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On the genetic involvement of apoptosis-related genes in Crohn's disease as revealed by an extended association screen using 245 markers: no evidence for new predisposing factors

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Abstract

Crohn's disease (CD) presents as an inflammatory barrier disease with characteristic destructive processes in the intestinal wall. Although the pathomechanisms of CD are still not exactly understood, there is evidence that, in addition to *e.g.* bacterial colonisation, genetic predisposition contributes to the development of CD. In order to search for predisposing genetic factors we scrutinised 245 microsatellite markers in a population-based linkage mapping study. These microsatellites cover gene loci the encoded protein of which take part in the regulation of apoptosis and (innate) immune processes. Respective loci contribute to the activation/suppression of apoptosis, are involved in signal transduction and cell cycle regulators or they belong to the tumor necrosis factor superfamily, caspase related genes or the BCL2 family. Furthermore, several cytokines as well as chemokines were included. The approach is based on three steps: analyzing pooled DNAs of patients and controls, verification of significantly differing microsatellite markers by genotyping individual DNA samples and, finally, additional reinvestigation of the respective gene in the region covered by the associated microsatellite by analysing single-nucleotide polymorphisms (SNPs). Using this step-wise process we were unable to demonstrate evidence for genetic predisposition of the chosen apoptosis- and immunity-related genes with respect to susceptibility for CD.

Introduction

Crohn's disease (CD) is a chronic inflammatory disorder characterized by destructive processes in the intestinal wall. Interactions between genetic and environmental factors potentially lead to an imbalance between the luminal bacterial flora, and the innate as well as the adaptive immune systems [1,2]. Epidemiological and genome wide studies have lead to the identification of factors establishing genetic involvement in CD [1,3,4]. Despite of fundamental findings, namely the variation in the CARD15 receptor and their association with CD, the causative instances regulating the exaggerated mucosal response remained elusive. The proposed pathomechanisms of CD are manifold. The dysregulated response of

Table 1: Genes investigated for CD association as represented by an intra- or juxtagenic microsatellite marker (for additional information see URL: http://www.ruhr-uni-bochum.de/mhg/marker_information_SENW.pdf)

apoptosis related	REQ	TNFSF12	CTLA4	Casp10	IL4
	RNF7	TNFSF14	DAP	Casp14	IL4R
	SMAC	TNFSF15	DAPK1	Casp2	IL6
AIF	TIAF1	TNFSF18	FADD	Casp3	IL8
APR3	TIAL1	TNFSF4	IKBKG	Casp4	IRF1
BCLG	TP73	TNFSF5	MADD	Casp5	NRG1B
BFAR	VDR	TNFSF6	MAP2K6	Casp6	PRL
CIDEB		TNFSF7	MAP3K14	Casp7	PRLR
CYBB	**Bcl2 related**	TNFSF8	MAP3K5	Casp8	
CYP51		TNFSF9	MAP4K4	CASP8AP2	**chromosome 6**
DAD1	BCL2A1	TOSO	NFKB1	Casp9	
DAP3	Bag1		NFKB2		No.1
DATF1	BAK	**innate immunity**	NSMAF	**apoptosis suppressor**	No.4
DAXX	BAX		PAWR		No.5
DEDD	BCL2	BPI	PIAS3		No.6
DHCR24	BCL2L1	CD14	PTEN	API5	No.7
EIF4G2	BCL2L11	CD5L	RARB	BIRC1	No.8
FASTK	BCL2L13	DEFB119/ DEFB121	RIPK1	BIRC2	D6S1014
FLIP	BID	DEFB127	RIPK2	BIRC3	D6S1959
FRZB	BIK	HBD1	RIPK3	BIRC4	D6S273
GSK3B	BNIP3L	IFNB1	RXRB	BIRC6	
GSR	MCL1	LY64	STK17A	BIRC8	**others**
GZMA		LY86	STK17B		
GZMB	**TNF superfamily**	LY96	TANK	**cytokine chemokines**	BPHL/TUBB
HLCS		NCF1	TRADD		TAPBPR
NME3	LTB (TNFSF3)	NCF4	Traf3		VEGF
NOL3	LTBR (TNFRSF3)	PGLYRP	Traf4	AXL	LGALS3
NOS1	TNFa	PLA2G4A	Traf5	CSF1R	BDNF
NOS2A	TNFRSF10A	PLUNC	Traf6	CSF2	NGFB
NOX1	TNFRSF10B	SerpinA1		CSF2RB	NGFR
NOX3	TNFRSF10C	SerpinB1	**cell cycle regulators**	CSF3	TrkC
NOX4	TNFRSF10D	SFTPA1		Dtk	
P2RX1	TNFRSF11A	SLPI	CCND2	erbB3	**positive control**
P53AIP1	TNFRSF11B	STAT3	CDC2	GAS1	CARD15
PDCD10	TNFRSF12	TGFB1	CDKN1A	IGF1	
PDCD2	TNFRSF17	TLR1	CDKN2A	IGF2R	
PDCD5	TNFRSF18	TLR2	PAK1B	IL10	
PDCD6	TNFRSF19	TLR3	RbAp48	IL10RA	
PDCD6IP	TNFRSF19L	TLR4	Rb2/p130	IL10RB	
PDCD8	TNFRSF1A	TLR5	RBP1	IL11RA	
PLA2G10	TNFRSF1B	TLR7	RBP2	IL12A	
PLA2G1B	TNFRSF21	TLR8	RBQ-1	IL12B	
PLA2G6	TNFRSF4	TLR9	RBQ-3	IL12RB2	
PTGS1	TNFRSF5	TLR10	TP53	IL13RA2	
REQ	TNFRSF6 (FAS)		TP53INP1	IL18	
RNF7	TNFRSF6B	**signal transduction**		IL18R	
SMAC	TNFRSF7		**caspase related**	IL1RL1	
TIAF1	TNFRSF8	Traf1		IL1B	
TIAL1	TNFRSF9	BCL10	ADPRT	IL2	
TP73	TNFSF10	CHUK	CARD4	IL24	
VDR	TNFSF11	CRADD	Casp1	IL2RA	

the innate immune system is supposed to present a crucial step in the pathogenesis of CD [5]. This fact has been confirmed genetically by several CD associations of genes such as CD14, TLR4 and in some instances the interaction of their variations with CARD15 [6,7]. In regard to the polarized T helper (Th) response, the adaptive immune system appears affected in CD as well [8-10]. Moreover, several studies implicated a role of programmed cell death in CD [11-15]. Apoptosis mediates 'self-tolerance', the elimination of autoreactive immune compartments. In addition, the thoroughly controlled termination of a physiological immune response is due to the process of programmed cell death. In CD mucosal T cells show less susceptibility to apoptosis [16]. In this context TNFα protein exerts multiple physiological effects, and anti-TNFα therapeutic strategies (*e.g.* infliximab) are effective in (maintaining) remission of CD [17]. In several studies it has been revealed that treatment of CD patients with infliximab leads to an activation of T cells rendering them susceptible for apoptosis [18,19]. Interestingly, the effect of this treatment may not be due to neutralisation of soluble TNFα (and its binding to the TNFRs), but rather it may be caused by its affinity to membrane-bound TNFα putatively changing the ratio of anti- and pro-apoptotic mediators towards induction of apoptosis [18,20]. Although the mechanisms of the causal role of T cells responses in CD remain to be determined in detail, there is substantial clinical evidence that suggests a role for uncontrolled activated T lymphocytes in the pathogenic process of CD [21-24]. Nevertheless, it is uncertain, whether a genetic basis for a decreased activation/apoptosis of T lymphocytes in CD patients exists, and whether increased anti-apoptotic markers, found in T cells of these patients are due to the mucosal inflammation, secondarily [18].

In such a complex situation we used extended association screening (EAS) with markers representing 245 apoptosis- and (innate) immunity-related genes. The majority of the investigated markers have been successfully utilized in respective studies before [25,26]. Our population based linkage mapping comprises a 3-stage analysis with pooled DNA in the initial phase and subsequently individual genotyping. In order to confirm such results, several tagging SNPs of the adjacent gene represented by the marker were analysed. Here, we investigated the role of distinct biological pathways for the susceptibility of CD.

Materials and methods

Patients

One hundred and fifty eight well-characterized patients with a clinical, endoscopical and histological diagnosis of CD were included. This patient cohort has been reported before [27,28]. All patients were of German origin and the diagnosis of CD was adjusted according to the diagnostic criteria of the European Community Workshop on Inflammatory Bowel Diseases (IBD). As controls a group of healthy northern German (NoG) and western German (WeG) origin were analysed. In the initial step a group of ~100 NoG individuals were used. In order to exclude population stratification, genotyping of chosen SNPs was performed in 180–460 NoG and WeG individuals.

Pooling of DNA

The DNA concentration from each individual of the patient and control cohorts was quantified by spectrophotometry, carried out four times, and then diluted accordingly to 100 ng/µl. In a second step the DNA was diluted to a concentration of 65 ng/µl and once more measured by spectrophotometry. Finally, DNA diluted to 50 ng/µl was adjusted to a final amount of 1000 ng for each individual in a pool of 50 persons. In the initial stage, marker analyses were performed with two patient and two control subpools, respectively.

Tailed primer PCR

Tailed primer PCR was performed as described before [25]: An 18 bp-tail was added to each sense oligonucleotide. PCR reaction included three oligonulceotides, two of which were target specific. The third one consists of the same sequence as the abovementioned tail that was additionally fluorescence-labelled.

Microsatellite markers

Intragenic microsatellite or markers located in the immediate vicinity (<50 kb) of the specific gene were included. Information on the oligonucleotide sequences and location of markers are given at the website (Additional file 1; see also Tab. 1). As reported before, only markers with equal "intra-subgroup" allele distributions with ≥ 2 alleles were considered in subsequent analyses [25]. Significantly associated markers were genotyped individually in order to exclude false-positive results due to possible pooling artefacts. All in all, 245 microsatellite markers representing distinct genes were analysed on an ABI377 slab-gel system (Applied Biosystems, Darmstadt, Germany).

Statistics for initial comparisons of allele frequencies

Raw data from ABI377 profiles were analysed by the Genotyper software (ABI) producing a marker specific allele image profile (AIP) which includes different heights of peaks reflecting the allele frequencies. In order to test differences of the AIPs between CD patients and the controls, all peak heights were summarized for each pool and set to 100 %. The total allele count for each distinct allele was then estimated. Thereupon, the AIPs of the case and control pools were compared statistically by means of contingency tables. Hence, P values are nominal and approximate, because estimated rather than observed counts were used for allele frequencies. The significance

Table 2: P values for microsatellite markers located intragenically or in the immediate vicinity of represented genes after the initial step and individual genotyping.

	p values				
gene (as represented by the respective marker)	after analysis with pooled DNA	after summation of alleles beneath 5%	after analyses of each single allele (most significant allele)	after individual genotyping[1] (p^c value)	after correction by Q-value of pooled data
FLIP	0.2871	0.1936	0.0100	0.0044 ($p^c > 0.05$; c = 9)	n.s.
BCL2A1	0.0948	0.0948	0.0275	n.s.	n.s.
BAG1	0.2541	0.2541	0.0163	n.s.	n.s.
BPI	0.0011	0.0011	0.0031	n.s.	n.s.
erbB3	0.0760	0.0932	0.0100	n.s.	n.s.
TP73	0.5928	0.3535	0.0302	n.s.	n.s.
TLR9	0.3004	0.3004	0.0300	n.s.	n.s.
TNFRSF17	0.0012	0.0014	0.0014	0.0012 ($p^c < 0.01$; c = 6)	n.s.
CARD15	0.0083	0.0247	0.0054	0.0050 ($p^c < 0.04$; c = 7)	n.s.

P values were generated using three different procedures as described in the methods' section. Briefly, data were analysed by means of contingency tables, initially comparing allele distributions represented by the AIF (after analyses with pooled DNA), then after summation of alleles < 5% in order to focus on the major alleles and, finally, after comparison of each single allele between the control and patient cohorts. For analysing the results of the individual genotyping χ^2 testing was utilised.
[1]Genotyping was performed with the same individuals used in the pooling procedure, and, when remaining significant, further individuals were added to the analyses (FLIP: CD = 134, controls = 150; *TNFRSF17*: CD = 147, controls = 135; *CARD15*: CD = 144, controls = 165).

level was set at p = 0.05. In order to focus the statistics on major alleles, all minor alleles with a frequency of less than 0.05 were summarized to a virtual allele. Subsequently, a second statistical analysis by means of contingency tables was undertaken. A third step for statistical testing each allele individually was accomplished (and the summation of all other marker alleles), whereby the respective value of the patient group was compared with those of the controls and subsequent χ^2 analyses. Despite of evidence that correction for multiple comparisons might eliminate 'real positive' results [26], Q value correction was performed with a cut off of 5% for the initial screening procedure [29].

Nevertheless, for selecting markers for further investigations, non-corrected P values were simply ranked according to their evidence for association including all performed statistical procedures.

Individual genotyping

Markers with significantly different allele distributions between patients and controls were controlled by genotyping individual DNA samples of patients and controls in order to exclude false-positive results due to pooling artefacts. Individual genotyping was performed by capillary gel electrophoresis by using the BeckmanCoulter CEQ8000 genetic analysis system (Beckman Coulter, Germany). Results were analysed by comparing each microsatellite allele frequency from the CD cohort with the corresponding allele frequency of the control group by χ^2 testing and corrected by the number of marker specific alleles according to Bonferroni (see Tab. 2 and URL: http://www.ruhr-uni-bochum.de/mhg/marker_information_SENW.pdf). Hardy-Weinberg equilibrium (HWE) was tested using the Genepop program http://wbiomed.curtin.edu.au/genepop.

SNP genotyping

SNPs in genes as represented by significantly associated markers after individual genotyping were investigated by analysis of restriction fragment length polymorphisms (RFLP; see Tab. 3). As the marker representing the *TNFRSF17* gene is located in ~1 MBp distance to the *MHC class II transactivator* (*MHC2TA*) gene, a functional variation (rs3087456, [30]) of *MHC2TA* was genotyped by RFLP analyses in 147 CD patients and 463 healthy controls from the abovementioned control populations (see Tab. 3). The results were evaluated by means of χ^2 -and HWE testing. Linkage disequilibrium (LD) between the marker alleles and the polymorphism was calculated by the Genepop program.

Results

Initial step

Microsatellites representing 245 genes involved in apoptosis regulation (see Tab. 1) were investigated by using EAS. None of the markers presented with significant intra-subgroup differences confirming the homogeneity of the pools. The statistical evaluation of the microsatellite frequencies in the CD patient and the control cohorts revealed 9 significantly different allele distributions of intra- or juxtagenic markers for *FLIP*, *BCL2A1*, *BAG1*, *BPI*,

Table 3: Investigated SNPs in genes as represented by significantly differing microsatellites of the individual genotyping step.

Gene	rs#	Allele 01/02	Oligonucleotides (sense/antisense)	RE	TM (°C)	Allele: fragment length (bp)
FLIP	Rs7583529	A/C	GGTGATTATTCGGACCCCA/ AACTACAGATCCCGTGTGGAG	TseI	57	01: 155 02: 103/52
	Rs2041765	T/C	GAACAAGGAGAGAACCTGGAC/ GAGCTGGAAGGCACAGTACA	MboII	56	01: 309 02: 188/121
TNFRSF17	Rs3743591	A/G	ATAAGCAGTTTCTGTTTCAGATGT/ CTCTACAAGAATTCCAGAGCA	BceAI	55	01: 223 02: 147/76
	Rs11570139	C/T	GCCCTGATATTTACACCCTGT/ CAGCCATCTGCAACATGAT	CaiI	54	01: 269 02: 161/108
	Rs373496	T/C	AGGAACTGAAACTCACAATAGC/ CAGCTCATTATCTGTCTGATGTT	AluI	55	01: 247 02: 100/90/54/3
MHC2TA	Rs3087456	G/A	* I GTGAAATTAATTTCAGAG**C**TGT/ CTCAGCTTCCCCAAGGAT	BfmI	58	01: 268 02: 231/37

Analyses were performed by using the RFLP method. The table depicts information on the used SNPs as well as RFLP/PCR conditions. * I A 5'-tail was added to the mismatch (bold letter) sense primer (5'-CATCGCTGATTCGCACAT-3'). PCR was performed with a third oligonucleotide with the equal sequence as the tail. RE: restriction enzyme; TM: melting temperature (used for annealing in PCR).

erbB3, *TP73*, *TLR9*, *TNFRSF17* and *CARD15* (summarized in Tab. 2).

Individual genotyping

Individual genotyping confirmed significant P values only for the 3 markers *FLIP* ($p = 0.0044$, $p_c > 0.05$, in HWE), *TNFRSF17* ($p = 0.0012$, $p_c < 0.01$, in HWE) and the positive control CARD15 ($p = 0.0050$, $p_c < 0.04$, in HWE). The additional associations for the other markers were rejected (see Tab. 2 and Additional file 1). There were no differences analysing $CARD15^+$ and $CARD15^-$ patients.

SNP genotyping

SNP markers (Tab. 3) were genotyped located in the respective genes in the vicinity of the microsatellites representing *TNFRSF17* and *FLIP*. Thus, SNPs were analyzed spread across the genes representing haplotypes as predisposed by the 'LD Select' method reported before [31]. RFLP analyses did not reveal any association of the selected SNPs, neither by comparing the $CARD15^+$ nor the $CARD15^-$ patients with the control group.

Comparison of TNFRSF17 *microsatellite alleles*

The genotypes of the *TNFRSF17* microsatellite alleles were compared between the patient and control cohorts. Analyses revealed evidence either for a predisposing (allele 3) and a protective allele (2) or linkage between these alleles and the marker alleles, respectively.

Genotypes including allele 2 are overrepresented in the control cohort, whereas those with the apparently predisposing allele 3 are more frequent in the CD cohort, thus confirming the results of individual genotyping (see Fig. 1).

MHC2TA *analyses*

The analyses of the functionally significant polymorphism rs3087456 revealed a marginal association in our CD patients when allele or genotype frequencies were compared between the combined control (WeG and NoG did not differ in allele frequencies) and the patient cohorts (see Tab. 4). Analyses for LD between *TNFRSF17* and *MHC2TA* alleles, however, did not reveal any significant deviations from equilibrium.

Discussion

The pathomechanisms of CD are still not exactly understood, albeit certain *CARD15* variations appear especially frequent in CD patients; thus genetic involvement is proven. These genetic predisposition factors, however, are neither sufficient nor explain they the pathogenesis in all CD patients. In this study we present an association screen mainly for apoptosis and immunity related genes by microsatellite markers as investigated in a 3-step approach.

Our initial analyses revealed 9 significantly different allele distributions of intra- or juxtagenic markers for *FLIP*, *BCL2A1*, *BAG1*, *BPI*, *erbB3*, *TP73*, *TLR9*, *TNFRSF17* and *CARD15* (see Tab. 2). Yet, after correction by Q-value, none of those markers remained significant. On the other hand, a recent study raised the question, whether the correction for multiple comparisons should be applied at all in EAS [26]. For example, in these analyses a previously significantly associated microsatellite (representing the *TNFα* gene), which has been used as a positive control such as *CARD15*, would have been rejected by the correction procedure. Therefore, it remains conceivable that the abovementioned markers represent rather hints for additional predisposing factors/loci with low effect size.

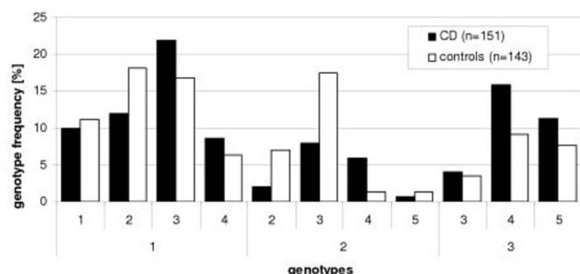

Figure 1
Genotype frequencies of the microsatellite representing the *TNFRSF17* gene. Only genotypes with a frequency of > 0.01 are included. Alleles of the respective microsatellite are indicated as numbers in the X-axis according to their length in bp. For example: 1–1 (read from the number below the numerical series and the first number of the numerical series) means homozygous genotype for microsatellite allele number one and 1–4 heterozygous genotype for allele 1 and 4. Genotypes comprising allele 2 are over-represented within the control group (47% *vs*. 29%; $p_c = 0.0042$ with c = 2), whereas allele 3 genotypes are more frequent in the patient cohort (58% *vs*. 52% $p^c = 0.3130$; c = 2). Therefore, allele 2 might imply a protective effect and/or allele 3 a predisposing effect on CD. Interestingly, the genotype 2–3 is more prevalent in the control group. This result can be interpreted by a different effect size of allele 2 (↑) as compared to allele 3, or the significant difference of this microsatellite is only due to linkage of allele 2 with a protective factor.

The most promising markers (reflected by a significant p-value) were included in further analyses regardless of the correction procedure. Individual genotyping rejected most markers found to be significantly different in the initial step of our approach and only three markers remained significant representing the *TNFRSF17*, *FLIP*, *CARD15* genes (Tab. 2). Obviously, pooled and individual genotyping yield somewhat contradictory results. Eight microsatellites revealed significantly differences between the patient and control cohorts after the pooling procedure, whereas individual genotyping results in the confirmation of 'only' 2 markers. These conspicuous differences might be due to several artefacts caused by analyses with pooled DNA. For example, a typical artefact is the length-dependent amplification of short alleles or the presence of null-alleles. Additionally, consistency of the analyses by a slab-gel system might reflect a further hindrance in this subtle procedure. Nevertheless, individual genotyping eliminates false positive results due to pooling artefacts and, in case of significant results, enables the thorough analyses of the marker alleles in detail (see Fig.1). In order to confirm the aforementioned positive results further markers (SNPs, Tab. 3) were genotyped located in the respective genes in the vicinity of the microsatellites representing *TNFRSF17* and *FLIP*. Yet, RFLP analyses did not reveal any association of the selected SNPs and, therefore, the microsatellite data were not confirmed. On the other hand, these SNPs might not represent regions properly that encompass regulatory elements.

In some instances, the LD of distinct microsatellite alleles covers long genetic distances, thus further gene variations might be in linkage with these alleles. Since the significantly associated '*TNFRSF17*' marker is located at the IBD8 region with 1MBp distance to the major histocompatibility class (MHC) II transactivator (*MHCIITA*), a previously reported functional variation of the *MHC2TA* gene was analysed (see Tab. 4; [30]). MHC2TA regulates the expression of *human leukocyte antigen* (*HLA*) genes regulating the adaptive immune system by presenting antigens to CD4+ T cells, thereby re-activating these cells. The *HLA* region has been implicated in IBD [32]. In addition to the localisation of *MHC2TA* at IBD8 and the associated marker in the adjacent region, the putative biological relevance of the functional rs3087456 polymorphism for CD motivated us to genotype this variation. The analyses did reveal a marginal association in our CD patients when allele or genotype frequencies were compared between the combined control and patient cohorts (see Tab. 4). Yet

Table 4: Allele and genotype frequencies of the functional *MHC2TA* polymorphism (rs3087456).

	Allele frequencies		p value	OR (CI)	Genotype frequencies		p value
CD (n = 147)	C	0.32	0.05	1.33 (0.90–2.01)	CC	0.08	0.54
	T	0.68			CT	0.48	0.06
					TT	0.44	0.03
controls (n = 463)	C	0.26			CC	0.07	
	T	0.74			CT	0.39	
					TT	0.54	

OR: odds ratio; CI: 95% confidence interval

there was no evidence for LD between *TNFRSF17* and *MHC2TA* alleles. In order to validate these data further patient cohorts comprising more individuals must be scrutinised. In addition, other genes that might be linked with the '*TNFRSF17*' marker must be analysed (at least 15 RefSeq genes in the region are encompassed by the microsatellite marker and *MHC2TA*).

In conclusion, this study did not reveal overt evidence for CD predisposition factors in apoptotic (and immune) pathways. Certainly, our approach depends on the LD between the investigated microsatellites and putative predisposing or protective alleles, depending on functional relevance to the disease. Thus, in some instances microsatellites might not be entirely representative for the adjacent genes. Furthermore, the investigated genes only cover part of the factors which coordinate programmed cell death. Yet, future information about haplotype blocks may facilitate more far-fetched interpretations of our analyses.

Additional material

Additional File 1

This file provides detailed information on the sequence of used oligonucleotides, represented gene, marker distance to gene and kind of nucleotide repeat (di, tri, etc.). Furthermore, the file includes graphical information on individually genotyped microsatellites markers with significant differences in allele distributions.

Click here for file

[http://www.biomedcentral.com/content/supplementary/1477-5751-4-8-S1.pdf]

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