 - 1.67
			AG	0.44	0.38		GG vs AA	0.007	1.91	1.19 - 3.05	0.017	1.82	1.11 - 2.96
			GG	0.08	0.05								
CDKN2A/B	rs10811661	733/588	CC	0.02	0.01	0.037	CT vs CC	0.473	0.71	0.28 - 1.81	0.486	0.71	0.27 - 1.87
			CT	0.22	0.28		TT vs CC	0.972	0.98	0.39 - 2.47	0.952	0.97	0.37 - 2.52
			TT	0.76	0.70								
HHEX	rs1111875	729/590	AA	0.33	0.32	0.841	AG vs AA	0.632	0.94	0.74 - 1.20	0.973	1.00	0.77 - 1.29
			AG	0.48	0.49		GG vs AA	0.598	0.92	0.67 - 1.26	0.796	0.96	0.68 - 1.34
			GG	0.18	0.19								
	rs7923837	724/589	AA	0.30	0.30	0.862	AG vs AA	0.768	1.04	0.81 - 1.34	0.474	1.10	0.84 - 1.44
			AG	0.50	0.49		GG vs AA	0.806	0.96	0.70 - 1.31	0.783	1.05	0.75 - 1.46
			GG	0.20	0.21								

Table III. Multivariate logistic regression of alleles and genotypes of *CDKAL1*, *CDKN2A/B* and *HHEX* genes

Gene	SNP	Allele†	adjusted OR	95% CI	*P* value	Genotype	adjusted OR	CI (95%)	*P* value
CDKAL1	rs7756992	**G** vs A	1.42	1.1.8-1.72	<0.001*	AG	1.43	1.13-1.81	0.003*
						GG	1.91	1.17-3.10	0.009*
CDKN2A/B	rs10811661	**T** vs C	1.21	0.96-1.53	0.101	CT	0.75	0.29-1.92	0.546
						TT	1.00	0.40-2.54	0.994
HHEX	rs1111875	**G** vs A	0.84	0.71-0.99	0.034	AG	0.96	0.75-1.24	0.767
						GG	0.88	0.63-1.21	0.420

*significant after bonferroni correction for multiple testing
†risk allele bold
OR, odds ratio

first two genes with significant odds ratio of 1.36, 1.35 and 1.28 yielded statistical power (1-β error probability) of 99, 99 and 97 per cent, respectively. Considering the non-significant association of SNPs of *HHEX* gene, we tried to estimate if our study had the adequate statistical power to detect the true association if existent in the population. We estimated power of study to detect true association by considering the significant odds ratio obtained for a north Indian sample by Chauhan *et al*[4] for one of the two SNPs (rs1111875) of *HHEX* gene as reference. Taking the log of odds ratio (log 1.27 = 0.104) from the above study as the effect size and given our sample size of 1379, the power of 97 per cent was obtained for our study (*P*=0.05), which implied that our study was sufficiently powered to detect true association of *HHEX* SNPs if existent in our population. We further estimated minimum effect size necessary to detect association with 90 per cent power, which turned out to be 0.087 for the two SNPs of *HHEX* gene. The observed effect size was virtually zero (-0.01), for both the SNPs (rs1111875 and rs7923837) of *HHEX* gene, ruling out the possibility of any association of these SNPs with T2DM in this population. Alternatively, based on prevalence rate of T2DM in Hyderabad city[1], power of study was estimated using Quanto software. Given the estimated prevalence of 16 per cent for T2DM[1], minor allele frequency of 0.27 and 0.30 for the two SNPs (rs7754840, rs7756992) of *CDKAL1* gene and with odds ratios of 1.36 and 1.35, we obtained power of about 95 and 99 per cent, respectively, which were qualitatively similar to the results obtained by using G power. This implied that given the high prevalence of T2DM in our population and with the sample size of 1379 our study was sufficiently powered to detect any true association of these genes with T2DM.

Comparison with earlier studies among Indian populations: Despite unique Indian population structure with large number of endogamous groups and enormous ethnic, linguistic and geographic heterogeneity, very few studies have hitherto focused on replication of earlier findings of genetic association of T2DM[2-5]. The risk allele frequencies of all the SNPs considered in our study were compared with other Indian studies (Table IV). A significant allelic heterogeneity was observed for the *CDKAL1* and *CDKN2A/B* genes among the few Indian populations studied, primarily because of the differences in the allele frequency between the northern and southern populations. Because of this heterogeneity in allele frequency and given that the data were available for only a couple of other Indian populations further analysis could not be performed.

Discussion

A significant association of all the three SNPs of *CDKAL1* and *CDKN2A/B* genes was observed with T2DM, as observed earlier among the European populations[10-16]. However, neither of the two SNPs of *HHEX* gene showed significant association. The logistic regression analysis of the alleles of *CDKAL1* and *CDKN2A/B* gene yielded significant odds, conferring risk towards developing T2DM. The biological significance of the observed genetic association can be explained by the already established role of *CDKAL1* in insulin secretion, which acts through impaired pancreatic beta cell function[26] and of *CDKN2A/B* in the beta cell function and regeneration. The non-significant association of *HHEX* gene in our population could be due to differential genetic predisposition of Europeans and Indian populations towards T2DM besides the fact that the Indian subcontinent itself is characterized by vast genetic differences between the

Table IV. Comparative presentation of risk allele frequencies (RAF) given as proportion of the SNPs of *CDKAL1*, *CDKN2A/B* and *HHEX* genes in different Indian regional populations

Gene	SNP	RAF in present study		RAF Chennai study		RAF for three north Indian studies[#] $\chi 2$ *P* value		Total cases			Total controls		Total Cases+Controls	
								df	$\chi 2$	*P* value	$\chi 2$	*P* value	$\chi 2$	*P* value
		Cases	Controls	Cases (N=926)	Controls (N=812)	Cases	Controls							
CDKAL1	rs7754840 (G/C)[*]	0.27	0.22	0.25	0.21[3]	0.45	0.27[2]	2	69.6	<0.001	5.36	0.07	66.39	<0.001
CDKAL1	rs7756992 (A/G)	0.30	0.24	0.27	0.22[3]	-	-	1	1.8	0.18	0.76	0.38	2.68	0.1
CDKN2A/B	rs10811661 (C/T)	0.87	0.85	-	-	0.91 0.89 0.82	0.89[2] 0.86[4] 0.76[5]	3	17.07	<0.001	22.6	<0.001	38.69	<0.001
HHEX	rs1111875 (A/G)	0.42	0.44	-	-	0.46 0.47	0.45[2] 0.43[4]	2	5.93	<0.05	0.68	0.71	2.32	0.31
HHEX	rs7923837 (A/G)	0.45	0.46	0.41	0.46[3]	-	-	1	2.7	0.1	<0.05	0.95	1.26	0.26

[*](Non-risk/risk allele)

[#]North Indian samples are from three different studies whose reference numbers are shown as superscript. Ref. 2: 532 cases + 386 controls; Ref. 4: 2486 cases + 2678 controls; Ref. 5: 217 cases + 246 controls. Superscript numerals denote reference numbers

regional populations[27]. In support to this, Cia *et al*[20] conducted a sub-grouped meta-analysis by ethnicity and observed a non-significant association of the *HHEX* gene with T2DM in the Indian population, suggesting that ethnicity was the main cause of the heterogeneity among the studies. With reference to other Indian studies, our results on *CDKAL1* gene were consistent with Chennai study[3], whereas one of the northern Indian studies did not show significant association of rs7754840 with T2DM[2]. Although another study constituting samples from northern and western India[4] showed strong association of *CDKAL1* with T2DM, the single SNP studied was different from those of the present study. The rs10811661 of *CDKN2A/B* also showed strong association with T2DM in our population as was the case with north and western Indian studies[4,5]. However, the variant near *HHEX* gene (rs1111875) was not significantly associated with T2DM in our study similar to other Indian populations studied[2,3]. The other SNP of this gene (rs7923837) was also not associated with T2DM in our population, although it was found significantly associated in a study from Chennai[3]. This may suggest that the T2DM susceptible genes vary with the ethnic background. This draws support from an Indian GWAS study which provides evidence for possible genetic heterogeneity between Indo-Europeans and Dravidian ethnic groups within India[22]. Overall, there is an inconsistency in the pattern of association of these genes with T2DM among Indian populations. Therefore, it is important to explore a number of geographically and ethnically heterogenous populations within India, not only to validate already known variants but also to see if any new genetic variants that might be unique to Indian populations can be found, before one can project genetic susceptibility/protective profile that would be representative of the Indian population in general.

Despite large sample size and sufficient statistical power of the study, we could not find significant association of *HHEX* gene with T2DM in our population. In spite of the biological significance of *HHEX*, it does not seem to play a significant role in the manifestation of T2DM in this Indian population. This can be explained by the possibility of these SNPs not being causal variant for T2DM in this population and/or probably not tagged to the same causal variants as in the case of Western populations. Further studies are required to cross-validate our findings in relatively larger samples as well as to explore novel SNPs of *HHEX* gene that might be associated with T2DM in this Indian population.

***Conflicts of Interest*:** None.

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