

Using Heat Shock Protein 70, Glypican 3 and Glutamine Synthetase for Early Detection of Hepatocellular Carcinoma in Cirrhotic Patients with Atypical Small Hepatic Focal Lesions

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Abstract: Background: Hepatocellular nodules in cirrhosis include a group of heterogeneous lesions ranging from benign macro regenerative nodules (MRN) to low and high grade dysplastic nodules (LGDN and HGDN) to hepatocellular carcinoma (HCC). The differential diagnosis among these lesions constitutes a major task, needing further sensitive immunocytochemical markers. **Objective:** This study was undertaken to select the most sensitive and specific markers for early detection of hepatocellular carcinoma in cirrhotic patients with atypical small hepatic focal lesions using Heat Shock Protein 70, Glypican 3, and Glutamine Synthetase. **Patients and Methods:** Thirty cirrhotic cases from the outpatient HCC clinic of National liver institute, Menoufiya University, from January 2013 to June 2015 with atypical small hepatic focal lesion (less than 3 cm in diameter), detected by imaging (U/S and tri-phasic CT) with alpha fetoprotein less than 200ng. Liver biopsy was done and H&E stained sections were prepared for histopathological diagnosis. Immunohistochemical expression of heat shock protein 70 (HSP-70), glypican-3 (GPC3) and glutamine synthetase (GS) were used to confirm or exclude the diagnosis of HCC. The sensitivity and specificity of the individual markers and combination of markers for the detection of HCC were measured. **Results:** Ten out of 30 examined cirrhotic patients with atypical hepatic focal lesions were provisionally diagnosed to have HCC by histopathology and were confirmed by immunohistochemistry. The sensitivity and specificity of the individual markers for the detection of HCC were both 90% for HSP70; 70% and 65% for GPC3; 100% and 80% for GS. All negative expression was seen in 66% MRN and LGDN and 60% HGDN, whereas all positive expression was detected in 60% of HCC. The sensitivity and specificity of the combination of 3 markers were 60% & 100% respectively, meanwhile the sensitivity and specificity raised up to 100% when at least 2 of the markers used were positive irrespective of which. The most sensitive combination was HSP70 and GS which gave better results than that of other combination. **Conclusion:** We demonstrated that immunohistochemical expression of HSP70, GPC3, and GS were very useful in distinguishing benign from malignant hepatocellular nodules arising in cirrhosis, if at least two of these markers were positive.

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1. Introduction

Hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death, affecting approximately half a million person each year worldwide (1). Liver cirrhosis is the most important predisposing factor for it (2). HCC is a rapidly fatal cancer that mostly affects persons in developing countries where hepatitis B virus (HBV) and hepatitis C virus (HCV) are endemic. Hepatitis B and C viral infection is the most common underlying cause of chronic liver disease leading to liver cirrhosis. Aflatoxin B1 and alcohol are also well-known risk factors (3).

Around 20–30% of the estimated 170 million HCV-infected individuals worldwide will develop

cirrhosis. Once cirrhosis is established, the annual incidence of HCC is of 3–5%, and one third of them will develop a HCC over their lifetime (4).

Hepatocellular carcinoma is a lethal cancer, and improved survival relies on the detection of early tumors smaller than 2 cm, which are less likely to produce dissemination (5). HCC patients have poor long-term survival, partly due to the recurrence which is experienced in up to 80% of the patients even after curative resection (6).

Nowadays, early HCC diagnosis is feasible in 30-60% of cases in developed countries and this enables the application of curative treatments such as resection, transplantation and percutaneous ablation.

Thus there is an urgent need to identify better tools to characterize these lesions (7).

Although serum alpha-fetoprotein (AFP) level is a useful marker for the detection and monitoring of HCC, AFP levels may remain normal in up to 30% of the patients with advanced HCC (8).

Some HCC lesions especially less than 2-3cm in diameter lacking typical haemodynamic changes by radiology can make diagnosis of HCC a great challenge. Histopathological criteria alone still pose problems for the differential diagnosis of high-grade dysplastic nodules versus early HCC, especially because the pathological hallmark of HCC, stromal invasion, can be absent or difficult to identify in biopsy specimens (9).

Tissue markers provide a more accurate modality for differential diagnosis between dysplastic nodules and HCC (10). We selected to study heat shock protein 70, glypican 3 (GPC3), and glutamine synthetase (GS) because it has recently been proposed that these markers are very promising in the distinction between malignant and nonmalignant hepatocellular lesions (11-13).

Heat Shock Protein 70 belongs to a class of genes (heat shock proteins) implicated in tumorigenesis, in the regulation of cell-cycle progression and apoptosis (14).

Glypican-3 is a membrane anchored heparin sulfate proteoglycan normally expressed in fetal liver and placenta, but not in normal adult liver (12). It is an oncofetal antigen that is a reliable serum and histochemical marker for hepatocellular carcinoma (15).

Glutamine synthetase it is also a target gene of β -catenin, a major driver of human HCC. The immunoreactivity of GS protein was shown to be increased from precancerous lesions to early or advanced stages, indicating a role in the progression of HCC (13). Several reports have suggested that GS is an early marker of hepato-carcinogenesis (10).

Combination of different immune-histochemical markers (HSP70, GPC-3 and GS) in hepatocellular nodules can help in diagnosis of HCC.

The objective of this work was to select the most sensitive and specific marker/or markers for early detection of HCC in cirrhotic patients with atypical small hepatic focal lesions.

2. Patients and Methods

Selected patients with liver cirrhosis were diagnosed on the basis of clinical presentation, liver function studies, complete blood picture, hepatitis C virus antibody, hepatitis B surface antigen, serum alpha fetoprotein less than 200ng, imaging procedures including ultrasonography and triphasic CT which showed atypical enhancement pattern; all of which

were collected from outpatient HCC clinic National liver institute, Menoufiya University, from January 2013 to June 2015. Ultrasound guided core needle liver biopsy was taken, processed and stained with H & E together with immuno-histochemical expression of heat shock protein 70, glypican-3 and glutamine synthetase to confirm the diagnosis of HCC.

Inclusion criteria:

Cirrhotic patients with hepatic focal lesions not more than three cm detected by ultrasound and showed atypical enhancement pattern on triphasic CT scan.

Exclusion criteria:

Hepatic focal lesions more than 3 cm, multi focal lesions, typical HCC criteria on triphasic CT, portal vein thrombosis, extrahepatic lymph node metastasis, metastatic lesions, alpha fetoprotein more than 200ng or previous HCC treatment.

Immunohistochemical staining:

Immunohistochemistry was performed on formalin-fixed paraffin-embedded 4 mm thickness sections mounted on positively charged slides. The slides were stained for HSP-70, GPC3 and GS to confirm the diagnosis of HCC.

Tissue sections were deparaffinized and rehydrated in graded alcohols to distilled water, next they were incubated in 3% hydrogen peroxide for 10 min to block the endogenous peroxidase. Slides were immersed in acetic acid and heated in microwave at 95° C for 30 min for antigen retrieval then left to cool down at room temperature and rinsed with phosphate buffered saline (PBS) then they were incubated overnight at room temperature with the following primary antibodies: HSP-70 (NBP1-77455 Novus Biologicals, USA; 0.1ml, 1:400), GPC3 (NBP2-12491 Novus Biologicals, USA; 0.1ml, 1:500) and GS (NBP2-02125 Novus Biologicals, USA; 0.1ml, 1:100). The staining was completed using the streptavidin-biotin complex detection method. D.A.B. was applied for 10 to 15 minutes and reaction was stopped by using distilled water. Counter stain Hematoxylin was applied for 2 minutes and washed with distilled water.

HSP-70 gave nucleocytoplasmic staining; while both GPC3 and GS gave cytoplasmic staining. The slides of negative controls were prepared by excluding the primary antibody and replacing it with PBS.

Interpretation and assessment of immunohistochemical staining of the studied markers:

Individual cases were considered immunoreactive (IR) for individual antigens when more than 5% of cells were IR. IR cases were further sub-classified as follows: + = 5%-10% IR cells (low expression); ++ = 11%-50% IR cells (intermediate expression); +++ > 50% IR cells (high expression) (10).

The sensitivity and specificity of different immunohistochemical reagents (HSP-70, GPC3 and GS) were calculated. Sensitivity was defined as: the number of positive malignant lesions as a percentage of the total number of malignant lesions. Specificity was defined as the number of benign lesions with negative results as a percentage of the total number of benign lesions (16).

3. Results:

The study was conducted on 30 cirrhotic patients with atypical small hepatic focal lesion (less than 3 cm in diameter). The studied patients were 23 with hepatitis C and 7 with hepatitis B. The patients included 19 males representing 63.33% and 11 females representing 36.67%. Patient's age ranged from 39-72 years old with mean age 58.23 ± 9.52 . Fourteen patients had tumor size 1-2 cm and the remaining 16 had tumor size 2-3 cm. Eighteen cases had focal lesions in the right lobe while the remaining 12 were in the left lobe.

Results of Pathological examination:

Twenty cases were benign focal lesions; of them, 12 were MRN, 3 patients were LGDN and 5 were HGDN (**Fig.1A,B**). Macroregenerative nodules had portal tracts which sometimes showed mild chronic inflammation. Hepatocytes looked normal with no atypia. There was fatty change in seven of these cases. Dysplastic nodules were of two types LGDN and HGDN based on histologic features. Low-grade dysplastic nodules had hepatic plate architecture preserved at two cells thick. Nuclei showed mild atypia with nuclear enlargement and irregularities and some cells showed steatosis. High-grade dysplastic nodules showed obvious cytological and architectural atypia but fall short of malignancy. Increased cellular density 1.5–2 times of normal was seen. There was mild thickening of the hepatic plates, up to three cells thick in 2 cases. Hepatocytes nuclei were atypical with hyperchromasia, variation in nuclear size and irregular contours.

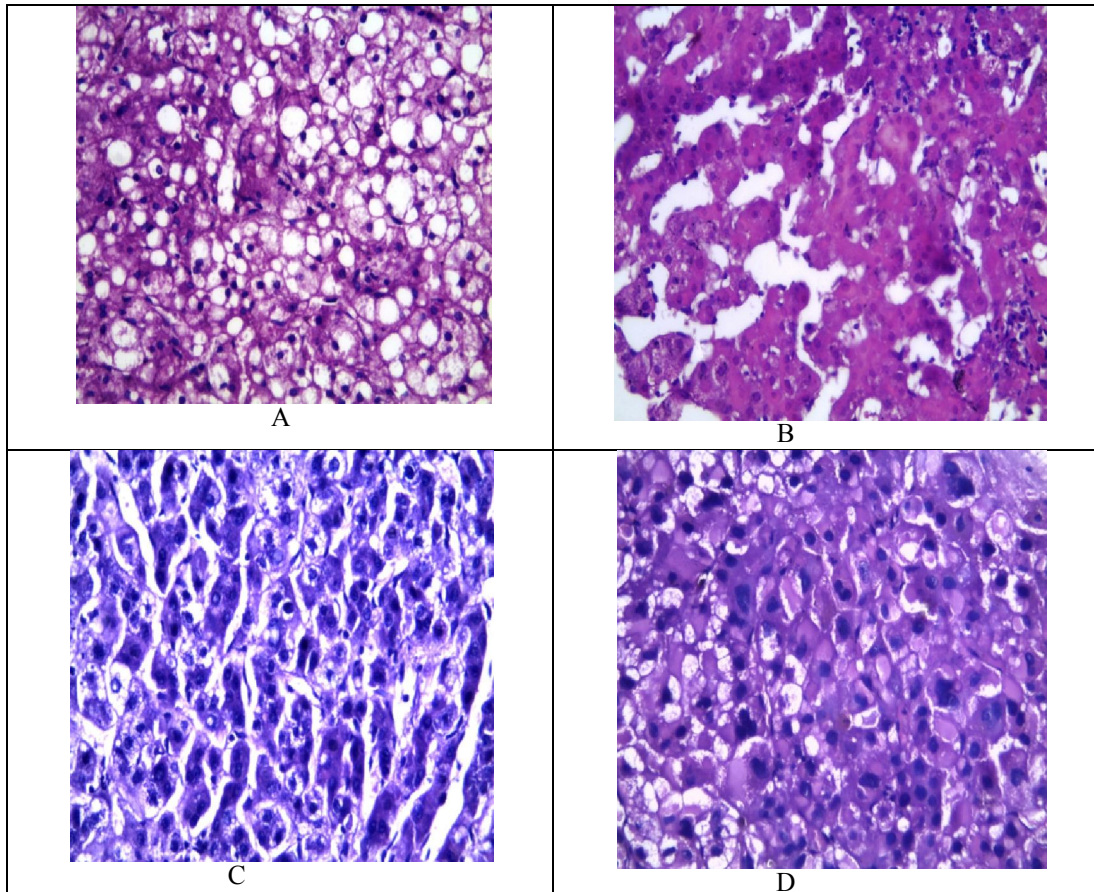


Fig.(1): Histopathological features of: (A) Macroregenerative nodules showing proliferated hepatocytes with increased fat content; (B) High grade dysplastic nodules showing proliferated hepatocytes with dysplastic nuclear changes; (C) well differentiated HCC showing mainly trabecular pattern; (D) poorly differentiated HCC showing highly pleomorphic malignant hepatocytes (H&Ex 400).

Ten patients showed characteristics of HCC (**Fig.1C,D**), out of them 4 were well differentiated, 4 patients were moderately differentiated and 2 were poorly differentiated HCC nodules. Early HCC showed only well-differentiated features with mild to moderate cytological atypia and trabecular thickening with loss of portal tracts within the lesion. Two out of four well-differentiated HCC cases were provisionally diagnosed by H&E to be suggestive of HCC and were confirmed by immunohistochemistry. Moderately and poorly differentiated HCCs showed apparent cytological atypia (nuclear irregularities, variation in nuclear size, prominent nucleoli, and occasionally multinucleation). Bile production was seen in a subset of cases together with increased cytoplasmic basophilia. There were hypercellularity with definite trabecular thickening (typically three cell or more), in addition to pseudoacinar or solid sheet formation. Stromal invasion was difficult to identify in core biopsy specimens where only two cases one

moderately differentiated and one poorly differentiated showed stromal invasion.

Results of Immunohistochemistry:

The slides were stained for HSP-70, GPC3 and GS to confirm the diagnosis of HCC.

HSP70 nucleocytoplasmic immunoreactivity was seen in the vast majority of HCCs (9 of 10 cases); including 4 well, 3 moderate and 2 poorly differentiated HCCs. Its expression in malignant cases was mainly intermediate or high. In nonmalignant nodules, HSP70 immunostaining was seen in none of dysplastic nodules, while 2 out of 12 MRN showed low expression. The sensitivity and specificity of HSP70 for HCC cases versus benign were 90% for both.

GPC3 cytoplasmic immunoreactivity was seen in most of HCCs (7 of 10 cases), including 3 well, 3 moderate and 1 poorly differentiated HCC. In nonmalignant nodules, GPC3 immunostaining was seen in 2 HGDNs, one LGDN and 4 MRNs. The sensitivity and specificity of GPC3 for HCC detection were relatively low (70% and 65%, respectively).

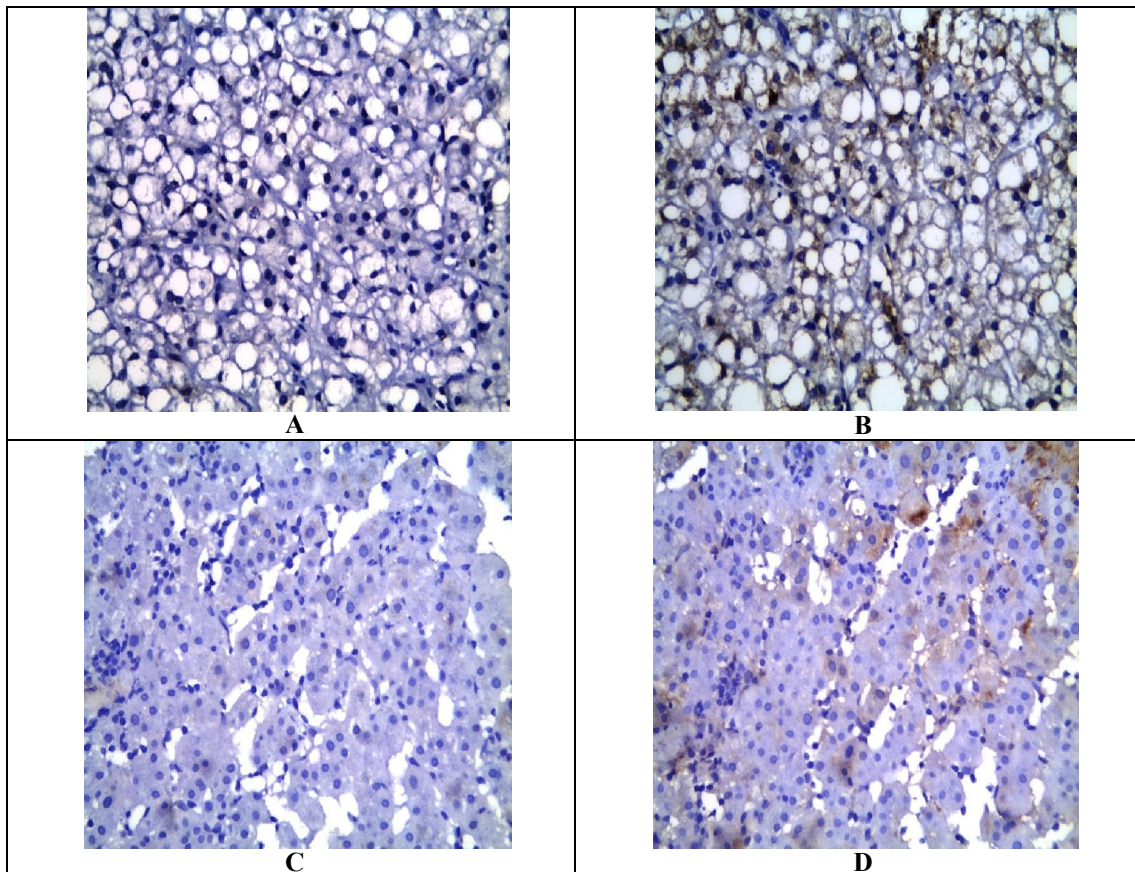


Fig.(2):Immunohistochemical staining of: (A) Macroregenerative nodules showing -ve HSP70 expression (this case was also negative for GS); (B) Macroregenerative nodules showing ++ intermediate cytoplasmic GPC3 expression; (C) High grade dysplastic nodule showing: -ve HSP70 expression (this case was also negative for GPC3); (D) High grade dysplastic nodule showing: + low cytoplasmic GS expression; (x400).

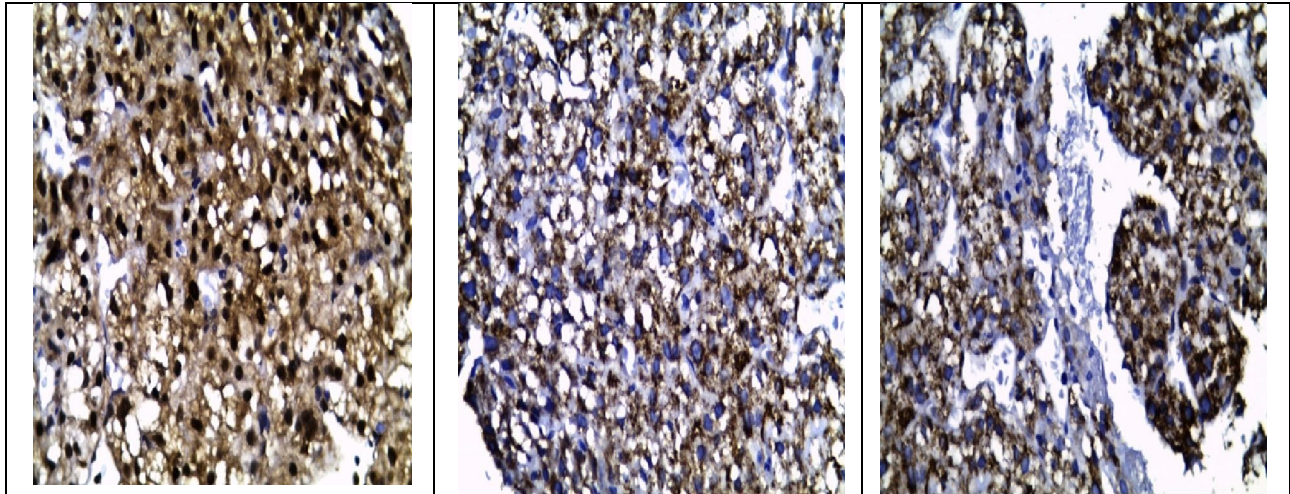


Fig.(3): Immunohistochemical staining of well differentiated HCC showing: (A) high +++ nucleocytoplasmic HSP70 expression; (B)high +++ cytoplasmic GPC3 expression; (C) high +++ cytoplasmic GS expression (x400).

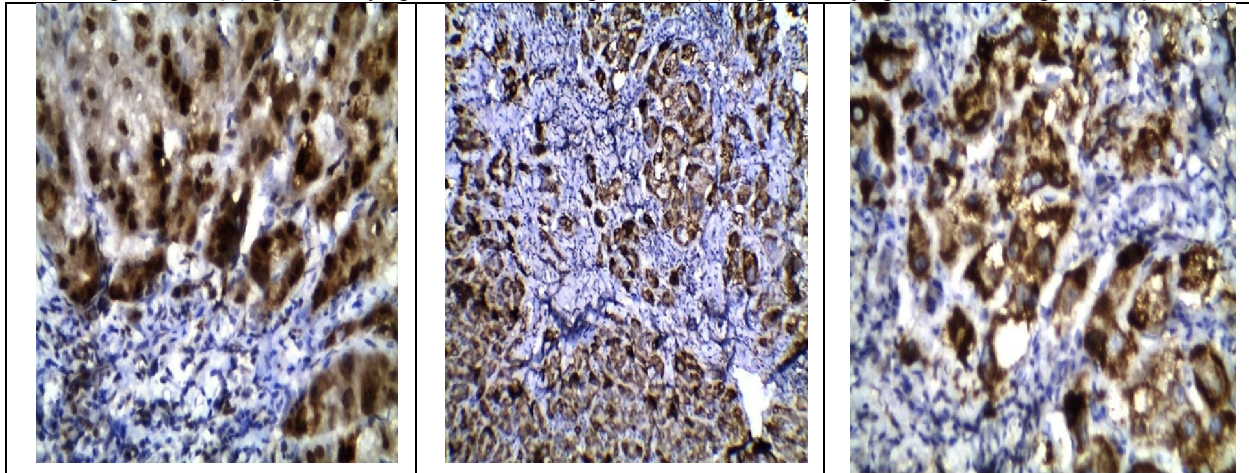


Fig.(4): Immunohistochemical staining of poorly differentiated HCC with stromal invasion showing: (A) high +++ nucleocytoplasmic HSP70 expression (x400); (B)high +++ cytoplasmic GPC3 expression (x200); (C) high +++ cytoplasmic GS expression (x400).

Table(1):Immunohistochemical expression of the studied markers in hepatocellular nodules in cirrhotic patients.

Hepatocellular nodules	N =30 (%)	HSP70				GPC 3				GS			
		-	+	++	+++	-	+	++	+++	-	+	++	+++
MRN	12 (40)	10	2	---	---	8	2	2	---	10	2	---	---
LGDN	3(10)	3	---	---	---	2	---	1	---	2	1	---	---
HGDN	5(16.7)	5	---	---	---	3	1	1	---	4	1	---	---
WDHCC	4(13.3)	---	---	2	2	1	2	--	1	---	---	2	2
MDHCC	4(13.3)	1	---	---	3	1	---	1	2	---	---	1	3
PDHCC	2(6.7)	---	1	---	1	1	1	---	---	---	---	---	2
Sensitivity	HCC	90%				70%				100%			
Specificity	vs. Benign	90%				65%				80%			

N= Number; MRN = Macroregenerative nodules; LGDN= Low grade dysplastic nodules; HGDN= High grade dysplastic nodules; HCC = hepatocellular carcinoma; WDHCC= Well differentiated; MDHCC= moderately differentiated; PDHCC= poorly differentiated.

HSP70= Heat shock protein 70; GPC3= Glypican 3; GS= Glutamine synthetase.

+ = 5%-10% (low expression); ++ = 11%-50% (intermediate expression); +++ >50% (high expression).

Table (2): Sensitivity and Specificity of markers combination for early detection of HCC.

Markers	HGDN(N=5)	HCC(N=10)	Sensitivity	Specificity
All negative	3	---	100%	60%
Three positive	----	6	60%	100%
At least two positive	----	10	100%	100%
At least one positive	2	10	100%	60%
HSP70+GPC3+	----	6	60%	100%
HSP70+GS+	----	9	90%	100%
GPC3+GS+	----	7	70%	100%
HSP70+	----	9	90%	100%
GPC3+	2	7	70%	60%
GS+	1	10	100%	80%

GS cytoplasmic immunoreactivity was seen in all HCCs (10 of 10cases). In nonmalignant nodules, GS low expression was seen in only one case of HGDN, one case of LGDN and 2 MRNs. On the other hand GS showed diffuse immunostaining (more than 50% of cells) in all HCC cases. The sensitivity and specificity of GS for HCC detection were 100%and 80%, respectively (**Table 1&Figs.2, 3, 4**).

Combination of HSP70, GPC3, and GS in the differential diagnosis between HCC and HGDN:

Table 2 illustrates the potential combinations of the markers under study for the distinction between HCC and HGDN. Negative expression of the three markers was detected in 3/5 (60%) of HGDN cases. The other two cases showed positivity only in one of the studied markers. Sensitivity and specificity of the combination of 3 markers (6/10 of HCC cases were positive for all 3 markers) were 60% & 100% respectively. Meanwhile the sensitivity and specificity raised up to 100% when at least 2 markers of the 3 used were positive irrespective of which. The most sensitive combination of two markers staining was HSP70 and GS which gave best results (90% sensitivity and 100% specificity).

4. Discussion

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and is the third leading cause of cancer-related death worldwide (**17**). The poor prognosis of patients with HCC is largely due to high rates of post-treatment recurrence and metastasis, in addition to resistance to systemic chemotherapy (**18**). It is reported that the 5-year overall survival rate of HCC is only 3-5% across the world (**19**).

Thus early detection of HCC lesions improved survival rate through treating patients before the occurrence of dissemination as had been previously reported by **Kudo**⁽⁵⁾. Since serum AFP level was taken previously as a useful marker for the detection and monitoring of HCC, however this level of AFP may remain normal as had been reported by **Soresi et al**⁽⁸⁾

in 30% of the patients with advanced HCC. Also, small hepatic focal lesions in cirrhotic patients having atypical enhancing pattern by U/S and tri-phasic CT with alpha fetoprotein less than 200ng making the diagnosis of HCC difficult.

Therefore, there is an urgent need to identify better tools to characterize HCC lesions. Histopathological criteria alone still pose problems for the differential diagnosis of certain types of benign hepatic nodules versus early HCC as usual criteria of malignancy may be absent or misleading. In particular, closely related lesions such as HGDN and well-differentiated HCC require a careful comprehensive morphological and phenotypical evaluation.

Different methodologic approaches have been used to select the most sensitive and specific markers for hepatocellular malignancy. Our present study was designed to evaluate the role of immunohistochemistry using 3 recognized putative markers of malignancy: HSP70, GPC3, and GS, taken either as individual tools or as a combination for early detection of HCC in cirrhotic patients with atypical small hepatic focal lesions. Immunohistochemistry is the most familiar and easily available technique and the reagents (specific antibodies) are now commercially available, reliable, and suitable to be applied on paraffin embedded and formalin-fixed tissue sections.

HSP70 low expression was seen in 2 out of 12 MRN but none of LGDN or HGDN was positive. It was overexpressed in 90% of malignant cases with 90% sensitivity and specificity. Such finding coincided with those of **Tommaso et al**⁽¹⁰⁾ who showed HSP70 immunoreactivity in the majority of HCCs including early and well differentiated forms but not in non malignant nodules, with sensitivity and specificity reaching up to 78% and 95% for detection of HCC, suggesting its use as a marker of malignancy. Also, **Sakamoto et al**⁽²⁰⁾ reported the significant overexpression of HSP70 in early HCC compared

with precancerous lesions. Also, HSP70 has been shown to be negative in other benign nodular lesions. Hence, HSP70 might work as a molecular marker to differentiate between benign and malignant liver nodules.

In our own study GPC3 has been mainly evaluated at the tissue level, although some studies reported that GPC3 can be found in the serum in about 55% of patients with HCC. GPC3 is also not detectable in serum of healthy subjects and in patients with liver cirrhosis without HCC (12). The sensitivity and specificity of serum GPC3 in diagnosing HCC was reported to be 51% and 90% respectively (21). In the present study the sensitivity and specificity for GPC3 at the tissue level was 70% and 65% respectively. GPC3 was detected in 7 HCC cases from 10, besides being positive in 7 benign lesions out of 20 (35%). Libbrecht *et al.* (22) showed that GPC3 stimulated hepatocytes growth by upregulating autocrine/paracrine signaling so could be used potentially as a molecular marker for the diagnosis of early HCC. In our study GPC3 positivity was seen in 33.3% of both MRN and in 40% of HGDN. This coincided with the findings of Mounajjed *et al.* (23) who detected GPC3 positive expression in about 50% of high-grade dysplastic nodules, but only in 5% of macro-regenerative/low-grade dysplastic nodules and benign hepatocytes in markedly inflamed livers. Therefore GPC-3 should not be used to diagnose hepatocellular carcinoma in isolation.

Glutamine synthetase catalyzes the synthesis of glutamine, which is the crucial energy source for the growth of both neoplastic and normal cells, and the up-regulation of GS is related to higher tumor growth and proliferation (24). In the present study GS was detected in all HCC cases besides being positive in 4 benign lesions out of 20 giving 100% sensitivity and 80% specificity; therefore making GS a good marker for HCC detection.

Osada *et al.* (13) had shown that immunoreactivity of GS protein increased from precancerous lesions to early or advanced stages; indicating a role in the promotion and progression of HCC. Other reports have suggested that GS is an early marker of hepatocarcinogenesis (10).

Previous studies discussed the significance of the GS expression level not only in the diagnosis of HCC but also in the prognosis of HCC patients but the results were controversial. Osada *et al.* (25) found that high GS expression HCC group had a significantly shorter disease-free survival time than the low-GS group. However, Dal Bello *et al.* (26) found that GS positive immunostaining was correlated with reduced specific and overall mortality. On the other hand, other study revealed that GS immunoreactivity was not related with clinicopathological parameters and

prognosis (27). These conflicting results indicate that the role of GS might vary in different patients or upon various treatments, and need the combination with another marker.

The sensitivity and specificity of the combination of 3 markers were 60% & 100% respectively, meanwhile the sensitivity and specificity raised up to 100% when at least 2 of the markers used were positive irrespective of which. Such finding coincided with previous findings of Tommaso *et al.* (10) and Mounajjed *et al.* (23) who found that overexpression of two of the three markers (HSP70, GPC3 & GS) in a given lesion is strongly associated with malignancy and may be used to support a diagnosis of hepatocellular carcinoma. Likewise, the Clinical Practice Guidelines of the European Association for the Study of the Liver recommended the use of these three markers to confirm HCC diagnosis (28).

All negative expression was seen in 66% MRN and LGDN and 60% HGDN and in none of HCC, whereas all positive expression was detected in 60% of HCC but in none of benign lesions. Only one marker was expressed, in cases of high-grade dysplastic nodule. The most sensitive combination in our study was HSP70 and GS which gave better results (90% sensitivity and 100% specificity) than that of other combinations. However, Tommaso *et al.* (10) showed that the best compromise between sensitivity and specificity was given by the combination HSP70 and GPC3 showing 59.38% sensitivity and 100% specificity.

In conclusion, we demonstrated that a panel composed of HSP70, GPC3 and GS was very useful in distinguishing between dysplastic and malignant hepatocellular nodules arising in cirrhosis. The all positive phenotype was restricted to 60% of HCC but never seen in dysplastic lesions, whereas the reverse phenotype (all negative) was a feature of the majority of nonmalignant nodules. Optimal sensitivity and specificity for HCC detection were obtained when at least 2 of all 3 markers used were positive regardless which.

Even if all of the stains are not available at time of investigation we recommend using the ones available which can still provide helpful supporting information in conjunction with the H&E findings.

Declaration of interest:

The authors report no declarations of interest.

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