Utility of an immunohistochemical panel in diagnosis of early hepatocellular carcinoma in post-hepatitis cirrhotic patients

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Abstract: Background: Although improved imaging techniques have made it possible to detect small liver lesions, differentiating benign lesions from hepatocellular carcinoma (HCC) still remains a challenge. There is an urgent need to support histological diagnosis on small biopsy specimens by further immunohistochemical analysis, especially dysplastic nodules and early HCC that differ only in subtle morphological changes. **Methods:** Heat shock protein 70 (HSP70), glypican 3(GPC3), and Enhancer of Zest Homologue 2 (EZH2) immunoreactivities were determined on formalin-fixed paraffin embedded tissues from 56 post-hepatitis cirrhotic patients, including 25 non-malignant nodules (7 large regenerative nodules, 7 low-grade dysplastic nodules, 11 high-grade dysplastic nodules) and 31 HCCs (8 early, 11 grade1, and 12 grade2-3). **Results:** The sensitivity and specificity for HCC detection were 77.4 % and 96 % for HSP70, 71 % and 92 % for GPC3, and 87 % and 88 % for EZH2.For diagnosis of early HCC-grade1 (eHCC-G1), the sensitivity and specificity were 78.9 % and 90.9 % for HSP70, 63.2 % and 81.8 % for GPC3, and 89.5 % and 72.7% for EZH2. When at least 2 markers, regardless which, were positive, a sensitivity of 73.7% with 100% specificity were found. **Conclusion:** A panel composed of HSP70, GPC3 and EZH2 is very useful in discrimination between dysplastic and early malignant hepatocellular nodules in cirrhotic patients.
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1-Introduction

Hepatocellular carcinoma (HCC) is the 5th most common cancer in the world and the second cause of cancer-related death. Even after resection, the overall survival rate of HCC patients is still unsatisfactory and its prognosis depends largely on early detection and management [1].

The main risk factors for HCC are chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections,which together, account for >80% of all cases worldwide [2]. In Egypt, the incidence of HCC has been doubled over the last decade, and Egypt has simultaneously been plagued with the highest prevalence of HCV infection (14.7%) in the world [3], while WHO estimated regional HCV prevalence is 5.3% in Africa [4].

Ultrasound and alpha-fetoprotein are most frequently used for surveillance of HCC in high-risk patients, but their sensitivities are still far from perfect [5].

Large regenerative nodule (LRN) may be the first step of hepatocarcinogenesis, which subsequently develops into advanced HCC through low-grade dysplastic nodule (LGDN), high-grade dysplastic nodule (HGDN) and early HCC [6]. HGDNs are the most advanced precancerous lesions of the liver with a risk of malignant transformation of about 30-40 % at 24 months [7]. Because of a significant overlap in the pathological and radiological features between HGDN and early HCC, various diagnostic markers were evaluated for detection of early HCC [8].

Heat shock protein 70 (HSP70) is one of the HSP family implicated in carcinogenesis, regulation of cell-cycle progression and anti-apoptosis [9]. HSP70 was reported as the most abundantly up-regulated gene in early HCC and significantly over-expressed in advanced HCC as compared to early HCC, and in early HCC as compared to precancerous lesions [10, 11]. Glypican 3 (GPC3) is a member of the glypican family of heparin sulfate proteoglycans that plays an important role in cell growth and differentiation. It is normally expressed in some embryonic tissues, including fetal liver but not in normal adult liver [12]. GPC3 protein levels are increased significantly in serum of HCC patients [13]. Several studies demonstrated that GPC3 is expressed in most HCCs but not in cirrhotic liver or benign hepatic lesions [14, 15]. Its expression is significantly different in early HCC, compared with HGDN [16].

Enhancer of Zest Homologue 2 (EZH2) is a key member of the Polycomb Group of proteins which are essential for embryonic development and stem cell renewal. EZH2 has a role in the development of highly malignant phenotypes and aggressive progression in a variety of cancers, including HCC, v with a reliability to differentiate malignant and benign hepatic tumors [17, 18].

The aim of this study was to evaluate HSP70, GPC3, and EZH2 immunoreactions in a spectrum of hepatocellular nodules ranging from cirrhotic large regenerative to low-grade and high-grade dysplastic to early and advanced HCC and to determine their sensitivity and specificity in the distinction between HGDN and early HCC.

2-Material and Methods

Liver tissue specimens were obtained from 56 post-hepatitis cirrhotic patients collected from Liver Disease File of Pathology Department, Faculty of Medicine, Zagazig University Hospital from 2007 to 2012. Cases included LRNs (n=7), LGDNs (n= 7), HGDNs (n= 11) and 31 HCCs (8 early, 11 grade 1, 12 grade 2-3). They were either surgically resected or core biopsy specimens. The diagnosis of hepatocellular nodules was performed according to criteria of the International Working Party [19] on hematoxylin and eosin stained sections. In HGDN, there was an increase in cell density (1.5-2 times), high nuclear cytoplasmic ratio, cytoplasmic basophilia, and irregular nuclear contour. If stromal or portal tract invasion seen, the nodule was considered early HCC. Criteria of small well- differentiated HCC of early stage (eHCC) are small size (up to 2 cm), increase in cellularity, nuclear cytoplasmic ratio and eosinophilia, irregular thin-trabecular pattern, retention of few portal tracts and replacing growth pattern at the tumor /non-tumor border [20]. Tumor grading was assessed according to criteria of Edmondson and Steiner [21].

Immunostaining

It was performed on 4 microns formalin-fixed, paraffin-embedded tissue sections using the standard avidin –biotin peroxidase complex (ABC) procedure. Antigen retrieval was performed for each section (microwave 750 w, 10mM citrate buffer, pH 6.0 for 15 minutes).Nonspecific binding was blocked with 10% normal rabbit serum. Commercially available antibodies used were as follows: anti-HSP70 antibody (Santa Cruz Biotechnology Inc., CA, SC-24, 1:250), anti-GPC3 (Bio-mosaics, Burlington VT, IG12, 1:100) and anti-EZH2 (BD Biosciences, CA, 11/EZH2, 1:100). The immunostaining was developed using 3,3'- diaminobenzidine as chromogen and Mayer's hematoxylin as counter stain.

As negative controls, the primary antibody was replaced by non – immune rabbit serum. Bile duct epithelium showed nucleo-cytoplasmic staining for HSP70 was used as an internal control whereas a HCC with strong immunoreaction to GPC3 and EZH2 was used as an external control [22, 23].

Evaluation criteria

All cases were semi-quantitatively scored according to the percentage of immunoreactive (IR) cells. Immunostaining of HSP70 (nucleo-cytoplasmic) and of GPC3 (cytoplasmic) were considered positive when showing >5% of IR cells. Positive cases were sub-classified as follows: + = 5-10% IR cells; ++ = 11-50% IR cells; +++ = > 50% IR cells. The reaction of EZH2 was detected as nuclear staining. Scores were assigned as follow: negative (score 0, no staining), weak (score 1, < 25% of nuclei stained), moderate (score 2, 25-75%) and strong (score 3, >75%) [18, 24].

Sensitivity was calculated as the proportion of affected cases resulting in positive tests. Specificity was calculated as the proportion of unaffected cases resulting in negative tests. Positive predictive value (PPV) was calculated as the proportion of positive tests that correctly identified affected cases. Negative predictive value (NPV) was calculated as the proportion of negative tests that correctly identified unaffected cases [22].

3-Results

Clinicopathological features of the studied cases were summarized in Table 1. Immunoreactivity of HSP70 was found in 77.4% (24/31) of the HCCs, including 6/8 (75%) of eHCC, 8/11 (72.7%) of HCCG1, and 10/12 (83.3%) of HCCG2-G3. Focal nucleo-cytoplasmic reaction was seen in scattered cells in most cases of eHCC and HCCG1, whereas most HCC G2-G3 showed strong and diffuse expression in > 50% of tumor cells (Fig. 1). In nonmalignant nodules, focal reactivity was seen only in one HGDN (Table 2).There was a positive relation between HSP70 reactivity and HCC grading but not with other clinicopathological parameters. The sensitivity, specificity, PPV and NPV of HSP70 for HCC distinction were 77.4 %, 96 %, 96%, and 77.4%, respectively (Table 4).

GPC3 immunoreaction was detected either as a cytoplasmic canalicular-like or cytoplasmic-membranous staining (Figs. 2&3).GPC3 reactivity was seen in 71% of HCCs (22/31), including 5/8(62.5%) of eHCC, 7/11 (63.6 %) of HCCG1 and 10/12 (83.3%) of HCCG2-G3. In non-malignant nodules, GPC3 immunostaining was found only in 2 HGDNs showing < 10% positive cells (Table 2). GPC3 reactivity was increased with HCC grading. The sensitivity, specificity, PPV and NPV of GPC3 for HCC detection were71%, 92%, 91.7 %, and 71.9 %, respectively (Table 4).

EZH2 nuclear immunostaining was detected in 27 /31 of HCCs (87 %), including 6 /8 of eHCC (75 %), 9/11 (81.8 %) of HCCG1 and 12 /12 (100 %) of HCCG2-G3 (Fig 4). In non-malignant nodules, weak (score 1) EZH2 reactivity was seen in 3/11 of HGDNs (Table 3). No correlation was found with any of the clinicopathological parameters. The sensitivity, specificity, PPV and NPV of EZH2 for HCC detection

were 87 %, 88 %, 90 % and 84.6 %, respectively (Table 4).

Sensitivity, specificity, PPV and NPV after grouping the nodular lesions into non-malignant and malignant groups were summarized in Table 4. When considering at least 2 positive markers, regardless this, the sensitivity for detection of HCC was 61.3 % with 100% specificity. Immunoreactivity for at least 1 marker showed increased sensitivity (90.3%) with reduced specificity (76 %). The best combination for detection of malignant nodules was GPC3/EZH2 (sensitivity 64.5% and specificity 100%).

Sensitivity, specificity, PPV and NPV of the three markers under study for detection of eHCC–G1 were reported in Table 5. A high sensitivity (89.5 %) with low specificity (45.5%) was found when at least 1 of the markers was positive, whereas low sensitivity (42.1%) with 100% specificity was detected for "all positive" phenotype. When at least 2 markers, regardless which, were positive, sensitivity increased to 73.7 % with 100% specificity. GPC3/EZH2 was the best combination for detection of eHCC-G1.

Table (1) Clinicopathological features of cases under study.

Variable	Total	Age (range)	Sex Male:Female	Viral status HCV:HBV	Lesion size (Range in cm)
Large regenerative nodules	7	40-60	5:2	5:2	1.2-2
Low-grade dysplastic nodules	7	51-69	4:3	6:1	0.5 -1.5
High-grade dysplastic nodules	11	61-75	7:4	8:3	0.8 -1.2
Early HCC	8	43-60	6:2	7:1	0.9-1.5
HCC G1	11	66-72	8:3	9:2	2.5-3.5
HCC G2-G3	12	58 – 71	8:4	10:2	3-5

Table (2): Immunohistochemical results of HSP70 and GPC3 as individual markers

Variable		*HSP70*				*GPC3*			
	Total	Positive cases (%)	+ (5 - 10%)	++ (11-50%)	+++ (>50%)	Positive cases (%)	+ (5-10%)	++ (11-50%)	+++ (>50%)
Large regenerative nodules	7	0	0	0	0	0	0	0	0
Low –grade dysplastic nodules	7	0	0	0	0	0	0	0	0
High -grade dysplastic nodules	11	1 (9%)	1	0	0	2(18.2%)	2	0	0
Early HCC	8	6 (75%)	4	2	0	5 (62.5%)	1	1	3
HCC G1	11	8 (72.7%)	2	3	3	7 (63.6%)	2	3	2
HCCG2-3	12	10(83.3%)	1	3	6	10(83.3%)	1	4	5

Table (3) Immunohistochemical results of EZH2 as individual marker.

Variable	**Total**	**Negative (score 0)**	**Weak (score 1)**	**Moderate (score 2)**	**Strong (score3)**
Large regenerative nodule	7	7	0	0	0
Low-grade dysplastic nodule	7	7	0	0	0
High-grade dysplastic nodule	11	8	3	0	0
Early HCC	8	2	4	2	0
HCC G1	11	2	5	4	0
HCC G2-3	12	0	3	4	5

Table (4) Sensitivity,specificity,positive predictive value (PPV) and negative predictive value (NPV) for distinction between nonmalignant (NM) and malignant (M) nodules using the panel of the markers under study

Variable	**MN(n= 25)**	**M(n= 31)**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
All 3 positive	0	9	29%	100 %	100%	53.2%
At least 2 positive	0	19	61.3%	100%	100%	67.6%
At least 1 positive	6	28	90.3%	76%	82.4%	86.4%
HSP70+/GPC3+	0	15	48.4%	100%	100%	61%
HSP70+/EZH2+	0	17	54.8%	100%	100%	64.1%
GPC3+/EZH2+	0	20	64.5%	100%	100%	69.4%
HSP70+	1	24	77.4%	96%	96%	77.4%
GPC3+	2	22	71%	92%	91.7%	71.9%
EZH2+	3	27	87%	88%	90%	84.6%

Table (5) Sensitivity,specificity,positive predictive value (PPV) and negative predictive value (NPV) for distinction between early and well differentiated hepatocellular carcinoma (eHCC-G1) and high-grade dysplastic nodule (HGDN)

Variable	eHCC-G1 (n =19)	HGDN (n =11)	Sensitivity	Specificity	PPV	NPV
All 3 positive	**8**	0	42.1%	100%	100%	50%
At least 2 positive	14	0	73.7%	100%	100%	68.8%
At least 1 positive	17	6 (54.5 %)	89.5%	45.5%	73.9%	71.4%
HSP70+/GPC3+	12	0	63.2%	100%	100%	61.1%
HSP70+/EZH2+	11	0	57.9%	100%	100%	57.9%
GPC3+/EZH2+	13	0	68.4%	100%	100%	64.7%
HSP70+	15	1	78.9%	90.9%	93.8%	71.4%
GPC3+	12	2	63.2%	81.8%	85.7%	56.3%
EZH2+	17	3	89.5%	72.7%	85%	80%

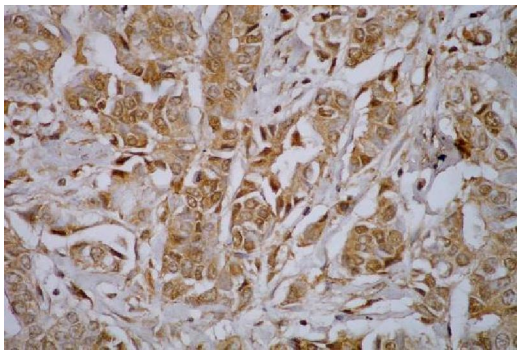


Fig (1): A case of poorly differentiated hepato-cellular carcinoma shows diffuse nucleo-cytoplasmic staining of HSP70 (original magnification, X400)

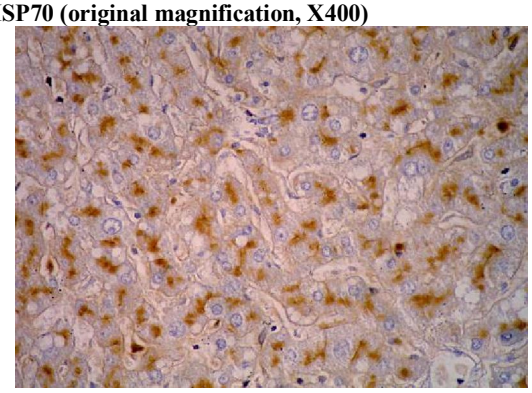


Fig (2): A case of well-differentiated hepatocellular carcinoma shows cytoplasmic canalicular staining of GPC3 (original magnification, X200)

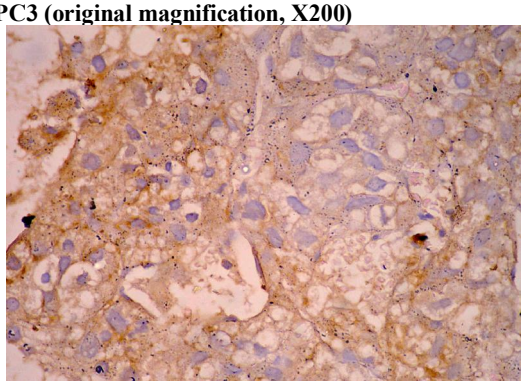


Fig (3): A case of poorly differentiated hepatocellular carcinoma shows diffuse cytoplasmic-membranous staining of GPC3 (original magnification, X400)

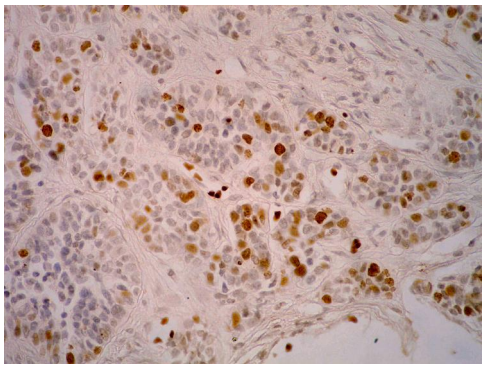
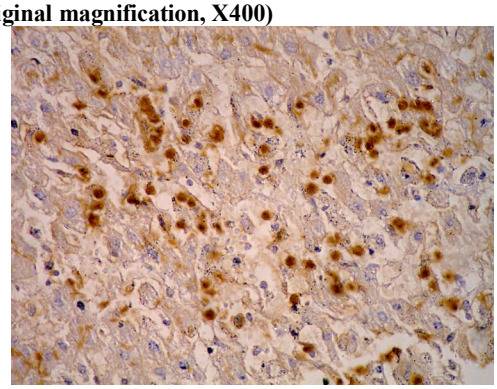
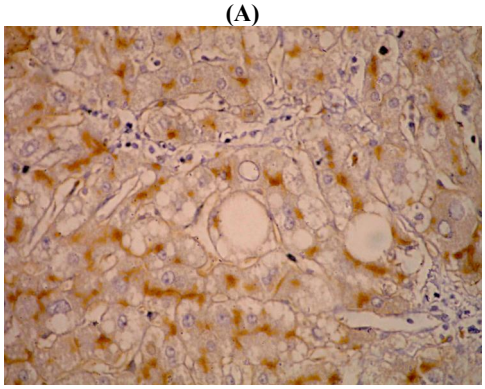


Fig (4): A case of poorly differentiated hepatocellular carcinoma shows diffuse nuclear staining of EZH2 (original magnification, X400)

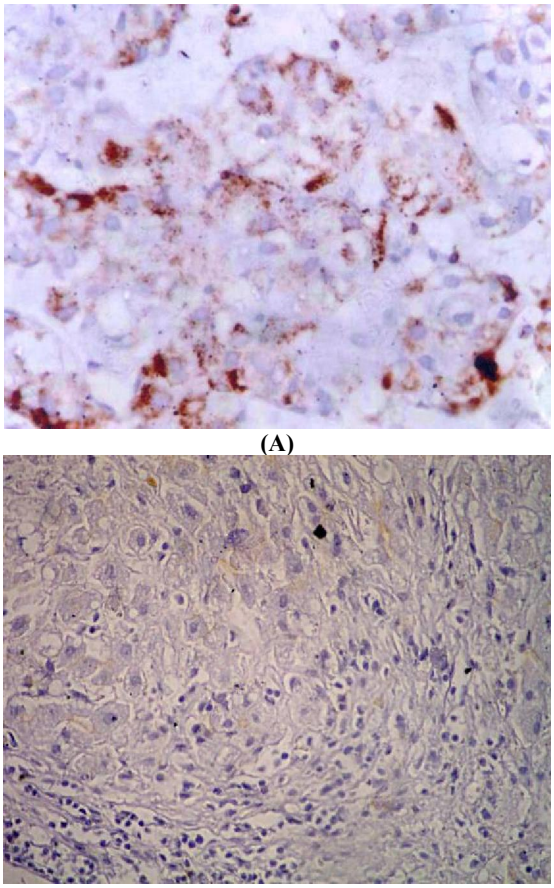


(A)



(B)

Fig (5): A case of early hepatocellular carcinoma is immunoreactive for both HSP70 (A) and GPC3. This immunophenotype supports diagnosis of malignancy (original magnification, X400)



(A)

(B)

Fig (6): A case of high grade dysplastic nodule shows focal cytoplasmic immunoreactivity for GPC3 while EZH2 is negative (original magnification,X400)

4-Discussion

Pathological examination of liver specimens has led to a clarification of the histological features of early HCCs. However, the morphologic criteria for confident diagnosis are still controversial [25].

Belonging to "borderline malignancy" category, HGDNs require an accurate distinction from early and well-differentiated HCC [26]. Pathologists and clinicians have to differentiate these lesions preoperatively by using clinical information, imaging techniques and biopsy specimens. However, most early HCC cases are asymptomatic with lack of typical radiological findings [27]. In this situation, immunohistochemical markers may provide additional diagnostic information, especially in small biopsies [28].

In the current study, 77.4 % (24/31) of HCCs were positive for HSP70. In contrast, focal staining only was observed in one out of 11 of HGDNs, indicating 77.4% sensitivity and 96 % specificity for HCC detection. In agreement with these results, Tremosini *et al.* [29] reported that sensitivity and specificity of HSP70 for HCC diagnosis were 57.5% and 85%, respectively. Among several clinicopathological parameters examined in the present study, advanced HCC grade was associated with higher immunoreactivity for HSP70 than early and well-differentiated HCC. This finding was in accordance with Shin *et al.* [30], who suggested that HSP70 is considered as a predictor of prognosis as well as a useful diagnostic marker of HCC.

Previous studies have been performed to evaluate the usefulness of GPC3 in distinction of early HCC. The results of Zhang *et al.* [31] and Honsova *et al.* [27] revealed GPC3 expression in 87.1% and 93% of HCCs, respectively. In contrast, all benign nodular lesions and cirrhosis were negative. The overall sensitivity and specificity of GPC3 for HCC diagnosis ranged between 57.5% and 95% & 83.4% and 100% as reported by Tremosini *et al.* [29] and Wang *et al.* [32], respectively. The results of the present study were consistent with the previous studies as 22/31 of HCCs showed GPC3 reactivity while none of non-malignant nodules were positive except two HGDNs showing only focal positive cells. The overall sensitivity and specificity of GPC3 for HCC detection were 71% and 92%, respectively. The extent of GPC3 immunoreactivity was affected by tumor grading, as most HCC G2-G3 showed greater number of reactive cells than eHCC-G1. Similar results were observed by Shirakawa *et al.* [33]. Using both transcript and immunocytochemical analyses, Llovet *et al.* [14] confirmed the diagnostic use of GPC3 in early HCC and its up-regulation in advanced HCC.

Hajosi-Kalcakosz *et al.* [18] detected EZH2 expression in most studied HCCs while all regenerative nodules, HGDNs and adenomas were negative. Also, Cai *et al.* [23] demonstrated that EZH2 is a valuable diagnostic biomarker of HCC with high sensitivity and specificity (95.8% and 97.8%, respectively). The present study results were close to these findings because the sensitivity and specificity of EZH2 for HCC detection were 87 % and 88 %, respectively. EZH2 reactivity was found in all HCC G2-G3 but in 78.9% of eHCC-G1 cases. Sasaki *et al.* [34] also concluded that over-expression of EZH2 is associated with aggressive biological behavior of HCC.

As regards nonmalignant nodules, hepatocytes of LRNs and LGDNs were never stained by any of the 3 markers in this study. This feature was in keeping with that reported by Di Tommaso *et al.* [22] supporting the concept that LGDNs are more related to regenerative rather than dysplastic premalignant nodules.

Regarding early and well-differentiated HCC (eHCC-G1) in the present study, the "all 3 positive" phenotype was found in 42.1% of eHCC-G1. Conversely, absence of reaction of all 3 markers in 100% of HGDNs supports the concept that these lesions are not only morphologically but also phenotypically distinct from eHCC-G1 as concluded by Di Tommaso *et al.* [24]. The phenotype of at least 2 positive markers, regardless which, was detected in 73.7% of eHCC-G1, but never seen in HGDNs. Lastly, only 1 positive marker was demonstrated in 89.5% of eHCC-G1, opposite to 54.5% of HGDNs.

The present study also investigated the diagnostic value of a panel composed of only 2 of the studied markers. The best combination for diagnosis of eHCC-G1 was GPC3/EZH2 showed 68.4% sensitivity with 100% specificity.

All present study results suggested using 3 markers panel because the immunodetection of at least 2 of them had 73.7% sensitivity and 100% specificity for diagnosis of early HCC. Similar results were reported by Cai *et al.* [23]. They concluded that the use of a 3 markers panel of HSP70, GPC3 and EZH2 can improve the rate of detection of HCCs in liver biopsies.

Conclusion

- A panel composed of HSP70, GPC3 and EZH2 is very useful in distinction between dysplastic and early malignant hepatocellular nodules arising in cirrhosis.
- The “all positive” phenotype is restricted to 42.1 % of eHCC-G1 but is never seen in HGDNs. The best sensitivity and specificity for eHCC-G1 detection are obtained when at least 2 of the 3 markers, regardless which, are positive.

So, we recommend that the use of HSP70, GPC3, and EZH2 immunostaining can effectively differentiate between HGDNs and eHCC-G1 in liver tissue specimens.

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