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Evaluation of death pathway genes *FAS* and *FASL* polymorphisms in chronic HBV infection

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Summary

This study was designed to determine the possible asssociation between selected *FAS* and *FASLG* polymorphisms and Hepatitis B virus (HBV) infection. *FAS*-670 G/A, *FAS*-1377 G/A, *FASLG*-844 T/C and *FASLG* IVS2nt-124 A/G polymorphisms were genotyped by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP). A total of age and sex matched 108 controls and a hundred chronic HBV patients were recruited to conduct a case–control study. *FAS*-670 polymorphism was associated with chronic HBV infection ($P = 0.03$) *FAS*-1377 GG, GA and AA genotypes among the cases (90%, 5% and 5%, respectively) were significantly different from those among the controls (68%, 31.5% and 5.6%; $P = 0.00$). *FASLG*-844 allele distribution was similar between the groups ($P = 0.17$) but TC genotype (67.3%) was frequent in chronic HBV patients, while CC genotype was found significantly higher (29.6%) in controls. No association between *FASLG* IVS2nt-124 polymorphism and chronic HBV infection could be identified ($P = 0.55$). *FAS*-670 polymorphism is associated with chronic HBV infection, while *FASLG* IVS2nt-124 A/G polymorphism is not. The *FAS*-1377G/A and *FASLG*-844 T/C genotypes are likely to play a substantial role in HBV infection. Further studies evaluating polymorphisms in other genes related with apoptosis are needed to elucidate the role of genetic variation in HBV infection.

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Introduction

Hepatitis B virus (HBV) infection is a major public health problem worldwide. The clinical features of HBV infection vary from clearance of the virus to fulminant hepatitis. The mechanism of susceptibility to chronic persistent HBV infection is not well clarified, while the outcome of HBV infection mainly depends on the host immune response. The persistence in time of HBV infection and the consequent chronic liver inflammation are characterized by an impairment of the immune response mediated by activated cytotoxic T lymphocytes (CTLs), which have an essential role in the immune clearance responsible for the destruction of HBV-infected cells via various pathways, including antiviral cytokines, perforin, granzyme b and the FAS/FASLG system (Bortolami *et al.*, 2008).

The FAS receptor–ligand system plays a key role in apoptotic signalling, in many cell types including cells of the immune system, and disruption of this pathway has been associated with tumorigenesis (Krammer *et al.*, 1994). FAS/CD95 belongs to the family of tumour necrosis factor receptors and binding by FAS ligand (*FASLG*) triggers apoptosis mediated by cytotoxic T lymphocytes (CTL) and natural killer cells. FAS ligand (*FASLG*) on the CTL can bind to a Fas molecule on the virus-infected cell and activate the enzymes that lead to apoptosis of the infected cell by means of destruction of its structural cytoskeleton proteins and by chromosomal degradation (Griffith *et al.*, 1995). The CTL response persists decades after clinical recovery from acute infection and that it can also be observed after resolution of chronicity (Bertoletti & Gehring, 2006). Altered levels of *FAS* and/or *FASLG* expression have been implicated in the pathogenesis of several liver diseases associated with immune regulation (Jung *et al.*, 2007). The *FAS*-1377A allele and *FAS*-–670G allele diminish promoter activity by disrupting Sp1 and STAT1 transcription factor binding sites, respectively, and decrease *FAS* gene expression (Miller *et al.*, 1988). The promoter of the *FASL* gene also has a functional single-nucleotide polymorphism a T or C at position −844, which is located in a binding motif of CAAT transcription factor (Fuks *et al.*, 2005). The basal expression of *FASL* in individuals carrying

the *FASL*–844C allele is higher than that in these carrying the *FASL*-844T allele (Fuks *et al.*, 2005).

The purpose of this study was to determine whether certain *FAS* and *FASLG* polymorphisms might be associated with HBV clearance. We examined *FAS* and *FASLG* polymorphisms in Turkish patient population who had recovered from HBV infections and those who had become chronic carriers.

Materials and methods

Patients

A hundred patients with chronic HBV infection followed up at the department of Infections Diseases and Clinical Microbiology were recruited in this study. Blood samples of healthy control group were collected from Konya State Hospital Blood Center. Healthy control group included sex- and age-matched 108 persons who were monitored for exposure to HBV, human immunodeficiency virus (HIV), hepatitis C virus (HCV) and hepatitis D virus (HDV).

Informed consent form was obtained from all patients, and the study protocol was approved by Meram Medical Faculty Ethical Committee (Approval number 2009/169).

The inclusion criteria of this study are given below:

Healthy control group

Participants must be seronegative anti-HBsAg, HBsAg and antiHBc and have no HBV vaccination story.
The ALT (IU L^{-1}) and AST levels must be in normal ranges.
To rule out confounding by coinfection with HCV or HDV, participants must be seronegative for anti-HCV, HDV antigen or anti-HDVAg and have no detectable HCV RNA.

Chronic Hepatitis B group

Chronic HBV infection is defined by two positive tests for HBsAg and antibodies to HBcAg at least 6 months apart and ALT and/or AST levels >60 IU L^{-1}. To rule out confounding by coinfection with HCV or HDV, participants must be seronegative for anti-HCV, HDV antigen or anti-HDVAg and have no detectable HCV RNA.
Patients must not have any clinical evidence relating to liver cirrhosis.
Patients were excluded from the study if they have one or more of the exclusion criteria: (i) evidence of past or current infection by HCV or HDV; (ii) other systemic disease not related to HBV infection (exp: autoimmune diseases); or (iii) with other hepatitis virus infection.

Molecular analysis

DNA extraction and analysis

Venous blood samples were collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Samples were stored at −20°C until assay. DNA was extracted from whole blood by salting out procedure (Aguilar-Reina *et al.*, 2005).

Genotype analysis of the FAS and FASL gene polymorphisms

Determination of the FAS (CD95, FAS/APO1)-670 G/A (rs 1800682), FAS-1377 G/A (rs2234767), FASL-844 T/C (rs763110), FASL IVS2nt-124 A/G (rs5030772) gene mutations was accomplished with polymerase chain reaction (PCR) and restriction fragment length polymorphism. The oligonucleotide primers used to determine these polymorphisms were described previously (Huang *et al.*, 1997; Stuck *et al.*, 2003; Sun *et al.*, 2004; Zhang *et al.*, 2005; Li *et al.*, 2006a,b; Park *et al.*, 2006).

PCR reaction was performed in a 25 μL volume with 100 ng DNA, 100 μm dNTPs, 20 pmol of each primer, 1.5 mM $MgCl_2$, 1x PCR buffer with $(NH_4)_2SO_4$ (Fermentas, Vilnius, Lithuania) and 2U Taq DNA polymerase (Fermentas). Amplification was performed on an automated Thermal Cycler (Techne Flexigene, Cambridge, UK). PCR conditions were 2 min for initial denaturation at 95°C; 35 cycles at 95°C for 45 s for denaturation, 1 min at 65°C annealing for FAS-670 G/A, FAS-1377 G/A, FASL-844 T/C and 60°C for FASL IVS2nt-124 A/G and 90 s at 72°C for extension, followed by 7 min at 72°C for final extension. The primer sequences are presented in Table 1.

The PCR products of FAS-670, FAS-1377 were digested with 10 U *Mva*I and *Bsh*1236I at 37°C for 14 h, while FASL-844 and FASL IVS2nt-124 PCR products were digested with 10 U *Bse*MI and 10 U *Bse*GI at 55°C for 4 h. The genotyping was determined by fragment separation at 120 V for 40–50 min on a 3% agarose gel containing 0.5 μg mL^{-1} ethidium bromide. A 100-bp marker (100-bp DNA Ladder; Fermentas) was used as a size standard for each gel lane. The gel was visualized under UV light using a gel electrophoresis visualizing system (Vilber Lourmat, Marne-la-Vallée, France). Length of the restriction fragments as listed in Table 1.

All procedures were conducted in a manner blind to the case status and other characteristics of the participants. Scoring of gels and data entry was conducted independently by two persons. We performed the PCRs and evaluated the results without knowing the groups of the subjects. At least 10% of the samples were retested, and the results were 100% concordant.

Statistical analysis

Statistical analyses were carried out using the SPSS statistical package, version 15.0 (SPSS NC., Chicago, IL, USA). The allele frequency distribution at each polymorphism locus was tested against the Hardy–Weinberg equilibrium under the Mendelian biallelic expectation by performing the chi-squared test and the Pearson's chi-squared and Fisher's exact test. Genotype

Table 1. Oligonucleotide primer sequences for PCR amplification, restriction enzymes used for RFLP analysis and length of restriction fragments

Gene and polymorphism	Direction	Primer sequence	Restriction enzyme	Genotypes and restricted fragment size
FAS-670 A/G (rs1800682)	Forward Reverse	5′-CTACCTAAGAGCTATCTACCGTTC-3′ 5′-GGCTGTCCATGTTGTGGCTGC-3′ (GenBank accession no: AY450925)	*MvaI*	GG, 184 bp/101 bp/47 bp, GA, 231 bp/184 bp/101 bp/47 bp AA, 231 bp/101 bp
FAS-1377 G/A (rs2234767)	Forward Reverse	5′- TGTGTGCACAAGGCTGGCGC-3′ 5′- TGCATCTGTCACTGCACTTACCACCA-3′ (GenBank accession no: AY450925)	*Bsh1236I*	GG, 104 bp/18 bp, GA, 122 bp/104 bp/18 bp AA, 122 bp
FASLG-844 T/C (rs763110)	Forward Reverse	5′-CAGCTACTCGGAGGCCAAG-3′ 5′- GCTCTGAGGGGAGAGACCAT-3′ (GenBank accession no: AF027385, Z96050)	*BseMI*	TT, 401 bp TC, 401 bp/233 bp/168 bp CC, 233 bp/168 bp
FASLG IVS2nt-124 A/G (rs5030772)	Forward Reverse	5′-GCAGTTCAGACCTACATGATTAGGAT-3′ 5′- CCAGATACAGACCTGTTAAATGGGC-3′ (GenBank accession no: AF027385, Z96050)	*BseMI*	AA, 219 bp AG, 219 bp/190 bp/29 bp GG, 190 bp/29 bp

associations were assessed by performing the Cochran–Armitage trend test. All tests were two-sided, and probability values <0.05 were considered statistically significant.

Results

The study population consisted of 100 patients with chronic HBV infection. The median ± SD age within the chronic HBV patients was 52.9 ± 12.3 years (age range 25–74 years). The male to female ratio was 54:46%. Healthy control group included sex- and age-matched 108 persons. The median age within the control group was 50.3 years (SD ± 10.7) and the age ranged from 24 to 74 years. No statistically significant differences were found between patients and control group subjects in terms of median age and sex distribution.

Allele frequencies and genotype distributions of *FAS* and *FASL* polymorphisms in patients and controls are shown in Table 2.

Genotype distribution and allele frequencies of *FAS* (*CD95, FAS/APO1*) −670 A/G (*rs*1800682) polymorphism

Allele frequencies difference of this polymorphism among patients and controls was statistically significant ($P = 0.03$). There was a significant difference between homozygous wild-type and mutant genotype frequencies among patients and controls ($P = 0.02$).

Genotype distribution and allele frequencies of *FAS* (*CD95, FAS/APO1*) −1377 G/A (*rs*2234767) polymorphism

FAS-1377 mutant allele frequency was significantly greater among chronic HBV group versus healthy controls ($P = 0.00$). However, homozygous genotypes did not differ between patients and healthy controls ($P = 0.45$). However, the genotype distribution of this polymorphism was against Hardy–Weinberg equilibrium in cases [$P = 5.253$(log likelihood ratio chi-squared=Llr)] not in controls [($P = 0.53$(Llr)]. So, it was planned to sequence all 208 samples at this SNP directly in a further study.

Genotype distribution and allele frequencies of *FASLG*-844 T/C (*rs*763110) polymorphism

Total allele frequencies of *FASL*-844 were not significantly different between the two groups ($P = 0$, 17) but when heterozygous and homozygous mutant genotypes were compared with each other, there was a significant difference ($P = 0.00$). The TC genotypes of *FASL*-844 were higher in chronic HBV group, while the CC genotype was higher in healthy controls. This indicates the susceptibility to chronic HBV infection.

Genotype distribution and allele frequencies of *FASLG* IVS2nt −124 A/G (*rs*5030772) polymorphism

There was no significant difference in the genotype or allele frequencies of the *FASL*-124 polymorphism among the groups ($P = 0.367$, Table.2). This polymorphism was not associated with chronic HBV infection.

Discussion

The FAS/FASLG system plays an important role in regulating extrinsic apoptotic pathway in liver. In chronically damaged liver tissues, FAS/*FASLG* system expression is upregulated, reaching a peak in patients with cirrhosis (Bortolami *et al.*, 2008). Polymorphisms in promoter region of the *FAS*-670 A/G, *FAS*-1377 G/A and *FASLG*-844 T/C have been shown to alter the transcriptional activities of these genes (Kanemitsu *et al.*, 2002). In this study, we examined whether genetic polymorphisms in death pathway genes *FAS* and *FASLG* were associated with the outcome of HBV infection. *FAS*-670 A/G, *FAS*-1377 G/A, *FASLG*-844 T/C and *FASLG* IVS2nt-124 A/G polymorphisms among the patients with chronic HBV infection and

Table 2. Genotype and allele frequencies of *FAS* and *FASL* polymorphisms in controls and chronic hepatitis B virus (HBV) patients

	Tests for deviation from Hardy–Weinberg equilibrium			Tests for association (C.I.: 95% confidence interval)				
SNP	Genotype	Controls	Chronic HBV patients	Allele freq. difference	Heterozygous	Homozygous	Allele positivity	Armitage's trend test
FAS position −670	AA	n_{11} = 38 (35.59%)	n_{11} = 45 (45.34%)	[A]<->[G]	[AA]<->[AG]	[AA+]<->[GG]	[AA]<->[AG+GG]	Common OR
	AG	n_{12} = 48 (52.81%)	n_{12} = 44 (43.31%)	OR = 0.644	OR = 0.774	OR = 0.384	OR = 0.651	OR = 0.642
	GG	n_{22} = 22 (19.59%)	n_{22} = 10 (10.34%)	C.I. = [0.431–0.962]	C.I. = [0.427–1.403]	C.I. = [0.162–0.910]	C.I. = [0.372–1.139]	
		f_a1 = 0.57 ± 0.035	f_a1 = 0.68 ± 0.033	χ^2 = 4.64	χ^2 = 0.71	χ^2 = 4.88	χ^2 = 2.27	χ^2 = 4.40
		F = 0.09116	*F* = −0.01586	p = 0.03125 (P)	p = 0.39843	p = 0.02714	p = 0.13208	p = 0.03587
		p = 0.343432 (Pearson)	p = 0.874624 (Pearson)					
		p = 0.343699 (Llr)	p = 0.874430 (Llr)					
		p = 0.331979 (Exact)	p = 1.000000 (Exact)					
FAS position −1377	GG	n_{11} = 68 (66.90%)	n_{11} = 90 (85.56%)	[G]<->[A]	[GG]<->[GA]	[GG+]<->[AA]	[GG]<->[GA+AA]	
	GA	n_{12} = 34 (36.20%)	n_{12} = 5 (13.88%)	OR = 0.300	OR = 0.111	OR = 0.630	OR = 0.189	OR = 0.501
	AA	n_{22} = 6 (4.90%)	n_{22} = 5 (0.56%)	C.I. = [0.161–0.556]	C.I. = [0.041–0.299]	C.I. = [0.184–2.150]	C.I. = [0.088–0.404]	
		f_a1 = 0.79 ± 0.029	f_a1 = 0.93 ± 0.024	χ^2 = 15.80	χ^2 = 24.41	χ^2 = 0.55	χ^2 = 20.79	χ^2 = 12.63
		F = 0.06087	*F* = 0.63964	p = 0.00007 (P)	p = 7.788e-07	p = 0.45698	p = 5.135e-06	p = 0.00038
		p = 0.527011 (Pearson)	p = 1.591e-10 (Pearson)					
		p = 0.534347 (Llr)	p = 5.253e-06 (Llr)					
		p = 0.564796 (Exact)	p = 9.629e-06 (Exact)					
FASL position −844	TT	n_{11} = 22 (22.23%)	n_{11} = 18 (26.54%)	[T]<->[C]	[TT]<->[TC]	[TT+]<->[CC]	[TT]<->[TC+CC]	
	TC	n_{12} = 54 (53.54%)	n_{12} = 66 (48.92%)	OR = 0.765	OR = 1.494	OR = 0.535	OR = 1.137	OR = 0.728
	CC	n_{22} = 32 (32.23%)	n_{22} = 14 (22.54%)	C.I. = [0.519–1.128]	C.I. = [0.728–3.067]	C.I. = [0.221–1.295]	C.I. = [0.568–2.274]	
		f_a1 = 0.45 ± 0.034	f_a1 = 0.52 ± 0.029	χ^2 = 1.83	χ^2 = 1.20	χ^2 = 1.94	χ^2 = 0.13	χ^2 = 2.19
		F = −0.00865	*F* = −0.34919	p = 0.17608 (P)	p = 0.27272	p = 0.16338	p = 0.71663	p = 0.13847
		p = 0.928392 (Pearson)	p = 0.000547 (Pearson)					
		p = 0.928388 (Llr)	p = 0.000473 (Llr)					
		p = 1.000000 (Exact)	p = 0.001067 (Exact)					
FASL position - 124	AA	n_{11} = 66 (66.90%)	n_{11} = 64 (62.41%)	[A]<->[G]	[AA]<->[AG]	[AA+]<->[GG]	[AA]<->[AG+AA]	
	AG	n_{12} = 38 (36.20%)	n_{12} = 26 (29.18%)	OR = 0.864	OR = 0.706	OR = 1.289	OR = 0.761	OR = 0.938
	GG	n_{22} = 4 (4.90%)	n_{22} = 5 (3.41%)	C.I. = [0.531–1.407]	C.I. = [0.385–1.293]	C.I. = [0.331–5.017]	C.I. = [0.427–1.356]	
		f_a1 = 0.79 ± 0.027	f_a1 = 0.81 ± 0.030	χ^2 = 0.35	χ^2 = 1.28	χ^2 = 0.13	χ^2 = 0.86	χ^2 = 0.34
		F = −0.04962	*F* = 0.10895	p = 0.55637 (P)	p = 0.25844	p = 0.71361	p = 0.35393	p = 0.56065
		p = 0.606114 (Pearson)	p = 0.288290 (Pearson)					
		p = 0.599604 (Llr)	p = 0.306302 (Llr)					
		p = 0.777168 (Exact)	p = 0.312903 (Exact)					

HBV, hepatitis B virus; $P < 0.05$; C.I., 95% confidence interval; OR, odds ratio; f a1, Frequency of allele 1 ± standard deviation; *F*, Inbreeding coefficient; p (Pearson), Pearson's goodness-of-fit chi-square (degree of freedom = 1); p (Llr), Log likelihood ratio chi-square (degree of freedom = 1); p (Exact), Exact test.

healthy individuals were analysed. *FASLG* IVS2nt-124 A/G polymorphisms exhibited no apparent relationship with the clearance of HBV infection.

Huang *et al.*, (1997) showed that *FAS*-670 A/G and *FAS*-1377 G/A polymorphisms disrupt the function of protein-binding elements of SP-1 (stimulatory protein 1) and STAT1 (signal transducer and activator of transcription). So, these polymorphisms put down promoter activity and reduce *FAS* gene expression (Miller *et al.*, 1988). It was reported in the same study that the presence of the −670A allele was associated with higher levels of necrosis in periportal areas in chronic hepatitis C patients (Miller *et al.*, 1988). This hypothesis was supported by a study reporting an association between the *FAS*-670 A/G polymorphism and systemic lupus erythematosus. In this report, it was demonstrated by electrophoretic mobility shift assay that the −670A allele had a higher binding ability of STAT1 than −670A/G (Sibley *et al.*, 2003). Aguilar-Reina *et al.*, (2005) suggested an association between the −670A/G polymorphism and the grade of necrosis in periportal areas in patients with chronic hepatitis C. On the contrary, there was no association between the variant −1377 G/A and the severity of liver damage (Li *et al.*, 2006a,b). With regard to the −1377 G/A polymorphism, Sibley *et al.* has shown that the −1377A allele has significantly reduced SP1 binding capacity compared with the G allele. They postulated that reduced SP1 binding in the *FAS* promoter resulted in a decrease in *FAS* expression (Huang *et al.*, 1999). According to our results, GG genotype was common in controls, and GA genotype was frequent in chronic HBV patients. When the results of the published data were compared, it was seen that the frequencies of the GG, AG and AA genotypes of the *FAS*-1377G/A among 252 southern Han Chinese control subjects were 32.1%, 49.2% and 18.7%, respectively, while among Caucasians in the study by Huang *et al.*, those were 76%, 22% and 2%, respectively (Wu *et al.*, 2003; Zhang *et al.*, 2005). The difference in our study may be due to racial characteristics or technical difficulties.

It was shown that *FASLG*-844 T/C polymorphism located in the 5′promoter of the FASLG gene lies within a putative binding motif for CAAT/enhancer-binding protein β(C/EBPβ), and a higher basal expression has been associated with the presence of the *FASLG*-844C rather than *FASLG*-844T allele (Fuks *et al.*, 2005). It was indicated that the −844 T/C polymorphism was not related with the susceptibility to HCV infection, the severity of liver damage and the progression of fibrosis in a cohort of Spanish patients (Ruiz-Ferrer *et al.*, 2007). In a Korean study, it was indicated that there were no associations between *FAS* or *FASLG* promoter polymorphisms and the HBV outcome and HBeAg clearance (Jung *et al.*, 2007). According to our results, the TC genotypes of *FASL*-844 were higher in chronic HBV group, while the CC genotype was higher in healthy controls.

FAS and FASLG are the primary inducers of membrane-induced (receptor-mediated) cell death. Administration of anti-FAS antibody in mice induces massive cell loss and hepatic failure (Ogasawara *et al.*, 1993), and there is increasing evidence that apoptosis contributes to liver injury, especially in alcoholic liver disease, viral hepatitis and in autoimmune liver disease (Kondo *et al.*, 1997; Pinkoski *et al.*, 2000; Natori *et al.*, 2001; Agarwal *et al.*, 2007). Given the roles of the FAS/FASLG system in carcinogenesis, it is biologically plausible that the *FAS* and *FASLG* polymorphisms may modulate risk of cancer. It has been shown that alterations of *FAS* and *FASLG* expression decrease the apoptotic capacity of cells and that many tumour cells might evade or suppress the immune system (Shimonishi *et al.*, 2000). The loss of *FAS* and gain of *FASLG* expression are common features of most human malignancies and are associated with progression of cancers (Lee *et al.*, 1999; Shimonishi *et al.*, 2000; Sun *et al.*, 2004; Zhang *et al.*, 2005). Bortolami showed a progressive increase in *FAS/FASLG* system expression with the severity of liver disease from chronic hepatitis to cirrhosis, followed by a decline from the latter to hepatocellular carcinoma in chronic HBV and HCV liver disease (Bortolami *et al.*, 2008).

In conclusion, *FAS*-670 polymorphism is associated with chronic HBV infection, while *FASLG* IVS2nt-124 A/G polymorphism is not. The *FAS*-1377G/A and *FASLG*-844 T/C genotypes are likely to play a substantial role in HBV infection. Further studies evaluating polymorphisms in other genes related with apoptosis are needed to elucidate the role of genetic variation in HBV infection.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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