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RESEARCH ARTICLE

Combined Assessment of EZH2, GPC3 and SUOX could Improve Diagnosis of Regenerative Nodule, Liver Dysplasia and Small HCC in Cirrhotic Patients

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Abstract

Background: Hepatocellular carcinoma (HCC) has become the main cause of death in patients with liver cirrhosis. In Egypt, HCC has been increased with a doubling incidence in the last few years. Diagnosis of the disease at an early asymptomatic stage is the only mean to achieve long-term survival.

Aim of Work: To validate the diagnostic accuracy of the panel of biomarkers; enhancer of zeste homologue 2 (EZH2), glypican-3 (GPC3) and sulfite oxidase (SUOX) in series of biopsies from liver nodules of cirrhosis.

Methods: Sections from formalin-fixed paraffin-embedded tissues of 125 liver nodules were assessed for EZH2, GPC3 and SUOX expressions by immunohistochemistry.

Results: Liver nodules were classified as; 40 cirrhotic large regenerative nodules (CLRN), 35 high-grade dysplastic nodules (HGDN), 20 well differentiated hepatocellular carcinomas with nodules 3 cm or smaller (S-HCCs), and 30 HCCs with nodules larger than 3 cm (L-HCCs). The sensitivity and specificity of EZH2 expression levels for S-HCC and L-HCC detection versus non-malignant liver tissues were 65 % and 90.7 % vs. 83.3 % and 93.3 % respectively ($p < 0.001$). The sensitivity and specificity of GPC3 expression levels for S-HCC and L-HCC detection versus non-malignant liver tissues were (70% and 74.7% vs. 80% and 77.3%) respectively. SUOX expression significantly decreased with progression of hepatocarcinogenesis from CLRN to L-HCC ($p < 0.001$). By contrast, EZH2 and GPC3 were significantly increased with progression of hepatocarcinogenesis. A panel of EZH2, GPC3 and SUOX showed a high sensitivity, specificity and AUC (93.3%, 94.7%, and 0.96) respectively for L-HCC detection. In the diagnosis of S-HCCs, the sensitivity, specificity and AUC of a combination of the 3-markers panel were (90%, 78.7%, 0.893%) respectively.

Conclusions: The combination of the three biomarkers (EZH2, GPC3 and SUOX) could greatly improve the prospects of the early detection of small HCCs in liver biopsies

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major world health problem with a high incidence and mortality rate. It ranks fifth and seventh among men and women worldwide, respectively. The majority (85%) of the cases occur in

developing countries in Eastern and Southeast Asia, Middle and Western Africa. In Egypt, HCC represents the first and second among most common cancers in men and women respectively (1).

Advances in radiological techniques allow early detection of small hepatic nodules less than 2 cm in patients with chronic liver diseases. However, there is significant overlap in histopathological and radiological features between high-grade dysplastic nodules and early HCC making a precise diagnosis demanding. Early detection of HCC is essential for proper treatment and hence good clinical outcomes. Early lesions include premalignant and small HCCs with early or progressed malignancy. The histological diagnosis of premalignant lesions includes large regenerative nodule and dysplastic nodule (low-grade and high grade) (2). Dysplastic nodules (DNs) carry a high risk of malignant transformation, particularly the high-grade DNs (HGDNs). However, diagnosis of HGDNs, and their differentiation from well-differentiated HCC are sometimes challenging on the basis of clinical, imaging, and even morphological examination (3).

Polycomb group proteins including enhancer of zeste homolog 2 (EZH2), play an important role in the maintenance of the proliferative and self-renewal capacity of hepatic stem/progenitor cells and their differentiation and carcinogenesis (4). Hajósi-Kalcakosz et al., (5) confirmed that EZH2 is a sensitive marker of hepatocellular carcinoma, but its specificity is very low, since almost all investigated malignant liver tumours were positive regardless of their histogenesis. Deregulation of Wnt/β-catenin signaling is well-established in HCC but the molecular cause, in addition to β-catenin mutation, has not been fully resolved. EZH2 is involved in epigenetic silencing of Wnt pathway negative regulators and results in an activated Wnt/β-catenin signaling to promote HCC. EZH2 and Wnt/β-catenin signaling cooperated to promote HCC proliferation (6).

Sulfite oxidase (SUOX) is a homodimeric protein localized at mitochondria. The enzyme catalyzes the oxidation of sulfite to sulfate, the final reaction in the oxidative degradation of cysteine and methionine (7).

Glypican-3 (GPC3) is a member of heparan sulfate proteoglycan family, which is linked to the cell surface. It plays an important role in cell growth, differentiation and migration (8).

Although it has been reported that CD31, CD34, HSP70, glutamine synthetase (GS) and α-smooth muscle actin may serve as distinguishing biomarkers for HCC or well differentiated HCC and DN or HGDN, the sensitivity of the individual markers for distinguishing between well differentiated HCC and HGDN is still limited (9,10). This may influence the accuracy of the pathological diagnosis and subsequent therapy. Therefore, there is a need to evaluate other markers separately and in combination to detect their accuracy in the differential diagnosis of HGDN and S-HCC.

Aim of work: was to analyze the expression pattern of EZH2, glypican-3 and SUOX in cirrhotic large regenerative nodules (CLRN), HGDNs, S-HCC and L-HCC, in a trial to evaluate the diagnostic accuracy of panel of these biomarkers.

PATIENTS AND METHODS

Patients and samples

The current study was conducted on 125 liver biopsy specimens which were taken from patients with liver cirrhosis. Liver thin-core biopsy specimens were obtained using a 20-gauge sample needle under ultrasound guidance. This study was done in the Internal Medicine, Oncology and Pathology departments, faculty of medicine; Zagazig University, Egypt, during the period from May 2011 to May 2014. Patients without any exclusion criteria, who accepted to be included in our study, gave consent to be followed-up. All patients were subjected to pelviabdominal ultrasonography, triphasic computed tomography of the abdomen and Tru-Cut biopsy from the liver nodule or surgical excision of the liver mass. We excluded patients with liver metastasis, co-morbid diseases (advanced heart failure, advanced respiratory failure), or who refused biopsy or surgical resection. The size of the liver nodules was determined through radiological study or surgical resection.

Histopathological evaluation

Hematoxylin and eosin (H&E)-stained slides were made from each formalin-fixed paraffin-embedded tissue and were reviewed by two pathologists. Large regenerative nodule (CLRN) is a nodule with a minimum diameter of 5 mm, and more distinctive and larger than surrounding cirrhotic regenerative nodules. It is composed of hepatocytes that are cytologically bland without any architectural or cytological atypia. The criteria for HCC and HGDN diagnosis were referenced from the World Health Organization and International Consensus Group for Hepatocellular Neoplasia guidelines (11). HCC grading was divided into well-differentiated (G1) and moderately to poorly differentiated (G2/G3) (12).

Immunohistochemical staining procedure

All samples were fixed with neutral 4% formaldehyde solution. Formalin-fixed, paraffin-embedded tissues were cut into 4-μm thick sections. Then, sections were subjected to dewaxing, rehydration, blocking with hydrogen peroxide, and antigen retrieval with microwave in a 10 mM citrate buffer (pH 6.0) for 10 min and cooled to room temperature. After being blocked with 1% goat serum albumin, sections were incubated with the antibodies; GPC3 (clone 1G-12, dilution 1:100; Santa Cruz Biotechnology, Santa Cruz, CA), SUOX (Rabbit monoclonal, clone EPR7618, dilution 1: 100, Abcam, Cambridge, UK) and EZH2 (BD Biosciences, CA,11/EZH2, 1:100) overnight at 4°C, followed with horseradish peroxidase labeled secondary antibodies for 30 minutes at room temperature. The sections were incubated with diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. Hepatocellular carcinoma, liver tissues and tissue from breast cancer were used as positive control to confirm the specificity of staining with GPC3, SUOX and EZH2 respectively. Negative controls were made with primary antibodies replaced by PBS. Positive and negative control slides were included within each batch of slides.

Assessment of immunohistochemistry

- EZH2 nuclear scores were negative (score = 0, no staining), weak (score = 1, <25% of nuclei staining), moderate (score = 2, 25-75% of nuclei staining) and strong (score = 3, >75% of nuclei staining) (5).
- Granular brown reaction of GPC3 was seen in the cytoplasm and/or the membranes of hepatocytes. Its immunoreactivity was semiquantitively assessed by examining 200×fields, the staining was described as; 0 staining (negative), 1+ staining (<10% of cells), 2+staining (10%-25% of cells), or 3+staining (>25% of cells) (13).
- SUOX cytoplasmic immunostaining was scored on the basis of the percentage of positive cells: 0 (0–5%), 1 (6–25%), 2 (26–50%), and 3 (>51%). Cutoff point was 5%, above it was considered positive (14).

Statistical analysis

All data were collected, tabulated and statistically analyzed using SPSS 15.0 for windows (SPSS Inc., Chicago, IL, USA). Quantitative variables were expressed as the mean ± SD & median (range), and categorical qualitative variables were expressed as absolute frequencies "number" and relative frequencies (percentage). Continuous data were checked for normality by using Kolmogorov-Smirnov test. Kruskal-Wallis H (KW) test was used to compare between more than two groups of dependent non-normally distributed data. Categorized data were compared using the Chi-square (χ^2) test. The Spearman's rank correlation coefficient (r) was calculated to assess the relationship between EZH2, GPC3 & SUOX staining intensities. To combine the three markers (EZH2, GPC3 and SUOX), we found the linear coefficient to maximize AUC for the combination. Receiver operating characteristic (ROC) curves were obtained to calculate the optimized cutoff point for EZH2, GPC3, SUOX staining intensities & combinations of the three markers to reach the best compromise in the diagnosis of HCC. The cutoff point with maximum sensitivity and specificity (validity) was used as the recommended cutoff point. Area under curve (AUC) was calculated to compare between different markers. All tests were two sided with $p < 0.05$ to be considered statistically significant.

RESULTS

Patients and their clinicopathological parameters

125 patients, 90 males and 35 females were enrolled in this study, with age ranged from 28-73 years (mean : 49.2 ±9.1). Liver nodules were histopathologically classified into 40 CLRN, 35 HGDN, 20 S-HCCs with nodules 3 cm or smaller and 30 L-HCCs with nodules larger than 3 cm. The age of the studied cases of CLRN ranged from 28-62 years (mean: 40.3 ±6.8), while the age of cases of HGDN, S-HCC and L-HCC ranged from 41-73 years (mean: 57.4 ±8.1). Clinicopathological features of patients having HCC are summarized in table (1).

EZH2 expression in CLRN, HGDN, S-HCC and L-HCC

The degree of immunoreactivity of EZH2, which was observed mainly in the hepatocellular cell nuclei, ranged from 0% to 100%, (Figure 1). Fifteen (75%) of the examined S-HCCs stained positive, the amount of reactivity was graded as 1+ in 3 cases (15%), 2+ in 7 cases (35%), and 3+ in 5 cases (25%). Twenty six (86.7%) of L-HCC (>3cm) stained positively for EZH2. No correlation was found between the tumor grade, stage and staining scores (p =0.554, 0.668), (Table 1).

Semi-quantification of EZH2 staining intensity in CLRN, HGDN, S-HCC and L-HCC demonstrated a mean of 2.3 ±1.8, 17.03 ±13.7, 42 ± 34.9, and 50 ±31.8), respectively (Table 2). Overall, the sensitivity, specificity

of EZH2 expression levels for S-HCC and L-HCC detection versus non-neoplastic liver tissues were (65 %, 90.7 %; 83.3 % 93.3 %), respectively (p <0.001), (Table 3).

GPC3 expression in CLRN, HGDN, S-HCC and L-HCC

GPC3 staining was performed in 40 CLRN and 35 HGDN. The results of 7 CLRN (17.5%) and 17 HGDN (48.6%) were positive. The ratio of HGDN was obviously higher than that of CLRN. We found 13 cases (65%) of S-HCC stained positive for GPC3. The amount of reactivity was graded as 1+ in 3 cases (15%), 2+ in 5 cases (25%), and 3+ in 5 cases (25%). Concerning L-HCC, GPC3 was positive in 27 (90.0%); its expression was graded as 1+ in 4 cases (13.4%), 2+ in 13 cases (43.3%), and 3+ in 10 cases (33.3%), (Figure 2). Significant correlation was found between the tumor grade, and its staining scores (p =0.004), (Table 1).

Semi-quantification of CLRN, HGDN, S-HCC and L-HCC demonstrated a mean GPC3 staining intensity of (1.7 ± 4.6, 10.1 ± 15.2, 18.5 ± 22.2 and 31.5 ± 27.1) respectively, (Table 2). Overall, the sensitivity, specificity of GPC3 expression levels for S-HCC and L-HCC detection versus non-neoplastic liver tissues were (70%, 74.7%; 80%, 77.3%) respectively (p <0.001), (Table 3).

SUOX expression in CLRN, HGDN, S-HCC and L-HCC

SUOX expression was (92.5%, 82.9%, 30%, and 16.7%) in CLRN, HGDN, S-HCC and L-HCC respectively. SUOX reactivity in S-HCC and L-HCC was significantly lower than that in HGDNs, and CLRN, (Figure 3). Immunoreaction score distribution of SUOX significantly decreased with progression of hepatocarcinogenesis from CLRN to L-HCC (mean 42.6 ± 21.1, 6.5 ± 15.6 respectively (p <0.001). By contrast, EZH2 and GPC3 were significantly increased with progression of hepatocarcinogenesis. There was negative correlation between SUOX and GPC3 (Spearman's r = -0.311, p =0.051) and positive correlation between GPC3 and EZH2 (Spearman's r = +0.562, p =0.010), (Table 4; Figure 4).

Table (1): Association of EZH2, GPC3 and SUOX expressions with patients' clinicopathological features in HCC

	No	EZH2 expression			GPC3 expression			SUOX expression		
		-ve	+ve	p*	-ve	+ve	p*	-ve	+ve	p*
Age (years)										
< 50	15	3	12	0.872	4	11	0.699	8	7	0.017
≥ 50	35	6	29		6	29		31	4	
Sex										
Male	36	7	29	0.986	7	29	0.813	29	7	0.749
Female	14	2	12		3	11		10	4	
Child Pugh classification										
A	30	6	24	0.940	7	23	0.718	23	7	0.731
B	20	3	17		3	17		16	4	
Liver cirrhosis										
Yes	50	9	41	0.000	10	40	0.000	39	11	0.000
No	0	0	0		0	0		0	0	
α-fetoprotein (IU/ml)										
<400	10	2	8	0.782	3	7	0.658	2	8	0.000
≥ 400	40	7	33		7	33		37	3	
Tumour size										
≤3 cm	20	5	15	0.498	7	13	0.071	14	6	0.443
>3 cm	30	4	26		3	27		25	5	
BCLC										
0	10	2	8	0.668	3	7	0.114	7	3	0.299
A	10	3	7		4	6		7	3	
B	20	3	17		1	19		15	5	
C	10	1	9		2	8		10	0	
Tumour differentiation										
Well	27	6	21	0.554	10	17	0.004	20	7	0.501
Moderate	19	2	17		0	19		15	4	
Poor	4	1	3		0	4		4	0	

* Chi-square test P < 0.05 is significant

Table (2): Immunoreactive staining intensities for EZH2, GPC3 and SUOX in CLRN, HGDN, Small and Large HCC

	No	EZH2		GPC3		SUOX	
		Mean ± SD	Median (Range)	Mean ± SD	Median (Range)	Mean ± SD	Median Range)
CLRN	40	2.3 ± 1.8	2.2 (0 – 6)	1.7 ± 4.6	0 (0 – 20)	42.6 ± 21.1	45.5 (3 – 80)
HGDN	35	17.03 ± 13.7	13 (4 – 60)	10.1 ± 15.2	0 (0 – 50)	32 ± 21.9	30 (0 – 70)
SHCC	20	42 ± 34.9	39 (0 – 95)	18.5 ± 22.2	10.5 (0 – 65)	11.8 ± 17.9	4.0 (0 – 60)
LHCC	30	50 ± 31.8	59.5 (0 – 90)	31.5 ± 27.1	22 (0 – 90)	6.5 ± 15.6	1.0 (0 – 60)
K-W*			52.255		55.076		46.195
p			<0.001		<0.001		<0.001

* K-W Kruskal-Wallis H test

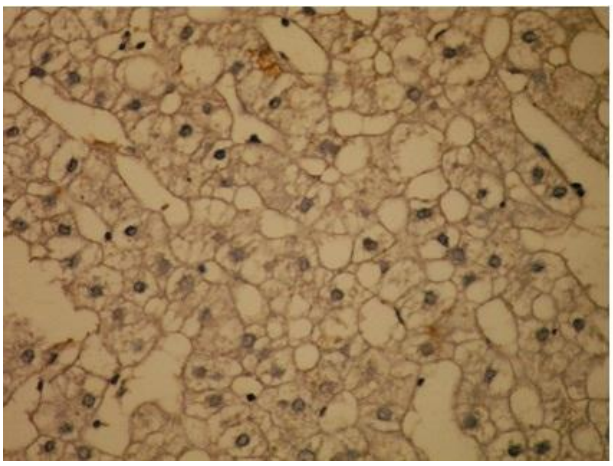
Table (3): EZH2, GPC3 & SUOX staining intensity as diagnostic tests for HCC; ROC curve Analysis

IHC	Cut-off values	Sens. % (95% CI)	Spec. % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	AUC (95% CI)
For small HCC						
EZH2	> 17	65 % (40.8-84.6)	90.7 % (81.7-96.2)	65 % (40.8-84.6)	90.7 % (81.7-96.2)	0.735 (0.634-0.820)
GPC3	> 4	70 % (45.7-88.1)	74.7 % (63.3-84.0)	42.4 % (25.5-60.8)	90.3 % (80.1-96.4)	0.726 (0.625-0.812)
SUOX	≤ 17	85 % (62.1-96.8)	76 % (64.7-85.1)	48.6 % (31.4-66)	95 % (86.1-99)	0.844 (0.755-0.910)
EZH2 & GPC3	> 14	90 % (68.3-98.8)	65.3 % (53.5-76)	40.9 % (26.3-56.8)	96.1 % (86.5-99.5)	0.820 (0.727-0.891)
EZH2 & SUOX	> - 5	90 % (68.3-98.8)	82.67 % (72.2-90.4%)	58.1 % (39.1-75.5)	96.9 % (89.2-99.6)	0.885 (0.803-0.941)
GPC3 & SUOX	> - 1	80 % (56.3 – 94.3)	86.7 % (76.8-93.4)	61.5 % (40.6-79.8)	94.2 % (85.8-98.4)	0.862 (0.776-0.924)
EZH2, SUOX & GPC3	> - 2	90 % (68.3-98.8)	78.67 % (67.7-87.3)	52.9 % (31.5-70.2)	96.7 % (88.6-99.6)	0.893 (0.813-0.947)
For large HCC						
EZH2	> 21	83.3 % (65.3-94.4)	93.3 % (85.1-97.8)	83.3 % (65.3-94.4)	93.3 % (85.1-97.8)	0.828 (0.742-0.895)
GPC3	> 5	80 % (61.4-92.3)	77.3 % (66.2-86.2)	58.5 % (42.1-73.7)	90.6 % (80.7-96.5)	0.798 (0.708-0.870)
SUOX	≤ 9	86.7 % (69.3-96.2)	85.3 % (75.3-92.4)	70.3 % (53-84.1)	94.1 % (85.6-98.4)	0.865 (0.785-0.924)
EZH2 & GPC3	> 21	96.7 % (82.8-99.9)	78.7 % (67.7-87.3)	64.4 % (48.8-78.1)	98.3 % (91.1-100)	0.940 (0.876-0.977)
EZH2 & SUOX	> 18	83.3 % (65.3-94.4)	97.3 % (90.7-99.7)	92.6 % (75.3-99.1)	93.6 % (85.7-97.9)	0.945 (0.883-0.980)
GPC3 & SUOX	> -1	86.7 % (69.3-96.2)	86.7% (76.8-93.4)	72.2 % (54.8-85.8)	94.2 % (85.8-98.4)	0.891 (0.815-0.943)
EZH2, SUOX & GPC3	> 21	93.3 % (77.9-99.2)	94.7 % (86.9-98.5)	87.5 % (71-96.5)	97.3 % (90.7-99.7)	0.958 (0.901-0.988)

Table (4): Correlation between immunoreaction staining intensity of EZH2, GPC3 and SUOX in CLRN, HGDN, SHCC and LHCC

	EZH2	GPC3	SUOX
CLRN (N=40)			
EZH2	---	r= +0.125, p= 0.411	r= -0.075, p=0.646
GPC3	r= +0.125, p= 0.441	---	r= -0.311, p=0.051
SUOX	r= -0.075, p=0.646	r= -0.311, p=0.051	---
HGDN (N=35)			
EZH2	---	r= +0.106, p=0.545	r= +0.087, p=0.619
GPC3	r= +0.106, p=0.545	---	r= +0.031, p=0.860
SUOX	r= +0.087, p=0.619	r= +0.031, p=0.860	---
SHCC (N=20)			
EZH2	---	r= +0.562, p=0.010	r= +0.008, p=0.972
GPC3	r= +0.562, p=0.010	---	r= +0.360, p=0.119
SUOX	r= +0.008, p=0.972	r= +0.360, p=0.119	---
LHCC (N=30)			
EZH2	---	r= +0.231, p=0.219	r= +0.202, p=0.286
GPC3	r= +0.231, p=0.219	---	r= -0.115, p=0.545
SUOX	r= +0.202, p=0.286	r= -0.115, p=0.545	---

r Spearman rank correlation coefficient. $P < 0.05$ is significant.



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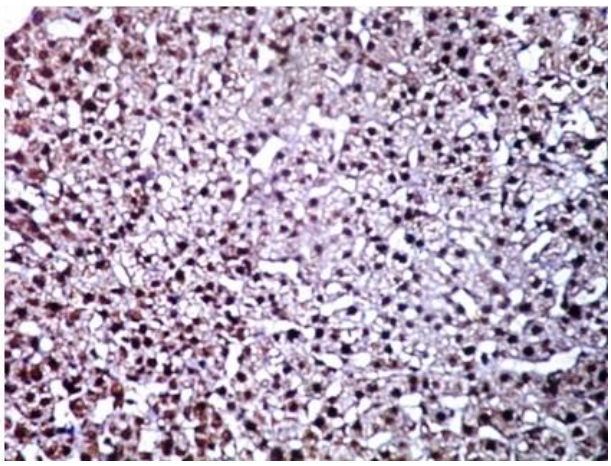

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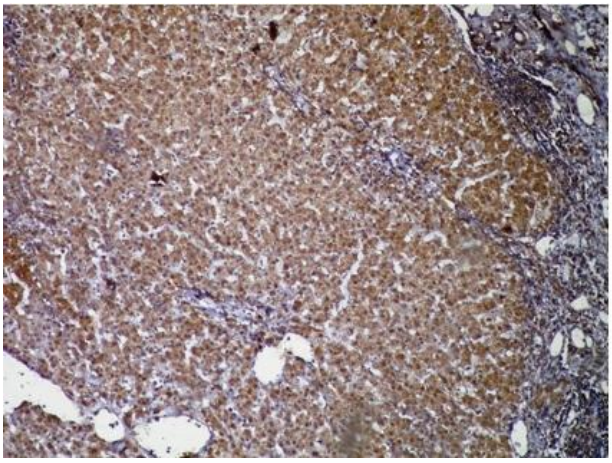

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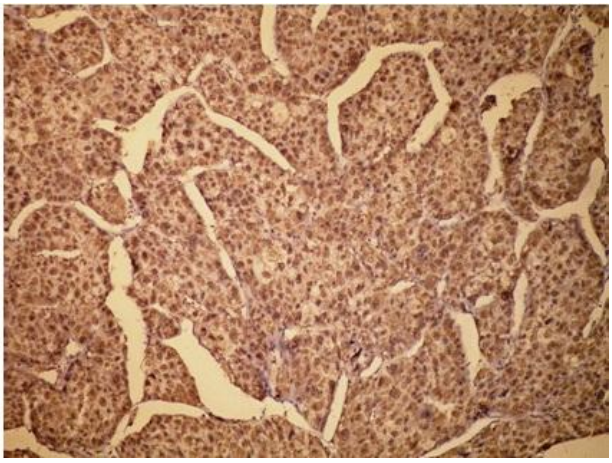

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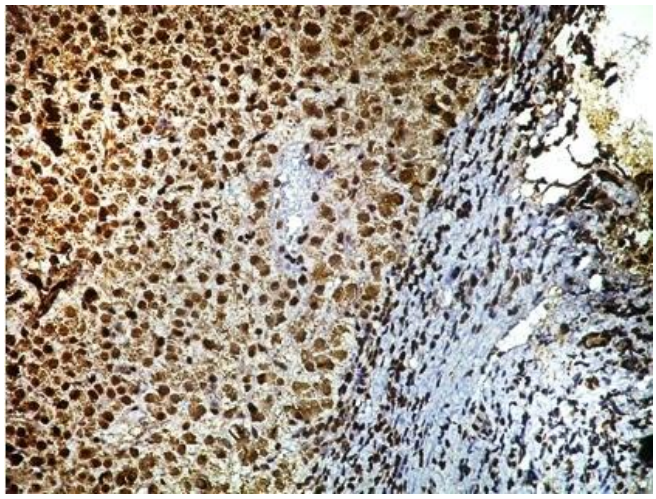
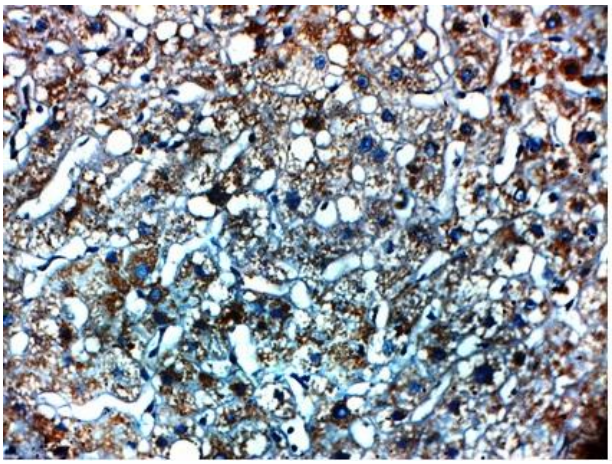


(e)

Figure (1): The expression patterns of (EZH2) examined by immunohistochemistry in liver biopsy tissues: (a) A large regenerative nodule biopsy case negatively expresses EZH2 (x400); (b) A dysplastic nodule biopsy case positively expresses EZH2 (x400); (c) A small HCC biopsy case shows positive immunostaining of EZH2(x200); (d) A moderately differentiated HCC biopsy case shows positive immunostaining of EZH2 (x200); (e) A poorly differentiated HCC biopsy case shows positive immunostaining of EZH2 (x400).



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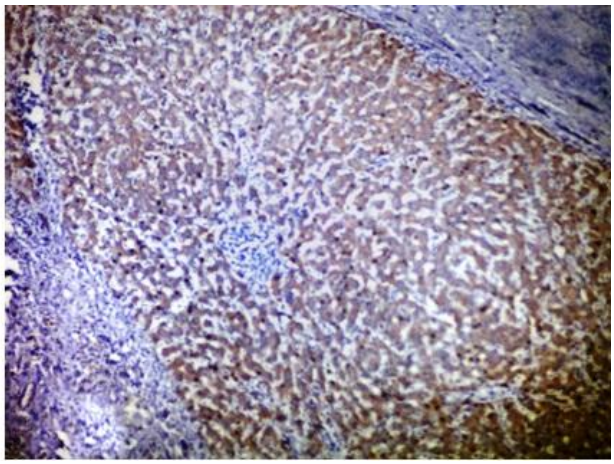

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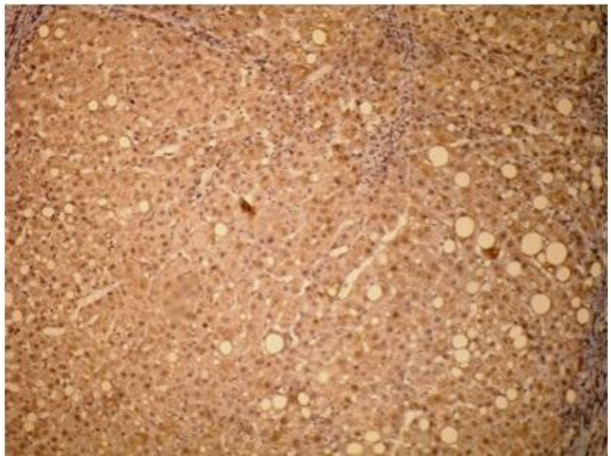

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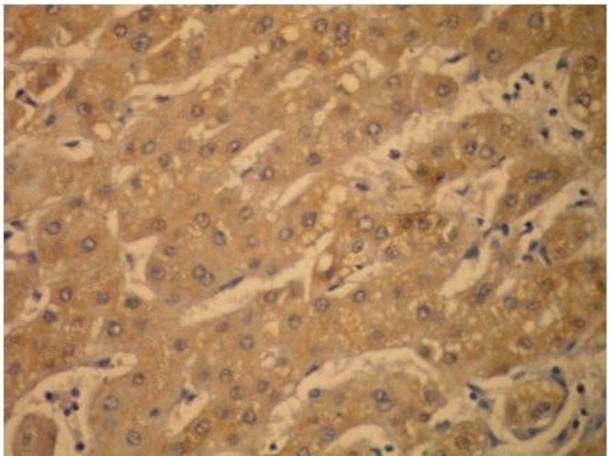

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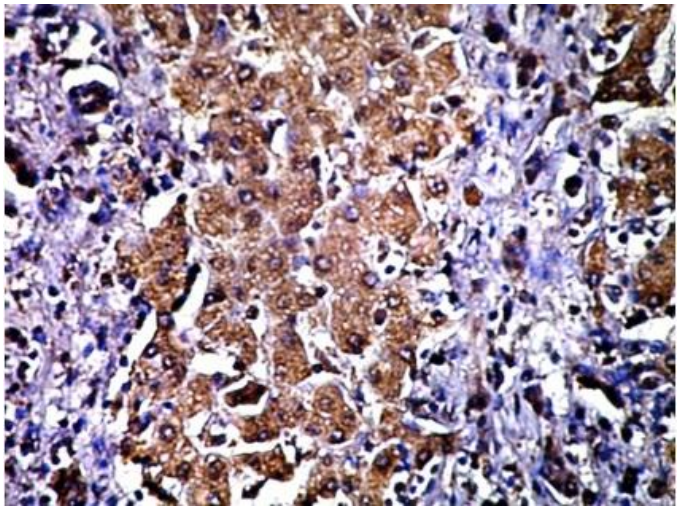
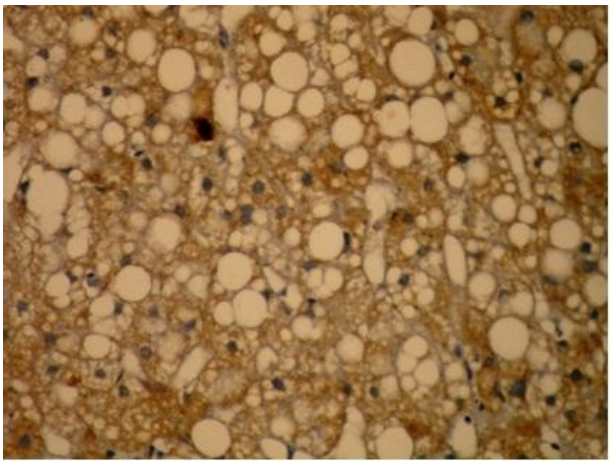
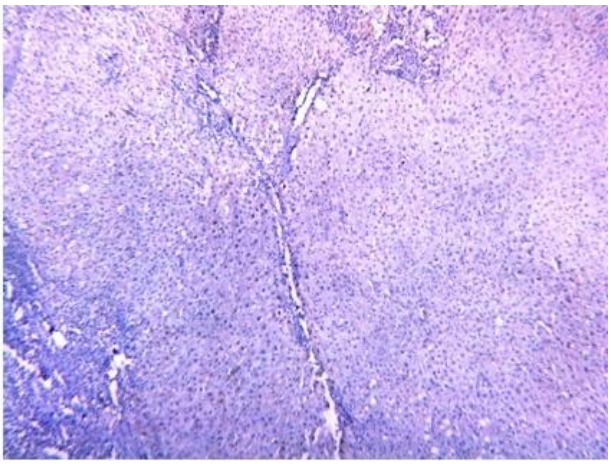


(e)

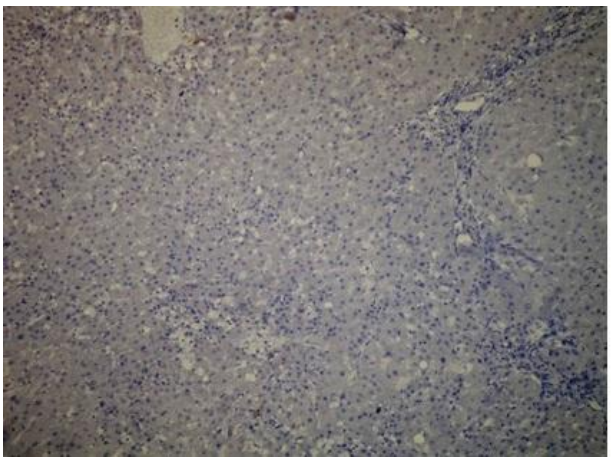
Figure (2): The expression patterns of (GPC3) examined by immunohistochemistry in liver biopsy tissues: (a) A large regenerative nodule biopsy case positively expresses GPC3 (x400); (b) A dysplastic nodule biopsy case positively expresses GPC3 (x100); (c) A small HCC biopsy case shows positive immunostaining of GPC3 (x200); (d) A moderately differentiated HCC biopsy case shows positive immunostaining of GPC3 (x400); (e) A poorly differentiated HCC biopsy case shows positive immunostaining of GPC3 (x400).



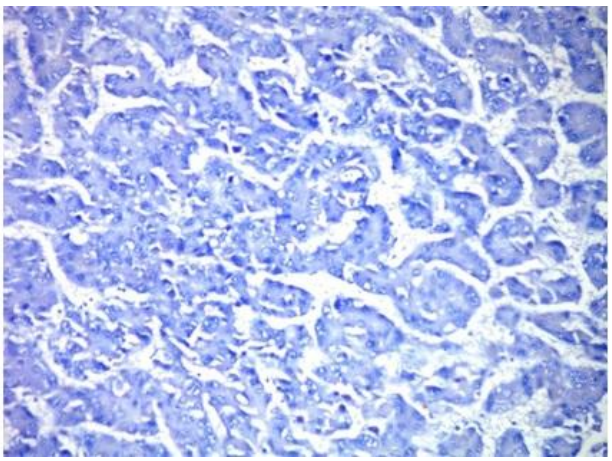
(a)



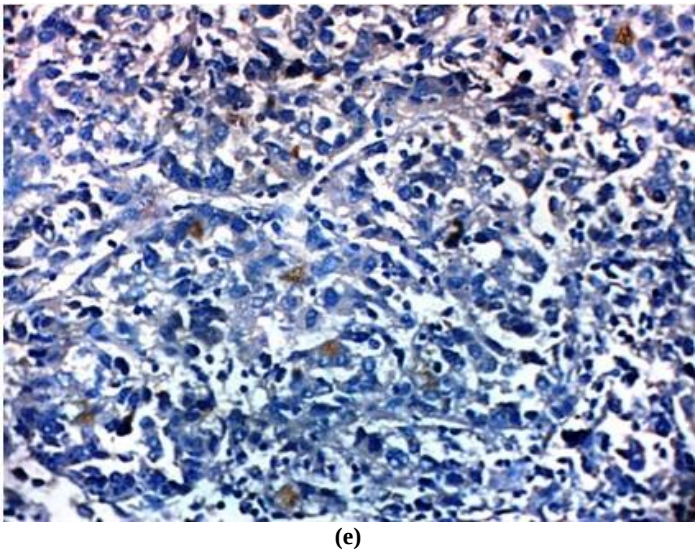
(b)



(c)



(d)



(e)

Figure (3): The expression patterns of (SUOX) examined by immunohistochemistry in liver biopsy tissues: (a) A large regenerative nodule biopsy case positively expresses SUOX (x400); (b) A dysplastic nodule biopsy case negatively expresses SUOX (x200); (c) A small HCC biopsy case shows negative immunostaining of SUOX(x200); (d) A moderately differentiated HCC biopsy case shows negative immunostaining of SUOX (x400); (e) A poorly differentiated HCC biopsy case shows negative immunostaining of SUOX (x400).

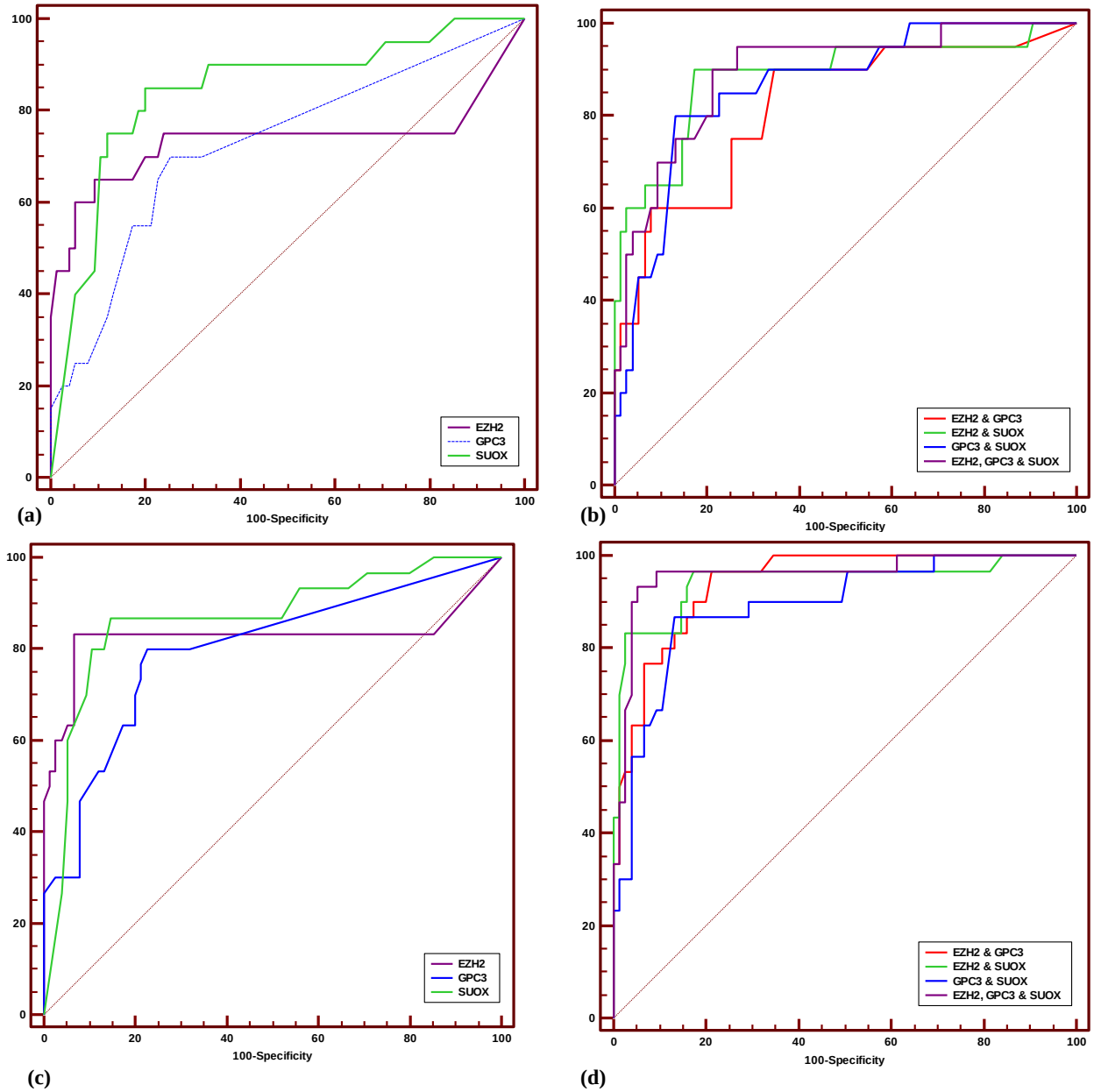


Fig. (4): ROC curves comparing EZH2, SUOX , GPC3 staining intensity & combinations of the three markers as a diagnostic tests for small & large HCC: (a) & (b) for small HCCs; (c) & (d) for large HCCs.

DISSCUSSION

There is marked overlapping in pathological and radiological features between early HCC and HGDN; this makes a confident diagnosis demanding. It also can be challenging, especially in case of highly differentiated tumors, to distinguish them from dysplastic nodules by morphological criteria alone, especially in needle biopsies where stromal invasion, can be absent. For this reason, tumor markers that could help to improve the diagnostic accuracy of histopathology, allowing a reliable differential diagnosis between high-grade dysplastic nodules and well-differentiated HCC before it develops an overt malignant phenotype, is urgently needed.

EZH2 has been suggested to play a role in the tumourigenesis of several types of human cancer, including HCC (15). In the present study, the mean staining intensity of EZH2 in S-HCC and L-HCC (>3cm) was significantly

greater than in non-neoplastic liver tissues; this result is in agreement with that of Cai et al. (15). The expression levels of EZH2 were able to distinguish HCCs from CLRN with very high sensitivity and specificity. Furthermore, the staining intensity of EZH2 in S-HCCs was significantly greater than that in HGDN. These observations strongly suggest that the immunohistochemical evaluation of EZH2 will enable us not only to differentiate HCCs from regenerative nodules, but also to distinguish S-HCCs from HGDN with a high degree of accuracy. Several studies had noted that EZH2 was over-expressed in most of the HCC resection specimens by IHC, whereas it was negatively expressed in almost all regenerative and dysplastic nodules (16-18).

GPC3 has roles in development and regulation of cellular proliferation and apoptosis. It is expressed in fetal liver and progenitor cells as well as in many cases of HCC. In our study, GPC3 was expressed in 17.5% of cirrhotic macronodules (CLRN); the stain was only cytoplasmic. Similarly, Llovet et al. (19) reported weak focal positivity in 26% of cirrhotic livers; Wang et al., (20) reported staining in 7% of non dysplastic cirrhotic cases; and Libbrecht et al., (21) noted focal positive staining in benign cirrhotic nodules near HCC in 8%. This is in contrast to results of Wang et al. (20) who reported that none of CLRN showed GPC3 positivity. GPC3 expression in large regenerative nodules supports that CLRN is an early step in the carcinogenesis pathways of HCC (22).

The present work reported high GPC3 expression (48.6%) in HGDN than previous results stated by Wang et al. (20) who reported that only 3% of HGDN cases showed low levels of GPC3 expression. Some factors might explain such discrepancies including: i) inadequate patient cohort, ii) different study designs, iii) different commercial antibodies used for the analysis, and iv) Racial factors.

We found that the sensitivity and specificity of GPC3 single staining for large HCC nodules were 80% and 77.3%, respectively, while for nodules 3 cm or smaller, the values were 70% and 74.7%, suggesting that GPC3 staining helps to achieve HCC diagnosis. These results are consistent with previous reports (9,20,23) that revealed the value of GPC3 in diagnosis of malignant nodules. GPC3, although useful in the diagnosis of HCC, can be detected by immunostaining in CLRN (17%) and HGDN(48.6%) lesions; and therefore its presence and significance as a cancer stain should be interpreted cautiously, especially in small biopsies; and it should be combined with other diagnostic immuno-marker as we stated in this work. GPC3 expression significantly increased in moderately and poorly differentiated HCC (P= 0.004). This result is similar to that done by Shirakawa et al. (24). In our panel, we have immuno-positive marker (SUOX) in benign liver lesions; this is in contrary to investigated biomarkers in which benign lesions were nearly negative for all markers of the panel. In the present study SUOX was gradually decreased from CLRN, HGDN, S-HCC, to L-HCC with significant difference (P value <0.001). Similar results have reported by Jin et al., (14), who stated that immunoreactivity score distribution of SUOX significantly decreased with the progression of hepatocarcinogenesis from CLRN to large HCC (P value <0.001). SUOX has the advantage that it gives high percentage of positive stain in non-neoplastic liver tissue (92.5%), so it's of great help especially in small liver biopsies. Also, we found a significant association between positive SUOX immunoreactivity and alpha fetoprotein less than 400IU/ml; this may indicate that SUOX is a favorable prognostic factor (Table 1). This result goes with that of Jin et al. (14) who found that SUOX-positive and AFP-negative patients had favorable overall survival compared with SUOX-negative and AFP-positive patients

Histopathological analysis isn't satisfied to achieve sufficient sensitivity and specificity for the diagnosis of hepatocellular carcinoma in small nodules; Immunohistochemical staining has been suggested to allow a confident diagnosis. To our knowledge, in this study, the diagnostic accuracy of combined EZH2, GPC3 and SUOX was first analyzed to diagnose CLRN, HGDN, S-HCC and L-HCC. As expected, a panel of EZH2, GPC3 and SUOX showed a high sensitivity, specificity and AUC (93.3%, 94.7%, and 0.96) for large HCC detection. Interestingly, the sensitivity, specificity and AUC for HCC diagnosis increased when EZH2 was used in combination with GPC3 and SUOX. In addition, for the diagnosis of S-HCCs, the sensitivity, specificity and AUC of a combination of the 3-markers panel were (90%, 78.7%, 0.893%) respectively. In case of use of only two biomarkers, the EZH2 and SUOX were the best combination for diagnosis of S-HCC (90%, 82.7%; sensitivity and specificity).

The sensitivity and specificity of EZH2 + GPC3+SUOX were higher than those of any single marker or any two-marker combination. Using the three-marker panel (EZH2, GPC3 and SUOX), immuno-positive cases (cases where any two markers showed abnormal staining pattern) were observed in none of the benign liver lesions, but were frequently observed in S-HCCs and L-HCCs. However, in HGDNs two markers showed abnormal staining pattern were observed in 9 cases. This means that HGDN is an independent risk factor for HCC development and close follow-up is highly needed. So, patients with HGDNs should be enrolled on liver transplant waiting lists, since they are associated with subsequent HCC development and decreased survival; the 5-year survival of patients with HGDNs was less than 50% (25).

Most HCCs develop in the background of advanced liver fibrosis and cirrhosis; this emphasizes on the multistep process of liver carcinogenesis through the progressive malignant transformation of cirrhotic nodules (26). Our study also confirms results of Wang et al. (27) and Cai et al., (28) who postulated GPC3 and EZH2 as early

markers of hepatocarcinogenesis. This is because a significant proportion of high grade dysplastic nodules and early HCC already displayed GPC3 and EZH2 expression.

CONCLUSIONS

Large regenerative nodules in liver cirrhosis represent an early step in HCC carcinogenesis pathways. HGDN is a risk factor for HCC development. Diagnosis of liver nodules by needle liver biopsy is based on the analysis of tiny fragments of the tissue, by which it is very difficult to distinguish between small well differentiated HCCs and certain benign and dysplastic liver nodules in patient with cirrhosis. The use of a three-biomarker panel (EZH2, GPC3 and SUOX) could improve the rate of detection of HCC $\leq$ 3 cm in liver biopsy tissues with better sensitivity and specificity. Moreover, it helps to distinguish S-HCC from non-malignant nodule with a high degree of accuracy.

Abbreviations

HCC: Hepatocellular carcinoma; S-HCC: Hepatocellular carcinomas with nodules 3 cm or smaller; L-HCCs: Hepatocellular carcinomas with nodules larger than 3 cm; EZH2: Enhancer of zeste homologue 2; GPC3: Glypican-3; SUOX: Sulfite oxidase; DNs: Dysplastic nodules; HGDNs: High-grade dysplastic nodules; CLRN: Cirrhotic large regenerative nodule; AUC: Area Under Curve.

Authors' contributions

All authors: Provision of study materials, design, collection, tissue processing techniques and assembly of data; HR and SA: Examination and histologic grading of the pathologic specimens. AH: Sharing in identification of the normal tissues and statistical analysis of results. EI, MA and SM evaluate the clinical profiles of the patients; HR wrote the manuscript; AH, EI, MA and SM critically revised the manuscript; All authors have read and approved the final manuscript.

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