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Polymorphisms rs763110 in *FASL* is linked to hepatitis C virus infection among high-risk populations

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ABSTRACT

Background: The Fas cell surface death receptor (FAS) and Fas ligand (FASL) can participate in the apoptosis of immune cells and target cells infected with a virus through the FAS-FASL signalling pathway. The decoy receptor 3 (DCR3) can competitively inhibit the binding of FAS to FASL. Our aim is to investigate the effect of single nucleotide polymorphisms (SNPs) in *FAS, FASL* and *DCR3* on hepatitis C virus (HCV) infection.

Methods: Four SNPs (rs763110 in *FASL*, rs1324551 and rs2234767 in *FAS* and rs2257440 in *DCR3*) were genotyped in 1495 controls free of HCV, 522 individuals with spontaneous HCV clearance and 732 patients with hepatitis C virus infection. The RegulomeDB database and RNAfold web servers were used to explore potential biological functions of SNPs.

Results: *FASL* rs763110 was associated with susceptibility to HCV infection, and not to CHC. The odds ratio (95% confidence interval) of HCV infection in high-risk populations carrying *FASL* rs763110-TT was 1.82 (1.36–2.51, $P < 0.001$) compared to that of CC genotypes and 1.93 (1.43–2.60, $P < 0.001$) higher than that of CC + CT genotypes. Based on computer simulation, *FASL* rs763110-T may affect the transcription of mRNA by affecting the binding of a transcription factor, leading to structural changes in mRNA.

Conclusion: The genetic variant in *FASL* is linked with HCV infection, but not to spontaneous HCV clearance.

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Introduction

Chronic infection with hepatitis C virus (CHCV) is one of the most common infectious diseases worldwide, mainly in high-risk populations such as intravenous drug users and paid blood donors [1,2]. Only 15% ~45% of infected people can self-clear hepatitis C virus (HCV) within 6 months of infection, and currently, about 1% of persons worldwide have chronic HCV infection (~71 million people) [2]. Chronic infection with HCV leads to fibrosis, cirrhosis of liver and hepatocellular carcinoma (HCC) [3]. There is currently no effective vaccine for preventing HCV infection [4]. Therefore, research on factors affecting HCV susceptibility and chronic HCV infection is a top priority.

The ability of the host to clear HCV is influenced by complex factors consisting of virus, host and environment [5]. Certain immune-related gene polymorphisms have been linked with HCV infection, such as interferon-lambda 3 (*IFNL3), HLA-DQB1* and *KIR2DS3* [6–9]. As an important member of the tumour necrosis factor superfamily (TNFSF) and TNF receptor superfamily (TNFRSF), the Fas cell surface death receptor (FAS) and Fas ligand (FASL) play crucial roles in antiviral immunity (both innate and adaptive) [10]. When the host is infected with the virus, FAS and FASL are involved in the regulation of apoptosis of immune and target cells through the FAS/FASL signalling pathway [11]. FAS is also a mediator of apoptosis-independent processes, such as induction of activation and pro-inflammatory signals [12]. In addition to FAS, FASL has another receptor, decoy receptor 3 (DCR3), which can inhibit FASL-mediated apoptosis by competitively binding to FASL over FAS. Apoptosis is a participant in processes eliminating viral infections [10,11]. Increased hepatocyte apoptosis and upregulated expression of death-inducing ligands, such as FAS/FASL, TRAIL and TNFα, are often observed in chronic HCV infected individuals [13]. Thus, FAS and FASL may play an important role in antiviral immunity,

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and this antiviral process may be affected by DCR3. Polymorphisms in *FAS* and *FASL* may inhibit apoptosis, so providing a favourable environment for HCV replication and increases the risk of HCV infection [14,15]. *FASL* gene mutations increase susceptibility to HCV infection by reducing *FASL* expression [16]. However, it is still unclear whether polymorphisms in *FAS, FASL* and *DCR3* have an effect on HCV infection.

In order to determine links between these pathways and HCV infection, we genotyped four SNPs in 1495 uninfected controls, 522 who had naturally cleared a HCV infection and 732 chronic HCV carriers to explore the association of *FASL, FAS* and *DCR3* variants with risk of both HCV infection and CHCV infection.

Methods

The study protocol conformed to ethical guidelines as reflected in approval by the Ethics Committee of the Eastern Theater Command Centers for Disease Control and Prevention, Nanjing, China. All subjects were informed of the purpose of the study and their consents were obtained. During 2006 to 2015, 1495 controls for of any hepatitis virus infection, 522 individuals with spontaneous HCV clearance and 732 patients with CHCV infection were recruited. Only subjects who neither infected with HBV or HIV nor suffered from other liver diseases were enrolled. All patients who had previously received antiviral therapy were excluded. According to the testing results of serum HCV antibody and HCV RNA (positive test results were indicated by '+' and negative by '-'), all qualified subjects were divided into three groups [Uninfected controls: HCV antibody (-), HCV RNA (-); Spontaneous HCV clearance: HCV antibody (+), HCV RNA (-); Persistent HCV infection: HCV antibody (+), HCV RNA (+)].

Ten ml venous blood was collected from each subject for testing for certain liver function tests, HCV antibodies and HCV-RNA loads within 4 h, and for DNA extraction. HCV antibody was tested by ELISA (Beijing Wantai Biological Pharmacy Enterprise Co, Ltd, Beijing, China) and HCV-RNA in the serum was isolated by RT-PCR kit (Takara Biotechnology Co, Ltd, Dalian, China). After centrifugation, genomic DNA in leukocytes was extracted by the Trizols method, with specific steps included proteinase K digestion, phenol-chloroform purification and ethanol precipitation. All DNA samples were stored in a −20°C freezer until further analysis. Regarding the selection of candidate SNPs, first, the linkage disequilibrium (LD) data of the HapMap Phase II CHB (Chinese in Beijing) obtained from the HapMap database (http://www.hapmap.org/) or the 1000 Genomes Project database (http://www.1000genomes.org/) was imported into the Haploview software (version 4.2; Broad Institute, Cambridge, MA, USA) to select the tag SNP ($r^2 \geq 0.8$). The SNPs with the minor allele frequency (MAF) value of the Chinese Han population ≥0.05 were selected in this study. Due to the potential regulation of adjacent sequences, this study also included 2000 bp upstream and downstream of the transcription initiation sites of *FASL* and *FAS* in the analysis. Then, we further combined existing literature on SNPs that met the primary screening criteria [17–20]. If previous studies confirmed that an SNP was associated with an immune disease or an infectious disease, the SNP would be included in our study for further analysis. Finally, four SNPs, *FASL* rs763110, *FAS* rs1324551, *FAS* rs2234767 and *DCR3* rs2257440, were included for further analysis.

Candidate SNPs were genotyped by Taqman real-time PCR assay in the Roche LightCycler 480 Sequence Detection System (Roche Diagnostics, Mannheim, Germany). The information of primers and probes is shown in Table 1 and the reaction initially underwent preheating at 50°C for 2 min and pre-denature at 95°C for 10 min, then 45 cycles (denaturation at 95°C for 15 s and annealing) and extension at 60°C for 1 min. The success rate for each SNP was above 90%. During the course of the experiment, 10% of the samples were randomly selected to repeat the experiment, with a consistent rate of 100%. All genotyping assays were performed without knowledge of the subject's clinical data and two blank controls were set up for each 384-well format for quality control.

The potential biological functions of SNPs were explored using RegulomeDB and RNAfold web server. Human RegulomeDB responds to the potential function of SNPs through the RegulomeDB score, whose

Table 1. Information of probes and primers used for FAS, FASL and DCR3 SNPs

rs763110 (C>T) in FASL (MAF 0.289/0.289)
Probe-A: HEX- CTGCTTTGTATTTCACA -MGB
Probe-G: FAM- TGCTTTGTATTTCGCA -MGB
Forward primer: CAAACCAGTGGAACCCACAGA
Reverse primer: GGGCAAACAATGAAAATGAAAAC
rs1324551 (G>A) in FAS (MAF 0.430/0.378)
Probe-T: HEX-TACTCGTTCTCACCGCA -MGB
Probe-C: FAM-TACTCGTTCCCACCGC-MGB
Forward primer: CCTTCTCTTTCTTCTTTTGCCCTTT
Reverse primer: CTTTTCAAAACAGGCTGCTCAAGTT
rs2234767 (G>A) in FAS (MAF 0.378/0.419)
Probe-A: HEX- AAGGCTGGCACACC-MGB
Probe-G: FAM- AGGCTGGCACGCC -MGB
Forward primer: ACCATCCTCCTTATCCCACTTCT
Reverse primer: AGACTGTTAGTGCCATGAGGAAGA
rs2257440 (T>C) in DCR3 (MAF 0.341/0.393)
Probe-G: HEX-ACTGGGCGCACAC-MGB
Probe-A: FAM-CACTGGGCACACAAC-MGB
Forward primer: GCAGCGCTCTAGGTAGTTCC
Reverse primer: TGGCAGAAACACCCACCTAC

TaqMan-MGB probe/primers sequences (5'-3'). Abbreviations: SNPs, single nucleotide polymorphisms; MAF, minor allele frequency: minor allele frequencies in controls group/minor allele frequencies from HapMap of Han Chinese in Beijing, China (CHB) or East Asia (EAS). (available at https://www.ncbi.nlm.nih.gov/projects/SNP/snp).

unique feature is its ability to comprehensively annotate, integrate and display the experimentally defined functional and biochemical regulatory elements of the human genome. Moreover, the RNA Web server (http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi) based on the latest Vienna RNA package (Version 2.3.1) was used to predict the RNA secondary structure and obtain its minimum free energy (MFE). And the effect of SNP variants on RNA secondary structure was determined by comparing MFE of wild-type and mutant secondary structures.

Differences in demographic characteristics were described by mean with standard deviation or count (proportion) and calculated by using the one-way analysis of variance (for continuous variables) or the chi-square (χ^2) test (for categorical variables). Four genetic models, including additive, dominant and recessive models, were used to analyse the association between each SNP and the outcome of HCV infection. The odds ratios (ORs) and their 95% confidence intervals (CIs) were computed by multivariate logistic regression analyses to estimate the association of SNPs with the outcome of HCV clearance. Stratified analysis and heterogeneity tests were further performed to rule out the effects induced by confounding factors on the final results of this study. The χ^2-based Q test was conducted to assess homogeneity among strata by selected variables. All the statistical analyses were performed by STATA 15.0 software (STATA Corp, College Station, TX, USA), and P-value <0.05 in a two-sided test was considered statistically significant. Bonferroni correction was used to analyse the genotypes distribution among groups, and P-value <0.0125 (0.05/4) in a two-sided test was considered statistically significant.

Results

Table 2 shows the demographic characteristics of the three groups, who were sex but not age matched. Serum levels of alanine aminotransferase (ALT), aspartate transaminase (AST) differed between the three groups. Of the three recruitment groups, paid blood donors were more likely to have a permanent HCV infection (33.1%), intravenous drug users were most likely to have cleared an infection (32.1%) whilst patients on haemodialysis were least likely to be infected (10.5%).

Table 2. Demographic and clinical characteristics among HCV controls, spontaneous clearance and persistent infection populations.

Variables	Controls n=1495	Spontaneous clearance n=522	Persistent infection n=732
Age [years]	53.2 (13.5)	50.3 (14.1)	51.4 (12.2)*
Gender			
Male	579 (39.9)	199 (38.1)	265 (36.2)**
Female	898 (60.1)	323 (61.9)	467 (63.8)
ALT [U/L]	13 (7-21)	25 (16-41)	36 (22-59)*
AST [U/L]	18 (12-25)	28 (20-40)	35 (25-54)*
High-risk population			
HD	568 (38.0)	89 (17.1)	77 (10.5)
IVDU	173 (11.6)	148 (28.4)	140 (19.1)
PBD	754 (50.4)	285 (54.6)	515 (70.4)

Abbreviations: ALT, alanine transaminase; AST, aspartate aminotransferase; HCV, hepatitis C virus; HD, hemodialysis; IVDU, Intravenous drug user, PBD, paid blood donors. *p<0.001, **p=0.229. Data is n (%), mean (SD) or median (IQR).

Allele frequency and genotype distribution of SNPs in the controls group were in Hardy–Weinberg equilibrium (all P>0.05). Genotyping of 1254 patients (Spontaneous clearance: 522, Persistent infection: 732) against the SNP assays had an average success rate of 93.54%. The genotype distribution of the four candidate SNPs and their association with HCV infection are presented in Table 3. The TT genotype of *FASL* rs763110 was associated with HCV infection in both the standard CC/CT/TT analyses and in the recessive TT vs CC+CT model. However, this failed to translate to a difference between those with an active infection and those who cleared the infection. There were differences in the frequencies of rs763110 genotypes the three patient groups in those aged above and below 50, in both men and women in those with ALT or AST <40 U/L, and in the haemodialysis and the paid blood donor groups (Table 4) .

In silico analysis of *FASL* rs763110 function was as follows. Rs763110 is located on near the 5′end of the *FASL*. The RegulomeDB score for rs763110 is 2a, meaning that rs763110 may have potential functions of transcription factor (TF) binding, matched TF motif, matched DNase Footprint and DNase peak. Based on SNPinfo web server, rs763110 may be a TF binding site. To further investigate the effects of mutations on TF and transcriptional regulation, RNAfold web servers were used to further predict the secondary structure of mRNA and calculate the minimum free energy of the centroid structure (a structure with minimal base pair distance). The MFE of the centroid mRNA secondary structure of the mutant U allele (corresponding to the T allele in this study) was −27.70 kcal/mol, which was lower than the wild C allele (corresponding to the G allele in this study, the MEF was −22.50 kcal/mol). In short, mutation in *FASL* rs763110 may affect the transcription of mRNA by affecting the binding of TF, leading to structural changes in mRNA and so potentially mediating the development of the disease.

Discussion

The primary result of our case-sectional study is that the TT genotype in *FASL* rs763110 is linked to infection with HCV. We can only speculate that this link is causative, as many factors over durations of months or years lead to infection, and susceptibility can only be determined by genotyping an uninfected population, followed by a long term follow-up and multivariate analysis to determine those cases who succumb to the infection.

Table 3. Genotype distributions of FAS, FASL and DCR3 genes among persistent infection, spontaneous clearance and controls group.

	Controls, n (%)	Spontaneous clearance, n (%)	Persistent infection, n (%)	(Spontaneous clearance & Persistent infection) *vs* Controls		Persistent infection *vs* Spontaneous clearance	
SNPs (genotype)	n = 1495	n = 522	n = 732	OR (95%CI)[a]	*P*[a]	OR (95%CI)[b]	*P*[b]
rs1324551							
GG	505(35.3)	168(34.6)	232(33.2)	1.00	–	1.00	–
GA	687(48.0)	218(45.0)	337(48.3)	1.03(0.86–1.23)	0.771	1.18(0.90–1.54)	0.232
AA	240(16.8)	99(20.4)	129(18.5)	1.13 (0.90–1.43)	0.293	1.02(0.73–1.44)	0.890
Dominant model				1.06(0.89–1.25)	0.536	1.13(0.88–1.45)	0.334
Recessive model				1.12(0.91–1.38)	0.303	0.93(0.69–1.26)	0.637
Additive model				1.06(0.96–1.19)	0.327	1.03(0.87–1.22)	0.702
rs2257440							
TT	586(42.5)	205(41.6)	276(40.7)	1.00	–	1.00	–
TC	645(46.8)	225(45.6)	331(48.8)	1.13(0.95–1.34)	0.165	1.10(0.85–1.41)	0.479
CC	148(10.7)	63(12.8)	71(10.5)	1.04(0.80–1.37)	0.759	0.83(0.56–1.22)	0.338
Dominant model				1.11(0.94–1.31)	0.203	1.04(0.82–1.32)	0.772
Recessive model				0.98(0.76–1.27)	0.872	0.79(0.55–1.14)	0.202
Additive model				1.06(0.93–1.19)	0.383	0.97(0.81–1.15)	0.690
rs763110							
CC	745(52.9)	260(53.6)	342(49.6)	1.00	–	1.00	–
CT	569(40.4)	179(36.9)	270(39.4)	0.91(0.76–1.08)	0.256	1.13(0.88–1.45)	0.345
TT	95(6.7)	46(9.5)	74(10.8)	1.82(1.36–2.51)	<0.001	1.34(0.88–2.02)	0.169
Dominant model				1.02(0.87–1.20)	0.773	1.17(0.92–1.48)	0.196
Recessive model				1.93(1.43–2.60)	<0.001	1.27(0.85–1.89)	0.242
Additive model				1.14(1.01–1.30)	0.036	1.15(0.96–1.37)	0.134
rs2234767							
GG	557(38.8)	189(38.2)	263(39.1)	1.00	–	1.00	–
GA	701(48.9)	241(48.7)	333(49.6)	0.96(0.81–1.14)	0.634	1.00(0.78–1.29)	0.988
AA	177(12.3)	65(13.1)	76(11.3)	0.95(0.73–1.24)	0.693	0.86(0.58–1.26)	0.442
Dominant model				0.96(0.81–1.13)	0.600	0.97(0.76–1.24)	0.818
Recessive model				0.97(0.76–1.24)	0.813	0.86(0.60–1.23)	0.404
Additive model				0.97(0.86–1.10)	0.615	0.95(0.79–1.13)	0.561

Abbreviations: CI, confidence interval; HCV, hepatitis C virus; OR, odds ratio; SNP, single nucleotide polymorphism. [a]The *P* value, OR and 95% CIs of Spontaneous clearance & Persistent infection *vs* Controls were calculated on the basis of the logistic regression model, adjusted by gender, age and high-risk population. [b]The *P* value, OR and 95% CIs of Persistent infection *vs* Spontaneous clearance were calculated on the basis of the logistic regression model, adjusted by gender, age, high-risk population. Bonferroni correction was applied and the *P* value was adjusted to 0.05/n. A pair of alleles such as A/G, if A is a less frequent gene, then Co-dominant model (GA *vs* GG; AA *vs* GG), Dominant model (GA+AA *vs* GG), Recessive model (AA *vs* GA+GG), Additive model (AA vs GA *vs* GG).

The protein encoded by *FAS* is a member of the TNFRSF. This receptor, containing the death domain, induces the transmission of a death signal through Fas-associated death domain proteins (FADD), caspase 8 and caspase 10 [21]. Genetic variations associated with *FAS* and *FASL* can participate in the development of a variety of malignancies (e.g. ovarian cancer), infectious diseases (e.g. human papillomavirus infection) and immune system diseases (e.g. VKH syndrome) by affecting the signal transduction of these pathways [12,22–26]. Abed et al. found that polymorphisms in *FAS/FASL* may influence the prognosis of HCV infection or HCC [27]. In addition, Bortolami et al. found that the expression of *FASL* mRNA in liver tissue of patients with HCV was significantly higher than that of uninfected controls, indicating a link between *FASL* and HCV infection [28]. Our genotypic data suggesting that mutation in *FASL* rs763110 is linked to HCV infection reflects that of Khalifa et al., who reported increased *FasL* −844 T > C in a small Egyptian cohort [16]. These data also confirm and extend results from Sung et al., who, in a Taiwanese cohort, found that the transcription of *FASL* mRNA in non-small-cell lung cancer tumours was higher in *FASL* rs763110 CC carriers than in *FASL* rs763110 TT+CT carriers [29].

FASL rs763110 is located in the *FASL* promoter on chromosome 1q23. The *FASL* rs763110 C allele and its flanking sequences are the binding site of CAAT Enhancer Binding Protein Beta (C/EBPβ) [30]. Based on the University of California Santa Cruz Genome Browser (genome-www@soe.ucsc.edu), the transcription factor (FOXA1 and C/EBPβ) ChIP-seq in liver tissue clusters in the region where rs763110 is located. Wu et al. [31] reported that the TT rs763110 genotype is linked to FASL expression in cervical cancer, speculating that apoptosis failure is partly due to altered *FASL* binding to transcription factors C/EBPβ and OCT1. In turn, we hypothesise that CAAT consisting of a variant (T) of *FASL* rs763110 may abnormally bind to C/EBPβ, thereby inhibiting downstream gene transcription and down-regulating mRNA expression.

Based on the RNA web server data, we also found that the mutation of rs763110 may affect the secondary structure of mRNA. Changes in mRNA secondary structure can further influence *FASL* mRNA translation, which may be one of the main reasons why Wu et al. found that rs763110 variant down-regulates *FASL* protein expression [31]. They also found that the basal *FASL* expression induced by *FASL* rs763110 C allele

Table 4. Stratified analysis of the association of *FASL* rs763110 with HCV susceptibility.

	Controls	Spontaneous clearance	Persistent infection	(Spontaneous clearance & Persistent infection) *vs* Controls		
Subgroups	n (CC+CT/TT)	n (CC+CT/TT)	n (CC+CT/TT)	OR (95% CI)[a]	P^a	P^b
Age (years)						0.565
<50	499/28	192/14	254/31	2.91(1.62–5.22)	<0.001	
≥50	815/67	247/32	358/43	1.65(1.12–2.44)	0.011	
Gender						0.894
Male	521/47	176/19	214/36	1.81(1.14–2.87)	0.012	
Female	793/48	263/27	398/38	1.99(1.27–3.12)	0.003	
ALT (U/L)						0.339
<40	1240/88	343/35	355/49	2.09(1.49–2.93)	<0.001	
≥40	62/6	95/11	255/25	1.11(0.43–2.90)	0.831	
AST (U/L)						0.527
<40	1239/88	354/33	366/48	2.01(1.44–2.81)	<0.001	
≥40	61/6	78/12	237/24	1.93(0.66–5.63)	0.228	
High-risk population						0.359
HD	497/49	76/11	51/19	2.13(1.24–3.66)	0.006	
IVDU	162/4	136/8	125/12	2.98(0.98–9.03)	0.054	
PBD	655/42	227/27	436/43	1.67(1.08–2.59)	0.021	

Abbreviations: CI, confidence interval; HCV, hepatitis C virus; OR, odds ratio; HD, haemodialysis patients; IVDU, Intravenous drug user; PBD, paid blood donors. [a]The *P* value, OR and 95% CIs of Spontaneous clearance & Persistent infection *vs* Controls were calculated on the basis of the logistic regression model, adjusted by gender, age and high-risk population. [b]*P*-value for the heterogeneity test.

was significantly higher than the T allele, increasing apoptosis activity induced by *Fas/FasL* pathway [21]. Due to the decreased activity of the Apoptosis Signalling Pathways, TT carriers cannot induce apoptosis of infected hepatocytes after HCV infection. Thus, HCV can continue to replicate in infected cells, eventually evading the host's immune system and leading to infection [13,32]. We suggest that the TT genotype of the *FASL* rs763110 may inhibit the apoptosis activity of the *Fas/FasL* pathway and increase the risk of HCV infection by reducing the expression of *FASL*. However, our results were based merely on literature research and computer simulation that will be needed to validate in larger sample population studies and functional experiments.

We must acknowledge some potential limitations in our research. Firstly, only bioinformatics analysis was used to verify the biological functions of candidate SNPs in the current study. Therefore, in our future research, functional experiments are needed to verify the action of these SNPs and to clarify the viral evasion strategy of the *Fas/FasL* pathway. Secondly, the study lacked information such as host *IFNL3* genotype and HCV genotype, which may affect the outcome of HCV infection and antiviral therapy response. However, previous epidemiological studies have shown that HCV genotype 1 was the most common genotype in Chinese high-risk populations [33]. Finally, our data apply only to Chinese high-risk populations, and may not be applicable to the general population or other racial or ethnic groups.

This work represents an advance in biomedical science because it demonstrates that genetic variation in the *FASL* rs763110 (the T allele) is linked to HCV infection in a high-risk Chinese population, which may provide new insights into the prevention, prediction, and treatment of HCV infection.

Summary table

What is known about this subject:
- Polymorphisms in *FAS/FASL* are linked to various malignancies
- *FAS/FASL* SNPs may influence the prognosis of HCV or HCC.
- SNPs in the TNF-related apoptosis-inducing ligand are linked to HCV infection

What this paper adds:
- *FASL* rs763110 is associated with HCV infection within a high-risk population, but not to spontaneous HCV clearance.
- SNPs *FAS* rs1324551, *FAS* rs2234767 and *DCR3* rs2257440 are not linked to HCV infection in high-risk populations.

Disclosure statement

No potential conflict of interest was reported by the authors.

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