AIM OF THE WORK

The aim of the present work is to know if BAFF Promoter polymorphism can be a host predisposing factor to develop mixed cryoglobulinimia in chronic HCV patients.

SUBJECTS

This study was conducted on ninety patients with hepatitis C positive chronic liver disease. Diagnosis of hepatitis C was based upon positivity for hepatitis C virus antibodies, and was then confirmed by polymerase chain reaction (PCR). Patients were recruited from internal medicine outpatient clinic as well as from inpatients admitted to medical wards in gastroenterology unit, Alexandria main university hospital. According to clinical presentation and positivity to cryoglobulin test patients were classified into two groups:-

- **Group I:** thirty HCV-MC patients.
- **Group II**: thirty HCV without MC.

Thirty age and sex-matched healthy volunteers were studied as the control group.

Exclusion criteria included concomitant autoimmune disorders such as sjogren syndrome, systemic lupus erythematosis, rheumatoid arthritis, or concomitant liver diseases (HBV)or Alcohol abuse, concomittent lymphoproliferative neoplasm and HCC.

All subjects enrolled in the study signed a written informed consent before participation. The study was approved by the Medical Ethics Committee of the Faculty of Medicine, Alexandria University.

METHODS

This study was carried out after taking informed written consent from the patients and after permission from the ethical committee of Alexandria University.

All patients were subjected to the following:

1- Thorough history taking.
2- Complete clinical examination with special stress on the presence of jaundice,ascites,ankle edema, lymphadenopathy, organomegaly and peripheral neuropathy, prurites ,ulcers, arthralgia.
3- Ultrasound examination for diagnosis of cirrhosis and detection of focal hepatic lesions

4- Laboratory investigations including

1- Complete blood picture.[105]
2-Liver function tests (ALT, AST, Albumin, Bilirubin and prothrombin time).[105]
3-HBs Ag, HCV Abs, PCR for HCV-RNA.[105]
4-AFP.
5-Rheumatoid factor(RF)

6-Cryoglobulin test [105]:

Ten ml of venous blood werecollected on plain tube under aseptic conditions for each patient and healthy control. The tubes and syringes were kept at 37^0C before sampling, clotting and serum separation. The warm serum was placed in a 4^0C refrigerator and examined for up to 7 days. If acryoprecipitate appear it was analyzed by capillary electrophoresis.

7-Capillary electrophoresis:

Figure(6): Sebia's minicap

Fresh serum samples are recomended for analysisfrom patients of cryoglobulintest+ve

Procedure:

- Barcode reading of sample tubes and sample racks;
- Sample dilution from primary tubes into dilution segments;
- Capillary washing;
- Injection of diluted samples;
- Protien analysis and direct detection on capillaries

Ahigh voltage protein separation was performed and charged molecules are separated by their electrophoretic mobility in alkaline buffer at 200 nm with the detection of proteins with specific PH and at anodic end of the capillary [106]

protiens are detected in following order:Albumin,alpha1-globulin,alpha2-globulin,beta1-globulin,beta2-globulin,gamma-globulin.[106]

All patients of cryoglobulin +ve revealed polyclonal hypergamaglobulinemia by capillary electrophoresis and this to prove that the type of cryoglobulinemia is mixed cryoglobulinemia

8-BAFF promoter polymorphism by (PCR-RFLP) PCR-restriction fragment length polymorphism.[105]:

Detection of BAFF promoter polymorphism by genomic DNA extraction from peripheral blood. A fragment of the gene region containing the polymorphism was amplified by PCR technique followed by restriction digestion (RFLP). This was done for both patients and healthy controls.

Specimen collection and storage [105]

Two ml venous blood was collected on EDTA tube under aseptic conditions for each patient and healthy control. EDTA whole blood was subjected to the DNA extraction protocol and then genomic DNA was preserved at -20^{0}C until genetic analysis.

DNA extraction [105]

DNA extraction was done using Thermo Scientific GeneJET Whole Blood Genomic DNA Purification Mini Kit Product number: #K0781 (Thermo Fisher Scientific, USA).

Basic principle

Samples were digested with Proteinase K and the supplied Lysis Solution. The lysate was then mixed with ethanol and loaded onto the purification column, where the DNA bound to the silica membrane. Impurities were effectively removed by washing the column with the prepared Wash Buffers. Genomic DNA was then eluted under low ionic strength conditions with the Elution Buffer.

Protocol for purification of genomic DNA from 200 µl whole blood[105]

Blood cell lysis and genomic DNA binding

Twenty µl of Proteinase K Solution were added to the bottom of 1.5 ml microcentrifuge tube then 200 µl of whole blood were added and mixed by vortexing. Four hundred µl of Lysis Solution were added and mixed thoroughly by vortexing. The sample was incubated at 56°C for 10 minutes while vortexing. Occasionally, till

complete lysis occurs.Two hundred µl of ethanol (96-100%) were added and mixed by pipetting. The prepared mixture was transferred to the spin column and centrifugated for 1 min at 8,000 rpm. The collection tube containing the flow-through solution was discarded and the column was placed into a new 2 ml collection tube.

Wash and dry

Five hundred µl of Wash Buffer WB I were added followed by centrifugation for 1 min at 10,000 rpm. The flow-through was discarded and the column was placed back into the collection tube.

Five hundred µl of Wash Buffer II were added to the column and centrifugated for 3 min at maximum speed (≥14,000 rpm).

The collection tube was emptied. The purification column was placed back into the tube and the column re-spun for 1 min. at maximum speed (≥14,000 rpm) to ensure complete dryness of the silica membrane.

The collection tube was discarded containing the flow-through solution and the column was transferred to a sterile DNA-ase free 1.5 ml microcentrifuge tube

Elution

Two hundred µl of Elution Buffer were added to the centre of the column membrane to elute genomic DNA. The column was incubated for 2 min at room temperature and centrifugated for 1 min at 10,000 rpm.

The purification columnwas discarded and purified genomic DNA was stored at -20°C

Method overview

Figure(7): Steps of genomic DNA extraction.[105]

The concentration in ng/µl and the purity of the extracted DNA were checked on nanodrop 2000 thermo scientific. 260/280 readings should read between1.8-2 ng/µl.

PCR Amplification

PCR (Polymerase chain reaction) was done using Thermo Scientific Dream Taq Green PCR Master Mix (2X) (Thermo Scientific code number: #K1081, USA)

Components of the Dream Taq Green Master Mix

Dream Taq DNA polymerase, optimized Dream Taq Green Buffer,$MgCl_2$,dNTPs, two tracking dyes,density reagent .

The reaction volume: 50 µl

Each reaction contained:

25 µlGreen Master Mix

0.1-1.0 µM ::of each primers in the following sequence

Forward5'-GGC ACA GTC AAC ATG GGA GT-3'

Reverse 5'-GCT AAG TGT TTT AGC ATT GAA TTG-3'

1-10 pg/µgGenomicDNA

DNA-ase free water: to 50 µl

The reaction mixture was subjected to the following PCR protocol

Sets of primers were derived from Sigma Aldrich, USA. PCR was performed on BIOMETRA thermal cycler (T professional thermocycler, Germany)

Protocol for PCR using BAFF primers for detection of BAFF polymorphism [105]

Initial denaturation: 95°C for 10 minutes
Denaturation: 95°C for 30 seconds
Annealing: 60°C for 1minute 35 cycles
Extension: 72°C for 1minute

Final Extension: 72°C for 15 minutes

Detection of polymorphisms using restriction enzymes [105]

The amplified PCR products were subjected to enzyme digestion by Thermo Scientific FastDigestBSrBI code number: #FD1274, USA.

Restriction enzyme, followed by electrophoresis through2% agarose gel and visualized by Dolphin-Doc gel documentation system (Wealtec, USA) after staining with ethidium bromide.

The total reaction volume for digestion using restriction enzyme: 30µl.

Each reaction contained:

- 17 µl nuclease free water.
- 2 µl 10X FastDigest Green Buffer.
- 10 µl PCR product.
- 1 µl FastDigest enzyme.

Restriction enzyme used [105]

BSrBI

The reaction mixture is incubated at 37 °C for 5 minutes in aheat block.
Finally electrophoresis in 2% agarose gel was run and ethidium bromide was used for staining the different bands to identify the polymorphism .
The expected amplicon for undigested PCR product was 468 bp in size.

The expected amplicon for homozygousC/C product was 116 bp and 352bp in size.

The expected amplicon forheterozygous C/T product was 468 bp,352 bp,116bp in size.

The expected amplicon for homozygous T/T product was 468 bp in size.

RESULTS

The age and sex of the 60 HCV positive patients with or without MC who were evaluated in this study are summarized in (Table 2).

No statistically significant differences were found between the two groups as regards age, however, female sex were significantly higher in group I (HCV-MC) than in group II (HCV).

Table (ii): Age and Sex of studied patients and controls

	Group I HCV -MC (n=30)	Group II HCV without MC (n=30)	P
Age (years)	54.90 ± 8.60	54.63 ± 9.58	0.910
Sex (no. male/no. female)	11/19	22/8	0.004*

*: Statistically significant at $p \leq 0.05$

In this study, cryoglobulinaemic manifestations detected in patients in order of frequency were: palpable purpura (60%), arthralgia(60%), peripheral neuropathy (33.3%) and/or myalgias (13.3%), with renal disease was detected in only 2 patients(6%) who presented by significant protienuria. (Table 3). 2 patients had no symptoms of cryoglobulinemia and were discovered by lab investigations. None of the patients had mesenteric, cardiac or retinal involvement.

Table (iii): Frequency of clinical manifestations of mixed cryoglobulinemia in studied patients

Clinical manifestation	Group I HCV-MC (n=30)	Percentage
Asymptomatic	2/30	6%
Palpable purpura	18/30	60%
Peripheral neuropaty	10/30	33.33%
Raynaud's phenomenon	4/30	13.3%
Renal involvement	2/30	6%
Arthralgia	18/30	60 %
Myalgia	4/30	12%

Laboratory investigations in studied groups are shown in (Table 4). As regards CBC, platelet count was significantly lower in group I (HCV-MC) than in group II(HCV without MC)(179±223versus114±172 respectively) ($P<0.05$) . Regarding liver function tests, Group II(HCV without MC) had significantly lower Aspartate transaminase than in group I(HCV-MC), significantly higher albumin and prothrombin activity than Group I(HCV-MC).

Rheumatoid factor was significantly higher in Group I than in Group II. Neither HCV-RNA level nor total bilirubin showed significant differences between the two groups.

Table (iv): Laboratory investigations in studied patients

	Group I HCV-MC (n=30)	Group II HCV (n=30)	P
Heamoglobingm/dl	11.2±0.9	10.8 ±0.7	>0.05
White blood cell ($x10^3$)	5.7±2.1	6.1±1.9	>0.05
Platelet count($x10^3$)	114±172	179±223	<0.05*
AST (IU/ml)	52.5±40.7	37.9±18.3	<0.05*
ALT (IU/ml)	61.57 ± 30.54	61.70 ± 35.45	>0.05
Prothrombin activity (%)	65±9.65	77±4.73	<0.05*
Albumin gm/dl	3.2±0.7	3.9±0.4	<0.05*
Bilirubin gm/dl	0.4±0.3	0.5±0.2	>0.05
AFP ng/ml	2.5±3.9	5.3±1.9	>0.05
PCR(IU/ml $\times10^6$)	2.60 ± 1.77	2.94 ± 1.60	>0.05
RF (normal<15 IU/ml)	293.31±102.57	30.33 ± 59.0	<0.001*

Z: Z for Mann Whitney test *: Statistically significant at $p \leq 0.05$

Sonographic findings in studied patients are summarized in (Table 5). Cirrhosis of the liver diagnosed by coarse echo texture and irregular borders was significantly higher in HCV patients without MC than in HCV –MC patients

Table (v): Sonographic findings in studied patients

Sonographic Finding	Group I HCV-MC (n=30)	Group II HCV (n=30)	P
Hepatomegaly	22	21	>0.05
Coarse liver with irregular borders	11	17	<0.05*
Bright liver	13	19	>0.05
Splenomegaly	18	17	>0.05
Hepatic focal lesion	-	-	>0.05

5-Genotype and allele distribution

BAFF

Amplification was done using BAFF primers for samples of cases and controlesThe resulting amplicon size was 468 bps as visualized using gel electrophoresis .

Digestion of the amplicon usingBSrBI restriction enzyme resulted in the following bands:

* C/C: Two bands with a size of 116 bp and 352 bp.
* C/T: Three bands with sizes 468, 352 & 116 bp.

* T/T: one band with sizes 468 bp.

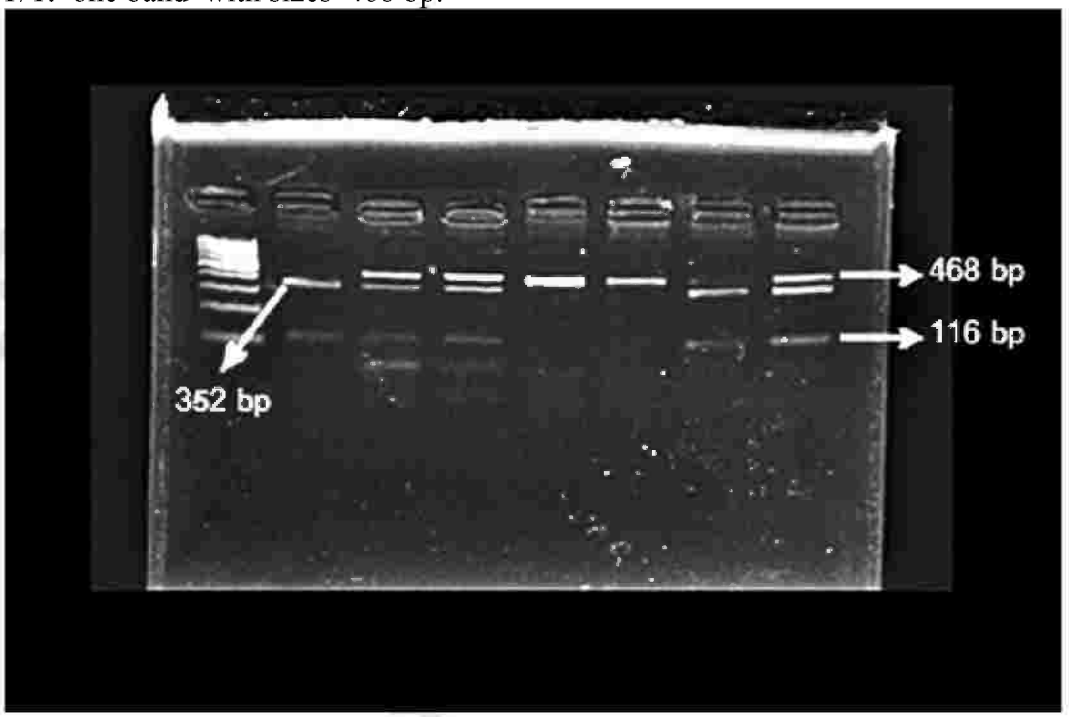

Figure (8): Gel electrophoresis representative results of -871C/T BAFF promoter genotyping. Lane 1: A 100-bp MW marker,amplicons digested with BSrBI restriction enzyme are shown. lanes 2,7: Homozygous C/C was cleaved in 2 fragments 116 bp and 352 bp.lanes 3,4,8: For C/T the presence of 3 bands 468 bp ,352 bp,and 116bp indicated heterozygosity.A single uncut band (468 bp) indicated T/T homozygosity as in lanes 5,6.

We compared the occurrence of C and T alleles among cases and controls using Chi-square test. Results were tabulated also in: (Table 6)

Table(vi): Distribution of the -871C/T BAFF promoter polymorphism genotypes and alleles in cases and control groups.

	Cases (n=60)		Control (n=30)		χ^2	P	OR	95% CI	
	No.	%	No.	%				LL	UL
Genotyping									
CC	14	23.3	10	33.3					
CT	22	36.7	16	53.3	6.637*	0.036*	0.982	0.349	2.768
TT	24	40.0	4	13.3			4.286*	1.129	16.266
Allele									
C	50	41.66	36	60.0	5.388*	0.020*			
T	70	58.33	24	40.0			2.100*	1.117	3.948

χ^2: Chi square test

*: Statistically significant at $p \leq 0.05$

Statistical analysis of genotypes and alleles of BSrBI restriction enzyme revealed the following:

1-Genotype distribution was 23.3% of cases showed wild type CC , 36.7 % showed heterozygous pattern CT and40.0 % showed homozygous TT. Among controls, 33.3 % were wild CC, 53.3% were heterozygous CT and 13.3% were homozygous TT. Figure (9)

2-Allele distribution was 41.66 % of cases showed allele C while 58.33 % showed allele T. Among controls, 60.0 % showed allele C while 40.0% showed allele T. Using Chi-square test, the present study showing that TT genotype was significantly increased in cases than controls (P 0.036*)also BAFF promoter T allele was significantly increased in cases than controls, P =0.020* .

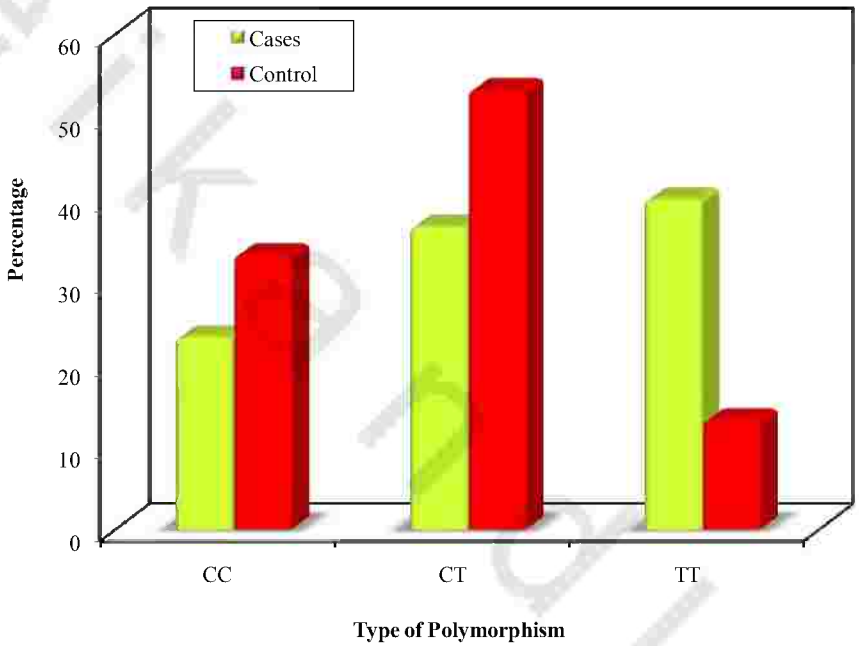

Figure (9): Comparison between the studied groups according to type of polymorphism

And when we compare the allelic variation between patients of HCV with MC and that of HCV without MC we found the results which tabulated in (Table7)

Table (vii): Distribution of the -871C/T BAFF promoter polymorphism genotypes and alleles in the HCV and HCV-MC.

	HCV (n=30)		HCV-MC (n=30)		χ^2	P	OR	95 % CI	
	No.	%	No.	%				LL	UL
Type of Polymorphism									
CC	12	40.0	2	6.7					
CT	14	46.7	8	26.7	19.446*	<0.001*	3.429	0.607	19.353
TT	4	13.3	20	66.7			30.00*	4.755	189.29
Wild	12	40.0	2	6.7	9.317*	0.002*			
Variant (CT + TT)	18	60.0	28	93.3			9.333*	1.866	46.684
Allele									
C	38	63.3	12	20.0	23.177*	<0.001*			
T	22	36.4	48	80.0			6.909*	3.037	15.720

χ^2: Chi square test
*: Statistically significant at $p \leq 0.05$

1- Regarding genotype

The analysis of the distribution of the −871 C/T alleles of the BAFFpromoter revealed higher prevalence ofTTgenotype in the group of patients with MC than in the group of HCV patients without MC (66.7% versus 13.3 %; P <0.001*) paralleled by reduction in the frequency of the CC genotype in the group of patients with MC in comparison to HCV patients without MC (6.7 % versus 40.0 %; P<0.001*).Figure (10)

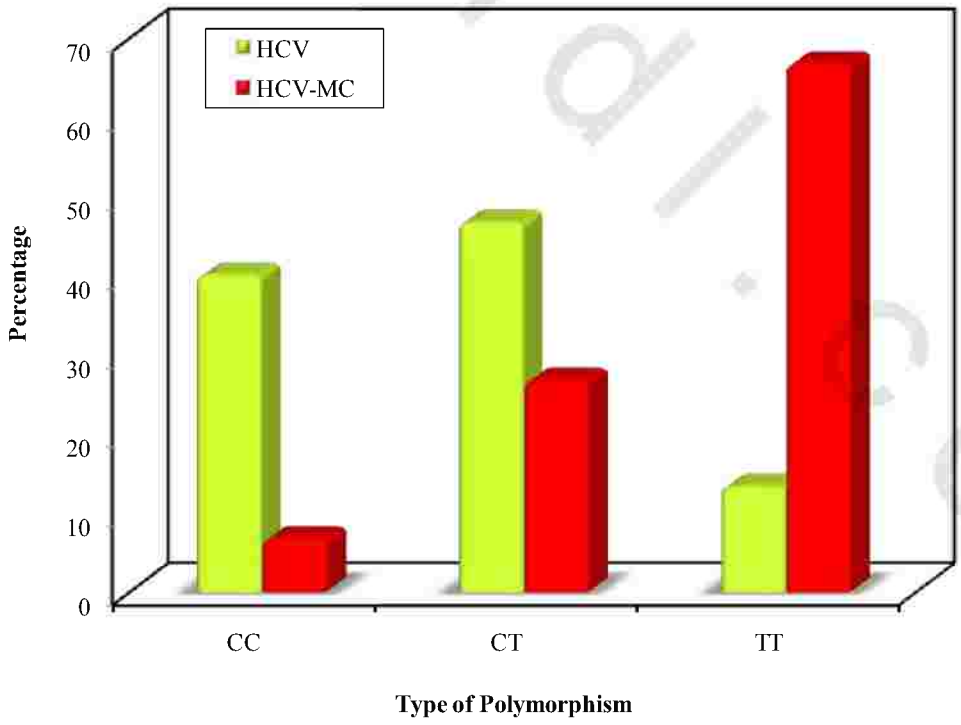

Figure (10): Genotype distribution for -871C/T BAFF promoter polymorphism in the HCV and HCV-MC.

Also homozygous TT plus heterozygous CT was significantly more prevalent in HCV-MC patients when compared to HCV carriers without MC (93.3 % versus 60.0 %; $P = 0.002^{*}$).

2- Regarding allele distribution,

The T allele was significantly increased in cases of HCV-MC compared with HCV carriers without MC(80.0% versus 36.4 %; $P<0.001^{*}$).also the C allele was significantly decreased in cases of HCV-MC compared with HCV carriers without MC(20.0% versus 63.3 %; $P<0.001^{*}$). **Figure (11)**

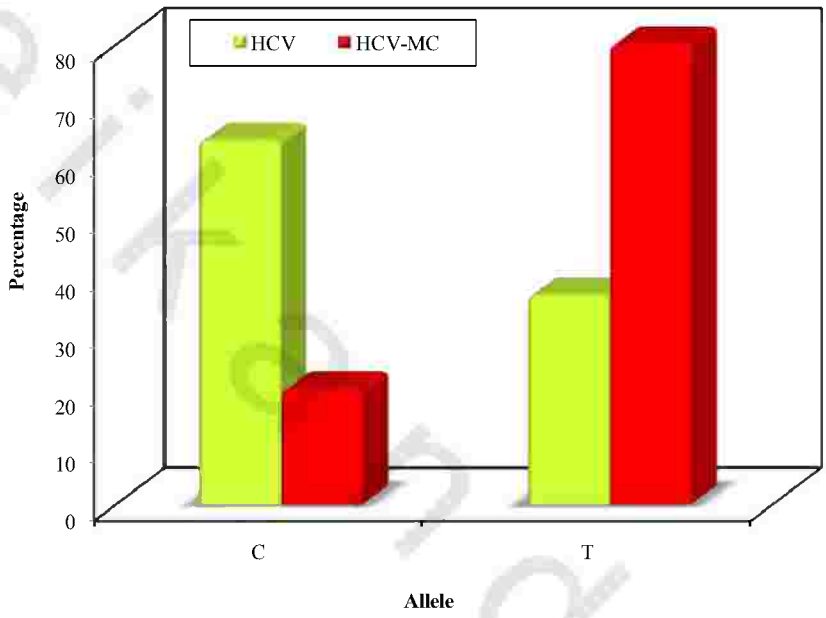

Figure (11):allele distribution for -871C/T BAFF promoter polymorphism in the HCV and HCV-MC