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Original article

Immunohistochemical Study of Arginase 1 and Glutamine Synthetase in Chronic Hepatitis C, Cirrhosis and Hepatocellular Carcinoma

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Abstract:

Background: Morphological distinction of HCC among advanced chronic liver diseases still poses a number of problems. Definite diagnosis can't be obtained by histologic evaluation alone in some cases, especially with small sample biopsies and in well differentiated tumors; in these cases, immunohistochemical staining is very useful. Therefore, more support is needed in the conventional pathological differentiation of HCC from chronic liver diseases, especially advanced cirrhosis and chronic hepatitis C. **Aim of the study**: to assess the expression of Arginase 1 (Arg-1) and Glutamine synthetase (GS) in chronic hepatitis c, liver Cirrhosis and HCC to evaluate their role in differentiation between them. **Methods:** A retrospective immunohistochemical study was performed on 74 liver cases; 17 cases of chronic hepatitis c, 25 cases of cirrhosis, and 32 cases of HCC. In addition to 8 cases of normal liver tissues obtained from donors for liver transplantation as a control group. **Results**: There was negative statistically significant relation between type of lesion (chronic hepatitis C, cirrhosis and HCC group) and Arg-1 expression ($P<0.001$). On the other hand, there was highly positive statistically significant relation between type of lesion (chronic hepatitis C, cirrhosis and HCC group) and the expression of GS ($P<0.001$), as GS expression showed gradual increase from zero in most cases of chronic hepatitis c and cirrhosis, to 62.5% were score 3+ in HCC cases. **Conclusion:** GS is a good marker in differentiating HCC from Cirrhosis and chronic hepatitis C, unlike Arg-1 which showed positive expression in HCC, cirrhosis and chronic hepatitis C.

Keywords: Arginase-1, Glutamine synthetase, Hepatocellular carcinoma, Chronic hepatitis C, Cirrhosis.

Abbreviations: Hepatocellular carcinoma (HCC), Arginase-1 (Arg-1), Glutamine synthetase (GS).

Introduction

The WHO estimates that 71 million people have chronic hepatitis C virus infection. A significant number of these cases will develop cirrhosis or liver cancer (1).

Cirrhosis is an increasing cause of morbidity and mortality in developed countries, being the 14th most common cause of death worldwide but fourth in central Europe (2). A significant number of patients with cancer concomitantly suffer from liver cirrhosis (3). HCC is the sixth and fourth common cancer in worldwide and Egypt, respectively. Egypt ranks the third and 15th most populous country in Africa and worldwide, respectively (4).

Morphological distinction of HCC among advanced chronic liver diseases still poses a number of problems. Therefore, more support is needed in the conventional pathological detection of HCC from chronic liver diseases, especially advanced cirrhosis (5). There are various approaches used to select the most sensitive and specific markers for diagnosis and differential diagnosis of HCC (6).

Arginase is a widely known enzyme of the urea cycle that catalyzes the hydrolysis of L-arginine to L-ornithine and urea. The action of arginase goes beyond the boundaries of hepatic ureogenic function, being widespread through most tissues. Two arginase isoforms coexist, Arg-1 predominantly expressed in the liver and Arg2 expressed throughout extrahepatic tissues (7).

Glutamine synthetase (GS) is an enzyme converting glutamate and ammonia into glutamine using adenosine triphosphate (ATP). GS is particularly highly expressed in the liver, kidney, skeletal muscle, and brain (8).

The present study aimed to assess the expression of Arg-1 and GS in chronic hepatitis C, liver cirrhosis and HCC.

Materials & Methods:

Study groups:

A retrospective study was performed on paraffin sections of 74 specimens of liver tissues including 17 specimens of chronic hepatitis C, and 25 specimens of cirrhosis and 32 specimens of HCC. In addition, 8 specimens of normal liver core biopsy obtained from donors for liver transplantation were used as a control group. They were obtained from the departments of pathology, Faculty of Medicine, Benha University and National Liver Institute, Menofia University, during the period between January 2014 and December 2020. This research plan was approved by the Research Ethics Committee of Faculty of Medicine, Benha University, Egypt.

The following data were collected from the patient files: age, gender, alpha fetoprotein level (when available), HCV antibody (anti-HCV) detected by ELIZA third generation.

All sections subjected to: Hematoxylin and eosin stain for histopathological assessment to confirm diagnosis and immunohistochemical staining usiny anti-Arg-1 and GS for evaluation of their expression.

Histopathological staining:

From each representative paraffin block of the studied cases, 4-μm thick sections were cut, stained by haematoxylin and eosin (H&E) and re-evaluated to confirm the diagnosis and to assess the following: for chronic hepatitis c cases (the grade of activity and stage of fibrosis using Metavir scoring system), for HCC (type, grade, stage and vascular invasion).

Immunohistochemical staining:

Immunohistochemical (IHC) procedure was performed according to manufacturer's instructions, using Arg 1 (Dilution 1:4,000, clone SL6ARG; Invitrogen, San Diego, CA, USA) and GS (NBP2-02125 Novus Biologicals, USA; 0.1ml, 1:100). Immunodetection was carried out using a standard labeled streptavidin-biotin system (Genemed, CA 94080, USA, South San Francisco). Antigen retrieval was done by using 10 mmol/L citrate monohydrate buffer (pH 6.0) and heating for 15 minutes in the microwave. Freshly prepared chromogen diaminobenzine (DAB, Envision ^{TM} Flex /HRP-Dako, REF K 8000) was used.

Negative control was used for each run of immunohistochemical staining for Arg- 1 & GS by omitting the primary antibody.

Positive control slides (as preferred by the data sheet for the antibody) were used in each run of immunohistochemical staining.

Immunohistochemical assessment:

Arginase-1: A positive result indicated by brown cytoplasmic staining with or without nuclear staining in tumor cells. The extent of positive tumor cells was classified into **1** (focal; ≤ 50% of tumor cells were positive), 2 (regional; 50: 90% of tumor cells were positive) and 3 (diffuse; ≥ 90 % of tumor cells were positive). The intensity of immunostaining scored as 0 (negative/ weak staining), 1+ (moderate staining) and 2+ (intense staining). The extent and intensity scores were multiplied to give a composite score (range: 0-6) for each tissue specimen. Composite scores of 0-3 were described as low Arg. 1 expression, and scores 4-6 were defined as high Arg.1 expression (9).

Glutamine Synthetase: A positive result was indicated by brown cytoplasmic staining. Cases were scored according to the number of immunoreactive (IR) cells. Individual cases were considered IR when more than 5% of cells were IR. Immunoreactive tissues were further subclassified as follows: + 1= 5–10% IR cells (low expresser, LE); +2 = 11–50% IR cells (intermediate expresser, IE); +3 ≥ 50% IR cells (high expresser, HE) (10).

Statistical analysis: Data were collected, tabulated and statistically analyzed using a personal computer with SPSS version 20 (SPSS, Inc., Chicago, IL, USA), p value is Statistically significant when ≤0.05. Receiver-operating characteristic (ROC) curve was used to predict sensitivity, specificity and accuracy of immunohistochemical score.

Results

Histopathological examination of 74 liver cases showed 17 (23%) cases of chronic hepatitis c, 25 (33.8%) cases of cirrhosis, and 32 (43.2%) cases of HCC. In addition 8 cases of normal liver tissues as a control group. All cases were examined histologically and immunohistochemically for Arg-1 and GS.

Clinicopathological results

For HCC cases, shown in table (1)

Table (1): Clinical and histopathological data of studied HCC group.

Variable	HCC group (NO.=32)	
	No.	%
Age		
Mean ±SD	59.81±9.33	
Range	45-82	
Median	57.5	
Gender		
Males	23	71.9
Females	9	28.1
M : F ratio	2.5:1	
AFP(ng/ml)		
Mean ±SD.	802.59 ± 1173.13	
Range	150-6050	
Median	431.5	
Tumor focality		
Single	26	81.3
Multiple	6	18.8
Tumor site		
Right lobe	17	53.1
Left lobe.	12	37.5
Right &left lobe	3	9.4
Tumor size		
Mean ±SD.	5 ±3.87	
Range	2-17	
Median	4	
Histopathological pattern		
Trabecular	16	50.0
Solid	7	21.9
Acinar	9	28.1
Grade of HCC		
Grade 1	10	31.2
Grade 2	15	46.9
Grade 3	7	21.9
Grade		
Low grade	25	78.1
High grade	7	21.9
Pathologic stage		
T1	10	31.2
T2	9	28.1
T3	6	18.8
T4	7	21.9
Stage		
Early stage	19	59.4
Advanced tumor	13	40.6
Vascular invasion		
Present	16	50.0
Absent	16	50.0

NO=Number, SD= Standard deviation, AFP= Alpha fetoprotein, HCC= hepatocellular carcinoma, HCV= Hepatitis C virus, M: F= male to female ratio**.**

Immunohistochemical results

Arg-1 expression:

-All cases of studied normal group showed high Arg. 1expression (scored 4+).

-There was negative statistical significant difference between type of lesion (chronic hepatitis C, cirrhosis and HCC group) and Arg-1 expression (P<0.001), as there was gradual decrease in Arg-1 from chronic hepatitis C group (94.1% showed high expression and 5.9% showed low expression) followed by cirrhotic group (72% showed high expression and 28% showed low expression) and the least is for HCC group (62.5% showed low expression and 37.5% showed high expression). Figure (1).

-In HCC group: There was positive statistically significant relation between Arg-1 expression and median AFP (p< 0.05). There was positive statistically significant relation between Arg-1 expression and focal lesions, site and size (p< 0.05). Table (2). While **t**here was no statistically significant relation between Arg-1 expression and grade of HCC.

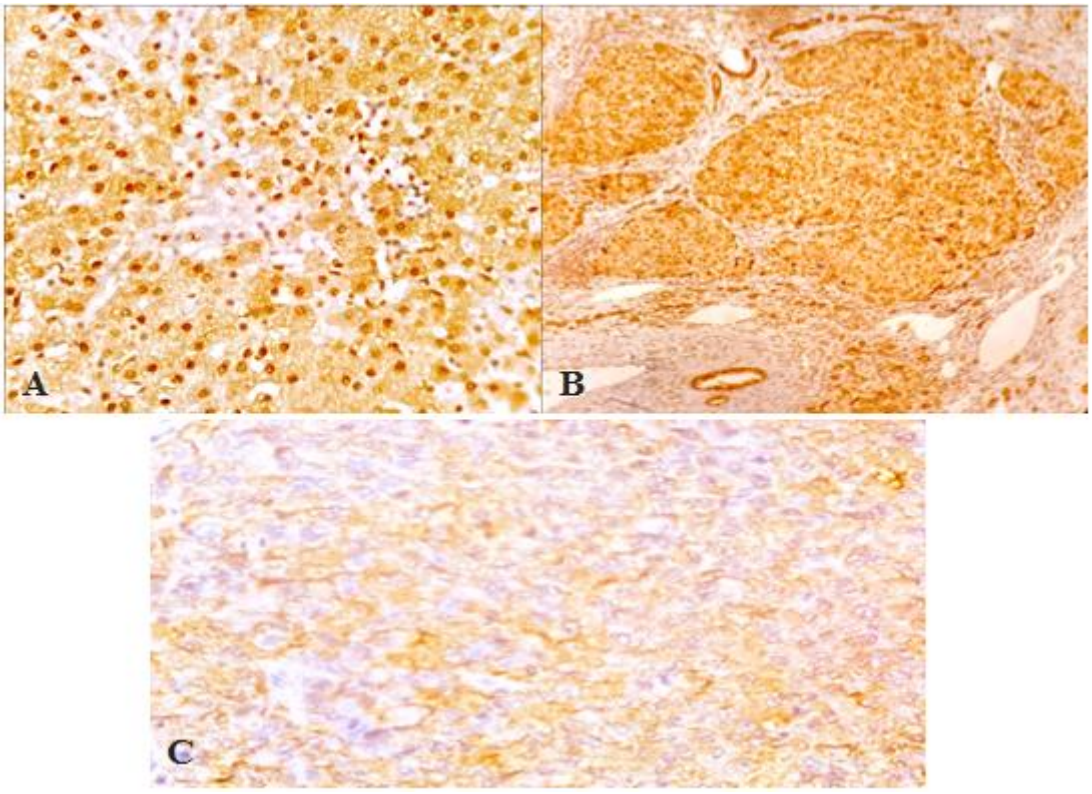

Figure (1): A) A case of chronic hepatitis C with moderate activity (A2) and moderate fibrosis (F2) showing positive cytoplasmic Arg-1 expression score (6+) (high expression) (IHC x100). **B)** A case of cirrhosis showing positive cytoplasmic Arg-1 expression score (6+) (high expression) (IHC X200). **C)** A case of hepatocellular carcinoma with cytoplasmic Arg-1 expression score (3+) (low expression) (IHC x400).

Table (2): Relation between Arg-1 expression and clinicohisto-pathological data of the studied cases:

VARIABLE	Arg- 1 expression				Statistical test	P value
	Low (n=20)		High(n=12)			
	No	%	No	%		
Lesion:					x^{2MC}=16.68	<0.001*
Chronic hepatitis C (NO=17)	1	5.9	16	94.1		
Cirrhosis (NO=25)	7	28	18	72		
HCC (NO=32)	20	62.5	12	37.5		
HCC						
Age (Median (IQR))	62 (53.5-64.75)		56 (50-66.5)		z= 0.840	0.401
Sex						
Male	13	65	10	83.3	x^2=1.25	0.264
Female	7	35	2	16.7		
AFP Median (IQR)	300(200-520)		330(250-537.5)		z=2.54	<0.001*
Focal lesion						
Single	20	100	6	50	x^2=12.31	0.0004*
Multiple	0	0.0	6	50		
Site						
Rt lobe	18	90	3	25	x^{22}=15.45	<0.001*
Lt lobe	2	10	7	58. 3		
Both	0	0.0	2	16. 7		
Size Median (IQR).	2.0(2-2)		7.5 (5.5-10.5)		z= 4.5	<0.001*
Histopathological pattern						
Trabecular	8	40.0	8	66.7	x^{2MC}=2.20	0.333
Solid	5	25.0	2	16.7		
Acinar	7	35.0	2	16.7		
Grade						
G1	5	25.0	5	41.7	x^2=1.02	0.602
G2	10	50.0	5	41.7		
G3	5	25.0	2	16.4		
Grade						
Low grade	15	75	10	83.3	x^2=0.305	0.580
High grade	5	25	2	16.7		
Vascular invasion						
Positive	9	45.0	7	58.3	x^2=0.533	0.465
Negative	11	55.0	5	41.7		
Stage						
T1	5	25.0	5	41.7	x^{2MC}=2.20	0.532
T2	7	35.0	2	16.7		
T3	3	15.0	3	25.0		
T4	5	25.0	2	16.7		
Stage						
Early stage	12	60	7	58.3	x^2=0.008	0.926
Advanced stage	8	40	5	41.7		
Glutamine synthetase						0.622
1+	1	5.0	0	0.0	x^{2MC}=0.950	
2+	6	30.0	5	41.7		
3+	13	65.0	7	58.3		

z=Mann Whitney test, MC: Monte Carlo test, KW= kruskal wallis.

GS expression:

-All cases of studied normal group showed low GS expression (scored 1+).

- There was highly positive significant difference between type of lesion (chronic hepatitis c, cirrhosis and HCC group) and GS expression (P<0.001), as there was increase in GS expression from chronic hepatitis C (100%) showed negative expression (0, 1+), and cirrhosis; (100%) showed negative expression (0, 1+); to HCC in which (96.9%) showed positive expression (2+, 3+) Figure (2).

-In HCC group: There was statistically significant positive relation between GS expression and grade of HCC (p= 0.05) Figure (2). There was no statistically significant relation between GS and age, sex, multiple focality, size, AFP, histopthological patterns, vascular invasion and stage of HCC (p > 0.05) Table (3).

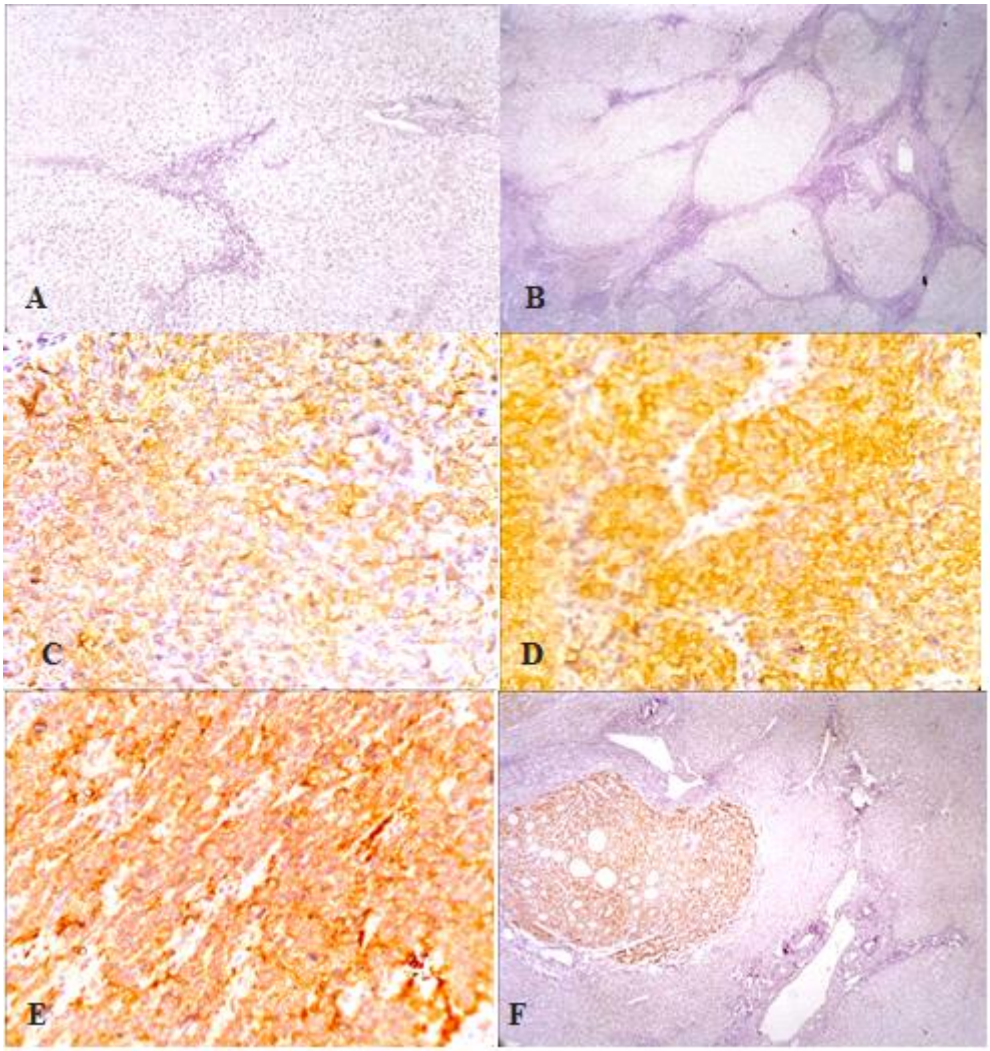

Figure (2): A) A case of chronic hepatitis c with moderate activity (A2) and moderate fibrosis (F2) showing negative cytoplasmic GS expression score (0) (IHC x100). **B)** A case of cirrhosis showing negative cytoplasmic GS expression score (0) (IHC X100). **C)** A case of well differentiated hepatocellular carcinoma showing cytoplasmic GS score (2+) (IHC x400). **D)** A case of moderately differentiated HCC showed cytoplasmic GS expression score (3+) (IHC x400). **E)** A case of poorly differentiated hepatocellular carcinoma (grade III) showing cytoplasmic GS expression score (3+) (IHC x400). **F)** A case of HCC (positive GS cytoplasmic expression; score 3) in hepatitis C background (negative GS expression; score 0) (IHC x400).

Table (3): Relation between GS expression and clinicohistopathological data of studied HCC group:

VARIABLE	GS expression							Statistical test	P value
	0	1+(n=1)		2+(n=11)		3+(n=20)			
	No. (%)	No	%	No	%	No	%		
Lesion:								71.95	<0.001*
Chronic hepatitis C (NO=17)	14 (82.4)	3 2	17.6 8	0 0	0 0	0 0	0 0		
Cirrhosis (NO=25)	23	1	3.1	11	34.4	20	62.5		
HCC (NO=32)	(92) 0								
HCC									
Age: Median (IQR)	0	56 (53-65)		62 (55-63)		57.5(53-67.5)		KW= 1.14	0.565
Sex								3.76	
Male	0	1	100	10	90.9	12	60.0		0.153
Female	0	0	0.0	1	9.1	8	40.0		
AFP Median (IQR)	0	436 (312-950)		310(235-570)		500		KW= 2.58	0.139
Focal lesion								0.254	0.881
Single	0	1	100	9	81.8	16	80.0		
Multiple	0	0	0	2	18.2	4	20.0		
Site								3.17	0.53
Rt lobe	0	1	100	9	81.8	11	55.0		
Lt lobe	0	0	0	2	18.2	7	35.0		
Both	0	0	0	0	0.0	2	10.0		
Size Median (IQR)	0	4.5(4.5-4.5)		3.5(2.38-4.75)		3.0(2.14-5.25)		KW= 2.69	0.125
Histopathological pattern	0	0	0.0	7	63.6	9	45.0	3.63	0.458
Trabecular	0	0	0.0	2	18.2	5	25.0		
Solid	0	1	100.0	2	18.2	6	30.0		
Acinar Grade									0.842
Grade 1	0	0	0.0	4	36.4	6	30.0	1.41	
Grade 2	0	1	100.0	5	45.5	9	45.0		
Grade 3	0	0	0.0	2	18.2	5	25.0		
Grade									0.05*
Low grade	0	1	100.00.	9	81.8	15	75.0	0.97	
High grade	0	0	0	2	18.2	5	25.0		
Vascular invasion									0.580
Positive	0	1	100.0	5	45.5	10	50.0	1.09	
Negative	0	0	0.0	6	54.5	10	50.0		
Stage									0.703
T1	0	0	0.0	4	36.4	6	30.0	3.80	
T2	0	1	100.0	2	18.2	6	30.0		
T3	0	0	0.0	3	27.3	3	15.0		
T4	0	0	0.0	2	18.2	5	25.0		
Stage									0.07
Early stage	0	1	100	6	54.5	12	60.0	0.794	
Advanced stage	0	0	0	5	45.5	8	40.0		

MC: Monte Carlo test, P=probability, KW= Kruskal Wallis.

Correlation between Arg- 1 and GS

There was significant inverse relation between Arg-1 and GS regarding their expression in studied chronic hepatitis C, cirrhosis and HCC group (P<0.001) Table (4).

ROC curve results:

Receiver-operating characteristic (ROC) curve was used to predict sensitivity, specificity and accuracy of Arg-1 & GS immunohistochemical score in chronic hepatitis c, Cirrhosis and HCC groups Tables (5, 6,7).

Table (4): Correlation between Arg- 1 and GS in studied cases.

	R	P value
Arg-1 and GS	-0.438	<0.001*

r: Spearman correlation co-efficient P=probability

Table (5): Validity of Arg-1 to predict HCC group from Cirrhosis one.

	HCC (32)		Cirrhosis (25)		Statistical test	P value
	No	%	No	%		
Arg- 1 score.						
≤4.5	27	69.2	12	48.0	8.59	0.003*
>4.5	5	15.6	13	52.0		
AUC (95% CI)			0.717 (0.581-0.853)			
Cut off point				4.5		
Sensitivity				**84.4**		
Specificity				**52.0**		
PPV				69.2		
NPV				72.2		
Accuracy				70.2		

AUC=area under the curve; PPV= positive predictive value; NPV= negative predictive value.

Table (6): Validity of Arg-1 to predict Cirrhosis group from hepatitis C one.

	Cirrhosis (25)		Hepatitis (17)		Statistical test (FET)	P value
	No	%	No	%		
Arg-1 score					5.98	0.014*
≤4.5	12	48.0	2	11.8		
>4.5	13	52.0	15	88.2		
AUC (95% CI)			0.771 (0.625-0.916)			
Cut off point				4.5		
Sensitivity				**48.0**		
Specificity				**88.2**		
PPV				85.7		
NPV				53.6		
Accuracy				64.3		

AUC= area under the curve; PPV= positive predictive value; NPV= negative predictive value.

Table (7): Validity of GS to predict HCC group from Cirrhosis one.

	HCC (32)		Cirrhosis (25)		Statistical test (FET)	P value
	No	%	No	%		
GS						
<2+	12	37.5	25	100.0	24.07	<0.001*
≥2+	20	62.5	0	0.0		
AUC (95% CI)	0.999(0.995-1.0)					
Cut off point	+2					
Sensitivity	**62.5**					
Specificity	**100.0**					
PPV	100.0					
NPV	67.6					
Accuracy	78.9					

AUC= area under the curve; PPV= positive predictive value; NPV= negative predictive value.

Discussion:

The hallmarks of cancer include biological capabilities as the results of genome instability and inflammation. Inflammation, a powerful component of the immune system, is one of the features of cancer and is involved in cancer occurrence and development. Currently, numerous studies have demonstrated that 15% to 20% of malignant tumors are the results of infections and uncontrolled inflammation. For example, inflammatory bowel disease is associated with cancer colon, and chronic hepatitis B virus infection leads to hepatocellular carcinoma. Development of research, energy metabolism reprogramming and evading immune destruction are also considered as important hallmarks of cancer. Accordingly, some anti-inflammatory and immune-related genes have garnered extensive attention in the therapy of cancers (11).

Differentiation of hepatocellular carcinoma from advanced chronic liver diseases may be problematic. In cases with poor tumor differentiation, especially in a small biopsy specimen may be additionally challenging. In such cases, immunohistochemical markers should be selected carefully (12).

In the current study, we attempted to search the expression of Arg-1 and GS in chronic hepatitis C, cirrhosis and HCC, and correlate their expression with clinic-histopathological data.

Arginase-1, which is involved in arginine hydrolysis to ornithine and urea in the urea cycle, is highly expressed in the liver at cytoplasmic and/or nuclear level. ARG1 encodes the Arg-1 isoform, which is confirmed to be located in the cytoplasm and highly expressed in liver and M2 macrophages. In addition to the metabolic enzyme activity in the hepatic urea cycle, Arg-1 also constitutes a pivotal immune cell component (11).

Many studies have demonstrated that Arg-1 is significantly involved in anti-inflammation, immune response, tumor immunity, and immunosuppression-related diseases for its metabolic enzyme activity in immune cells (11).

In agreement with previous study (13), Arg-1 expression showed high expression (score +4) in control group.

In agreement with previous study (9), there was positive statistically significant correlation between Arg-1 expression and size of HCC ($p< 0.05$), tumors with size > 5cm (86.7% were high score and 13.3% were low score) suggesting that Arg-1 might function as an oncogene in the carcinogenesis.

In agreement with previous study (9), there was positive statistically significant relation between Arg-1 expression and median AFP ($p< 0.05$), high Arg 1 score in 78% of cases with high AFP ($p< 0.05$).

In agreement with previous study **(**9), there was positive statistically significant relation between Arg-1 expression and focal lesions ($p< 0.05$).

In agreement with previous study (11), there was negative statistically significant difference between type of lesion (chronic hepatitis C, cirrhosis and HCC group) regarding Arg-1 expression ($P<0.001$), as there was gradual decrease in Arg-1 which showed highest expression among chronic hepatitics c group followed by cirrhotic group and the least is for HCC group.

In agreement with previous study (14), using ROC curve, we found that validity of Arg-1 to predict HCC group from Cirrhosis: AUC was 0.717 (good) for HCC, Sensitivity of Arg-1 was 84.4, Specificity was 52.0, Cut off value was 4.5, Positive predictive value was 69.2 and negative predictive value was 72.2.

In agreement with previous study (14), using ROC curve, we found that validity of Arg-1 score to predict Cirrhosis group from hepatitis one: AUC was 0.771 (good) for cirrhosis, Sensitivity was 48, Specificity was 88.2, Cut off value score was 4.5, Positive predictive value was 85.7 and negative predictive value was 53.6.

Glutamine synthetase, which is a well-recognized target of the Wnt/β- catenin pathway, is an enzyme of nitrogen metabolism and it catalyzes the conversion of glutamine to glutamate. This reaction also takes place in the control of many important cellular processes such as autophagia, activation of the mTOR pathway, and the release of inflammatory mediators (15).

In agreement with previous study (16), GS expression was seen in the cytoplasm of pericentral hepatocytes (zone 3). All 8 cases were scored (+1).

In agreement with previous study (17), regarding GS expression in chronic hepatitis C: 82.4% were scored zero and 17.6% were scored (1+).

In agreement with previous study (18), regarding GS expression of GS in cirrhotic groups, 8% of cirrhotic cases were scored (1+) and 92 were scored (0).

In agreement with previous study (18), there was positive statistically significant relation between GS expression and grade of HCC ($p = 0.05$).

In agreement with previous study (19), there was no statistically significant relation between GS and age, sex, focality, size, AFP, histopthological patterns, vascular invasion and stage of HCC.

In agreement with previous study (16), there was highly positive statistical significant difference between type of lesion (chronic hepatitis C, cirrhosis and HCC group) regarding the expression of GS ($P<0.001$), as GS expression was zero for most cases with chronic hepatitis C (82.4%) and 17.6% score 1+, for cirrhosis; 92% score 0 and 8% score 1+ and for

HCC; 62.5% were score 3+, 34.4% score 2+ and 3.1% score 1+.

In agreement with previous study (10), using ROC curve, we found that validity of GS to predict HCC group from Cirrhosis: AUC of GS was 0.999 (excellent) for HCC, Sensitivity of GS was 62.5 and Specificity was 100.0 for HCC, Cut off value of GS expression was 2+, Positive predictive value was 100 and negative predictive value was 67.6 for HCC.

Conclusion:

We demonstrated that GS may be a favorable marker in differentiating HCC from cirrhosis and chronic hepatitis C, unlike Arg 1 showed positive expression in HCC, cirrhosis and chronic hepatitis C.

Conflicts of interest:

No conflicst of interest.

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