RESEARCH ARTICLE

Genetic Polymorphisms of Fas/FasL Promoter Associated with Hepatitis C cirrhosis and HCC

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Abstract

Aim: The present study was performed to determine any associations of genetic polymorphisms of Fas/FasL promoter regions, at Fas670 and Fas1377 and FasL844, with hepatitis C cirrhosis and HCC, with a focus on severity of disease. **Methods:** Totals of 120 patients with cirrhosis and 101 with hepatocellular carcinoma (HCC) were enrolled. All had chronic HCV infection as indicated by positive anti-HCV antibodies and positive HCV RNA on real time PCR. One hundred healthy control subjects were also included in the study. Patients were subjected to full clinical, radiological and histopathological examinations. In addition to routine laboratory tests for liver function tests, Fas670 and Fas1377 and FasL844 genetic polymorphisms of Fas/FasL promoter regions were assessed by RFLP-PCR (restriction fragment length polymorphism with polymerase chain reaction). **Results:** Significant higher levels of the AG genotype in Fas670 and Fas1773 were observed in patients with cirrhosis and HCC (P=0.0001) as compared to control subjects. In addition, the CC genotype in FASL844 was also more common in patients (P=0.01). Furtehrmore, there was a significant association of substitution of A by G alleles in Fas670 and Fas1773 with advanced BCA staging (P=0.02, P=0.0001 respectively) and larger tumor size >5cm (P=0.01, P=0.0001 respectively) and in Fas670 with advanced pathological grading (P=0.0001). Moreover the CC genotype of FASL844 was significantly linked with advanced BCA, large tumor size >5cm and advanced pathological grading (P=0.0001). **Conclusion:** The findings of the present study highlight associations of genetic polymorphisms of promoter regions in Fas and Fas L with cirrhosis and HCC associated with chronic HCV. Support was also obtained for the conclusion that single nucleotide polymorphisms of the Fas/ FasL system impact on clinical and histopathological grading of HCCs. Further large scale studies are recommended for confirmation.

Keywords: HCV- HCC- genetic polymorphism in promoter regions of Fas/FasL- cirrhosis

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Introduction

Hepatitis C (HCV) continues to be a global health burden affecting around 170 million populations each year. Chronic HCV is associated with liver cirrhosis and hepatocellular carcinoma in around 20% of the affected patients (Wang et al., 2003; Ierardi et al., 2010).

There are several mechanisms that contribute to HCV associated hepatic complications that is attributed mainly to HCV persistence due to evasion of cell mediated immune response. The persistence of HCV in hepatocytes leads to repeated attempts by cytotoxic T lymphocytes (CTL) to clear infected hepatocytes (Mirouxet al., 2010). This eventually leads to the presence of histological necro-inflammation in chronic HCV.

The attempt of clearance of infected hepatocyte is mediated by several immune mechanisms among which are apoptosis of infected hepatocytes (Guidotti et al., 2006). The apoptosis is mediated by Fas and its ligand FasL, a transmembrane receptor that is a member of the tumor necrosis factor receptor superfamily, which plays an important role in embryogenesis, autoimmunity, liver cirrhosis and tumorigenesis. Fas/Fas-L system is composed from two Fas receptor isoforms and their natural ligands (Zeisel et al., 2005; Zaki et al., 2008).

In normal physiology, hepatocytes express low levels of the Fas receptors. Fas receptor is overexpressed on the hepatocytes in the presence of extrinsic or intrinsic provocative signals associated with inflammatory cytokines such as IL-1 and DNA damage due to the oxidative stress leading to hepatocytes apoptosis by the Fas system (Guicciardiet al., 2004),The apoptosis is mediated by the interaction between Fas receptor on hepatocytes and Fas ligand on cytotoxic T cell (Abe et al., 2001).

The main etiology of HCV persistence in the host body for years leading to the onset of chronic complications is due to HCV core protein that represses the apoptosis to

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escape the host immune response (Guicciardi et al., 2005).

Host factors such as the genetic polymorphisms with functional differences of Fas/FasL genes are reported to be associated with HCV related liver cirrhosis (Penget al., 2005; Feitelson et al., 2009).

Fas gene is located at chromosome 10. The most common polymorphisms of the gene involves single nucleotide at the promoter region namely Fas670A>G rs1800682 and Fas1377G>A rs2234767. This single nucleotide substitution has been shown to destroy stimulatory protein 1 and signal transducer and activator of transcription 1 (STAT1) protein binding element, respectively thus leading to decrease the promoter activity leading to the decrease fas expression (Huang et al., 1997; Sibleyet al., 2003). In regard to FasL gene it is located on the chromosome 1. The important polymorphism involves FasL844T>C (rs763110) in the promoter region. The presence of FasL844 C allele is associated with higher expression of FasL than T allele leading to the stimulation of the apoptotic activity of the Fas/Fas pathway (Wu et al., 2003).

The gold standard for diagnosis and staging of cirrhosis is histopathological examination of liver biopsy. However, this method has limitations mainly to the variability of the sampling (Ratziu et al., 2005). Radiological methods using magnetic resonance imaging (MRI), ultrasonography, computerized tomography (CT) can provide additional diagnostic data can be obtained from radiological methods like abnormality in hepatic texture or surface, rarefied hepatic central vein, an enlarged caudate lobe, splenomegaly or collateral veins (Martinez-Nogueraet al., 2002).

For diagnosis and staging of HCC various methods are used. The patients with cirrhosis are subjected to regular six months interval follow up by abdominal ultrasound and serum alpha-fetoprotein determinations. When there is an abnormality detected by follow up methods, advanced radiological methods such as helicoidal computed tomography and magnetic resonance are used for confirmation of the diagnosis and the tumor staging. The attempt to perform histopathological examination is reserved only in cases of the data shortage of the imaging methods to diagnose the lesion. Eight classifications are available for tumor staging for determination of the prognosis and to the therapeutic approach. Among those classifications, the Barcelona Classification (BCLC) attempts to correlate tumor stage with treatment (França1 et al., 2004).

The present study was performed to study the genetic polymorphisms of Fas/FasL promoter regions of Fas670 and Fas1377 and FasL844 associated with hepatitis C cirrhosis and HCC and the relation between the polymorphism and the severity of the diseases.

Materials and Methods

Patients

The study is retrospective case-control study. Patients were recruited from Mansoura University hospital, tropical medicine department, Egypt from January 2014 to January 2016. The patients were classified to two groups patients with hepatitis C virus associated cirrhosis and patients with HCC on top of HCV chronic infection. Exclusion criteria included patients with hepatitis B virus, HIV and patients with other cancers.

The study was approved by Mansoura Faculty of Medicine ethical committee. The participants signed written approval consents. In addition to patients, 100 age and sex matched healthy volunteers were included in the study.

Methods

All patients were assessed by clinical examination and pathology reports for liver biopsies from patients with cirrhosis. Lover biopsies were performed by the use of ultrasound guidedTruCutliver technique.Liver biopsies were analyzed according to a histological METAVIR scoring system (O'Brien et al., 2005).

The staging of patients with HCC was performed by the use of clinical data, laboratory data and radiological investigations including ultrasound and CT as indicated and reported according to BCLC.

Ten millimeter blood samples were obtained from each participant and divided into two aliquots. One aliquot over EDTA for genomic DNA separation from the monuclear cells and the other aliquot was plain aliquot for sera separation. Sera were subjected to routine laboratory study of complete liver functions by autoanalyzer (Dialab 450 system), determination of circulatory anti-HCC by Elecys system (Roche-diagnostic) and fetoprotein (AFP) measurement by enzyme linked immunosorbant assay (ELISA - DRG International Inc., USA.).

The presence of HCV-RNA in patients' sera was detected by real-time polymerase chain reaction.

Molecular Analysis of Fas/FasL Polymorphism
DNA extraction

Blood samples on EDTA were subjected to monuclear cells separation and DNA was extracted by the use of DNA mini extract kit from Qiagen(Hilden, Germany) according to the manufacturer's instructions.

PCR-Restriction fragment length polymorphism

Genotyping of FAS and FASL Polymorphisms was determined by polymerase chain reaction (PCR)–Restriction Fragment Length according to the method previously described (Zhang et al., 2007).

The primers used for PCR reaction and the restriction enzymes with bp products were summarized in Table 1.

Statistical Analysis

Data were collected, revised, coded and entered to the statistical package for social science (SPSS) version 20. The quantitative data were presented as mean, standard deviations and ranges. The comparison between the studied groups was done by using One Way Analysis of Variance (ANOVA).

Results

The patients enrolled in the study were 120 patients with cirrhosis and 101 patients with HCC all patients

Table 1. Primers Used for PCR and Restriction Endonuclease Enzymes and Bp of the Products

Gene	Primers	Restriction endonuclease enzyme	base pairs (bp)
FAS-670	F:ATA GCT GGG GCT ATG CGA TT	BstUI	FAS-1377A fragment of 122 bp
	R: CAT TTG ACT GGG CTG TCC AT		FAS-1377G fragments of 104 and 18 bp.
FAS-1377	F: TGT GTG CAC AAG GCT GGC GC	BstUI	FAS-1377A fragment of 122 bp
	R: TGC ATC TGT GTC ACT GC ACTT ACC ACC A		FAS-1377G with fragments of 104 and 18 bp.
FASL-844	F: CAG CTA CTC GGA GGC CAA G R:GCT CTG AGG GGA GAG ACC AT	BsrD	FASL-884C allele with 233- and 168-bp fragments

Table 2. Demographic, Clinical and Laboratory Findings of Patients with Cirrhosis (n=120)

Parameter	
Gender	
Male	93 (77.5%)
Female	27 (22.5%)
age	50.8± 5.5
Child-Pugh score	
A	18 (15%)
B	36 (30%)
C	66 (55%)
Splenomegaly	53 (44.2%)
Ascites	
Mild	8 (6.7%)
Moderate	13 (10.8%)
Severe	38 (31.7%)
Albumin (gm/dl)	3.0± 1.1
Total bilirubin (mg/dl)	3.4± 2.3
Direct bilirubin(mg/dl)	2.1± 1.1
AST(IU/l)	54.1± 20.9
ALT (IU/l)	40.1± 14.5
AFP (nglml)	11.9± 1.4

Table 3. Demographic, Clinical, Pathological, Radiological and Laboratory Data of Patients with HCC

Parameter	
Gender	
Male	74 (73.3%)
Female	27 (26.7%)
Age Mean± SD (years)	58.2± 10.5
Histopathological Grading	
I	31 (30.7%)
II	38 (37.6%)
III	24 (23.8%)
IV	8 (7.9%)
BCLA	
A	45 (44.5%)
B	30 (29.7%)
C	22 (21.8%)
D	4 (4.0%)
No. of masses	
1	78 (79.6%)
2	12 (12.2%)
multiple	11 (8.2%)
Size of the tumour	
<5cm	51 (50.5%)
>5cm	50 (49.5%)
Albumin (gm/dl)	3.2± 1.2
Total bilirubin(mg/dl)	1.3± 0.4
Direct bilirubin (mg/dl)	0.7± 0.4
AST (IU/l)	89.8± 9.2
ALT(IU/l)	70.8± 9.7
AFP (ng/ml)	922.5± 101.8

had chronic HCV as indicated by positive anti-HCV and positive HCV RNA by real time PCR. The mean age±SD of the patients with cirrhosis was 50.8± 5.5 years. They were mainly male93 (77.5%). Child-Pugh score classification of the patients showed they were mainly in score C (55%) and score B (30%).The majority of patients had ascites mainly severe (31.7%). The mean± SD value of ALF was 11.9± 1.4ng/ml, Table 2.

In Table 3, the demographic, clinical, pathological, radiological and laboratory findings of the patients with HCC were analyzed. The main age± SD of the patients was 58.2± 10.5 years and they were mainly male (73.3%). The pathological grading of HCC Tumor revealed that grade I and grade II were the most frequent among the patients (30.7%, 37.6% respectively). Regarding BCLA classification the patients were mainly in A and B stages (44.5%, 29.7% respectively). The majority of the patients had single mass (79.6%) and half of the patients (50.5%) had the tumor size less than 5cmm. The mean± SD value of ALF was922.5± 101.8 ng/ml.

Table 4 summarizes the polymorphism distribution of Fas/FasL genes among patients and control subjects. The distinguished finding the significant increase of AG polymorphism in Fas670 and Fas1773 in patients with cirrhosis and in patients with HCC (P=0.0001) when compared to control subjects. In the meantime there was significant increase in CC polymorphism in FASL844 in patients with cirrhosis and in patients with HCC when compared to the control subjects (P=0.01).

Though there was frequent presence of AG genotypes of Fas670, Fas1773 among higher grade of cirrhosis in Child-Pugh classification and CC genotype in FasL844, this increase was statistically insignificant (P=0.5, P=0.07,

Table 4. Genotypic Polymorphism of Fas/FasL among Patients and Control.

	Fas670			Fas1773			FASL844		
	AA	AG	GG	AA	AG	GG	TT	CC	TC
	No. %	No. %	No. %	No. %	No. %	No. %	No. %	No. %	No. %
Patients with cirrhosis (n=120)	27 (22.5%)	60 (50%)	33 (27.5%)	24 ((20%)	75 (62.5%)	21 (17.5%)	20 (16.7%)	79 (65.8%)	21 (17.5%)
Patients with HCC(n=101)	25 (24.5%)	54 (53.5%)	22 (21.8%)	21 (20.8%)	58 (57.4%)	22 (21.8%)	20 (19.8%)	61 (60.4%)	20 (19.8%)
Control(n=100)	35 (35%)	40 (40%)	25 (25%)	20 (20%)	55 (55%)	25 (25%)	25 (25%)	58 (58%)	117 (17%)
P		P=0.0001			P=0.0001			P=0.01	

Table 5. Association of Genotypic Polymorphism of Fas/FasL and Clinical Findings in Patients with Cirrhosis

	Fas670				Fas1773				FASL844			
	AA	AG	GG	P	AA	AG	GG	P	TT	CC	TC	P
Child Classification												
A (n=18)	3 (16.7%)	9(50%)	6(33.3%)	P=0.5	7(38.9%)	3(16.7)	8(44.4%)	P=0.07	5(27.8%	5(27.8)	8(44.4%)	P=0.06
B (n=36)	9(25%)	21(58.3%)	6(16.7%)		6(16.7%)	25(69.4%)	5(13.9.%)		5(13.9%)	26(72.2%)	5(13.9%)	
C (n=66)	15(22.7%)	30(45.5%)	21(31.8%)		11(16.7%)	45(68.2%)	10(15.2%)		10(15.2%)	46(69.7%)	10(15.2%)	
Splenomegaly (n=53)	20(37.7%)	20(37.7%)	13(24.5%)	P=0.8	18(33.9%)	18(33.9%)	17(32.1%)	P=0.9	5(9.4%)	30(56.6%)	18(33.9%)	P=0.9

P=0.06 respectively) (Table 5).

The other distinguished finding of the present study was the significant frequent association of substitution of A by G alleles in Fas670 gene and in Fas1773 in advanced BCA staging(P=0.02, P=0.0001 respectively) and in the presence of larger tumor with size >5cm (P=0.01, P=0.0001 respectively) substitution of A by G alleles in Fas670 in advanced pathological grading (P=0.0001). Moreover the CC genotype of FASL844 was the significant frequent gene associated with advanced BCA, large tumor size >5cm and advanced pathological grading (P=0.0001), Table 6.

Discussion

The effect of HCV on hepatocytes is related mainly to the immune mediated inflammatory response that can lead either to the clearance of the infection or destruction of the liver with development of HCC and cirrhosis. The balance between different immunological mechanisms is the principal guide to the prognosis of HCV (Ruggieri et al., 2003; Zeisel et al., 2011).

Among the immune response to HCV, apoptosis has the role of eliminating infected hepatocytes with HCV. Both exaggerated apoptosis and lack of it are associated with chronic HCV complications. Lack of apoptosis will lead to HCV persistence in the hepatocytes with

Table 6. Association of Genotypic Polymorphism of Fas/FasL and Pathological and Radiological Findings in Patients

	Fas670				Fas1773				FASL844			
	AA	AG	GG	P	AA	AG	GG	P	TT	CC	TC	P
BCA												P=0.000
A(n=45)	15(30%)	20 (44.4%)	10(22.2%)	P=0.02	7(15.6%)	30(66.7%)	8(17.8%)	P=0.000	4 (8.9%)	27(60%)	14(31.1%)	
B(n=30)	8 (26.7%)	14(46.7%)	8(26.7%)		4(13.3%)	14(46.7%)	12(40%)		6(20%)	18(60%)	6(20%)	
C(n=22)	2 (9.1%)	16((72.7%)	4(18.2%)		10(45.5%)	1254.5%)	0(0%)		10(45.4%)	12 (54.5%)	0(0%)	
D (n=4)	0 (0%)	4(100%)	0(0%)		0(0%)	2(50%)	2(50%)		0(0%)	4 (100%)	0(0%)	
Tumor size				P=0.01				P=0.000				P=0.000
<5 cm (n=51)	12(23.5%)	23(45.1%)	16(31.4%)		15(29.4%)	26(50.9%	10(19.6%)		9(17.6%)	26(50.9%)	16(31.4%)	
>5cm(n=50)	13(26%)	31(62%)	6(12%)		6(12%)	32(64%)	12(24%)		11(22%)	35(70%)	4(8%)	
No. of masses												
1(n=78)	22 (28.2%)	40 (51.3%)	16(20.5%)	P=0.4	18(23.1%)	42 (53.8%)	18(23.1%)	P=0.2	15(19.2%)	49(62.8%)	14(17.9%)	P=0.2
2(n=12)	1 (8.3%)	7(58.3%)	4(33.3%)		1 (8.3%)	7(58.3%)	4(33.3%)		2 (16.7%)	7 (58.3%)	3(25%)	
Multiple(n=11)	2 (18.2%)	7(63.6%)	2(18.2%)		2(18.2%)	8(72.7%)	1(9.1%)		3(27.3%)	5(45.5%)	3(27.3%)	
Grading												P=0.0001
I (n=31)	8(25.8%)	16(51.6%)	7(22.5%)	P=0.05	1 (3.2%)	20 (64.5%)	10(32.2%)	P=0.0001	3(9.6%)	18(58.1%)	10(32.3%)	
II(n=38)	10(32.3%)	16(51.6%)	12(38.7%)		6(15.8%)	22(57.9%)	10(26.3%)		12 (38.8%)	18(47.4%)	8(21.1%)	
III(n=24)	6(25%)	16(66.7%)	2(8.3%)		12(50%)	12(50%)	0(0%)		4(16.7%)	20(83.3%)	0(0%)	
IV(n=8)	0	6(75%)	2(25%)		2(25%)	4(50%)	2(25%)		2(25%)	4(50%)	2(25%)	

evasion of immune system. While exaggerated apoptosis is associated with marked inflammatory reaction in the liver leading to severe inflammation associated with HCV infection (El Bassiouny et al., 2008; Bortolami et al., 2008; Zaki et al., 2008).

Genetic polymorphisms that change the level of Fas expression in hepatocytes and Fas L on cytotoxic lymphocytes can affect HCV progress among the patients (McIlroy et al., 2005).

The distinguished finding in the present study was the significant increase of AG polymorphism in Fas670 and Fas1773 among patients with HCV associated cirrhosis with the increase in CC genotype in FASL844. Similar result was reported by previous study (Liao et al., 2016). The single nucleotide substitution of A by C at the promoter region of Fas gene is known to be associated with decease in the promoter activity thus decreasing Fas expression (Huang et al., 1997; Sibley et al., 2003). The decrease of Fas expression leads to viral persistence due to the evasion of immune system. In the meantime the over expression of FasL caused by the presence of FasL844 C allele is associated with higher expression of FasL (Wu et al., 2003). The overexpression of FasL may induce apoptosis of activated T lymphocytes and aid in HCV persistence (Nada et al., 2005).

Similar genotypes were noticed among patients with HCC in the present study. Previous studies had reported that down-regulation of Fasin the presence of up-regulation of FasL, were important in tumor evasion from immune surveillance in hepatic tumor development and this leads to the assumption that the use of the components of the Fas system as targets for anticancer therapy. In addition, the linear correspondence between liver tissue expression of Fas and its serum levels suggests that they could be considered as predictive markers for tumorigenesis in HCC (Hammamet al., 2012; Liao et al., 2016).

There was frequent presence of AG genotypes of Fas670, Fas1773 among higher grade of cirrhosis in Child-Pugh classification and CC genotype in FasL844, however, this increase was statistically insignificant. Previous studies reported significant association between Of some importance is that the correlationbetween Fas 1377G, and 670G with higher grade of cirrhosis activity (Wang et al., 2003; McIlroy et al., 2005; Liao et al., 2006). The data suggests thatthe SNPs in the Fas promoter regions may be associated with cirrhosis severity due to HCV infection. Large scale studies of patients with cirrhosis can make the data more significant.

The novel finding in the present study was the significant association of down regulation of Fas on hepatocytes and up regulation of FasL as demonstrated by the significant frequent association of substitution of A by G alleles in Fas670 gene and in Fas1773 in advanced BCA staging advanced pathology grading the presence of larger tumor with size >5cm. Previous study by Liao et al., (2016) demonstrated only significant association between high serum AFP level and FasL 844 T/C polymorphyism.

It is hypothesized that the Fas/FasL system had an important role in tumorgrowth and the grade of malignancy (Lai et al., 2005; Zakiet al., 2008; El Bassiounyet al., 2008; Liao et al., 2016).The overexpression of FasL on HCC tumor cells is thought tobe a mechanism that tumors employ to escape immune surveillance(Lee et al., 2004; El Bassiouny et al., 2008). There are no sufficient data regarding the SNPs of Fas/FasL system and its relation to the severity of HCC and its clinical grading.

The present results may reveal that the study of Fas/ Fas L expression related to its genotypes can be used to guide the patient clinical staging and can be regarded as a new prognostic marker.

However, to establish this new marker there should be further studies with a greater number of patients and follow up to identify the relationship between Fas/Fasl polymorphism, expression and the prognosis of patients with HCC related to HCV.

The findings of the present study highlight the association of genetic polymorphism of promoters regions in Fas and Fas L and the presence of cirrhosis and hepatocellular carcinoma associated with chronic HCV. The findings support that there are association between single nucleotide polymorphisms of Fas/ FasL system and the clinical and histopathological grading of hepatocellular carcinoma. Further large scale studies are recommended to establish this finding.

Conflict of Interest

There are no any conflicts of interests for any of the authors.

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