EZH2 protein: a promising immunomarker for the detection of hepatocellular carcinomas in liver needle biopsies

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ABSTRACT

Background and aims A previous study of ours indicated that enhancer of zeste homologue 2 (EZH2) plays an important role in hepatocellular carcinoma (HCC) tumorigenesis. The aim of the present study was to investigate the potential diagnostic utility of EZH2 in HCC.

Methods Immunohistochemistry was performed to examine the expression dynamics of EZH2 in two independent surgical cohorts of HCC and non-malignant liver tissues to develop a diagnostic yield of EZH2, HSP70 and GPC3 for HCC detection. The diagnostic performances of EZH2 and a three-marker panel in HCC were re-evaluated by using an additional biopsy cohort.

Results Immunohistochemistry analysis demonstrated that the sensitivity and specificity of EZH2 for HCC detection was 95.8% and 97.8% in the testing cohort. Similar results were confirmed in the validation cohort. For diagnosis of well-differentiated HCCs, the sensitivity and specificity were 68.9% and 91.5% for EZH2, 62.5% and 98.5% for HSP70, 50.0% and 92.1% for GPC3, and 75.0% and 100% for a three-marker panel. In biopsies, positive cases for at least one marker increased from large regenerative nodule and hepatocellular adenoma (0/12) to focal nodular hyperplasia (2/20), dysplastic nodule (7/25), well-differentiated HCC (16/18) and moderately and poorly differentiated HCC (54/54). When at least two positive markers were considered, regardless of their identity, the positive cases were detected in 0/12 large regenerative nodules and hepatocellular adenomas, 0/20 focal nodular hyperplasias, 0/25 dysplastic nodules, 11/18 well-differentiated HCCs, 32/37 moderately differentiated HCCs and 15/17 poorly differentiated HCCs.

Conclusion Our findings suggest that EZH2 protein, as examined by immunohistochemistry, may serve as a promising diagnostic biomarker of HCCs, and the use of a three-marker panel (EZH2, HSP70 and GPC3) can improve the rate of detection of HCCs in liver biopsy tissues.

Significance of this study

What is already known about this subject?

- Needle biopsy is recommended to identify the features of liver malignancies when small hepatic nodules are detected. Clinically, it is difficult to make a histological distinction between well-differentiated hepatocellular carcinomas (WD HCCs) and dysplastic nodules (DNs), especially in liver biopsy tissues. EZH2 plays a key role in the tumorigenesis of HCC. Increased expression of EZH2 has been frequently detected in HCC tissues, and has been correlated with the aggressiveness and poor prognosis of HCCs.

What are the new findings?

- The staining intensity of EZH2 by immunohistochemistry in WD HCCs is significantly larger than that in DNs.
- The evaluation of EZH2 expression enables us not only to discriminate HCCs and non-neoplastic liver tissue, but also to distinguish WD HCC from DNs with a high degree of accuracy.
- The combination of the three markers (EZH2, HSP70 and GPC3) could greatly improve our ability to diagnose HCCs with better sensitivity and optimal specificity.

How might it impact on clinical practice in the foreseeable future?

- The results reported here suggest that EZH2 can serve as a promising immunomarker of HCCs in the diagnostic work-up of the liver lesions, and that the use of a three-marker panel (EZH2, HSP70 and GPC3) enables HCCs to be detected more easily in liver biopsies.

INTRODUCTION

Hepatocellular carcinoma (HCC) is a major lethal malignancy. The disease has a high prevalence in southeast Asia and Africa, and the incidence of HCC has also been steadily increasing in Europe and America.[1 2] Due to the high prevalence of hepatitis B virus (HBV) infection in Chinese populations, HBV-related liver cirrhosis and/or HCC has become one of the main disease burdens in China. Unfortunately, the long-term prognosis of patients with HCC remains unsatisfactory in spite of recent advances in surgical techniques and medical management.[3] This poor outcome is, in part, attributable to the fact that HCC is generally diagnosed at a late clinical stage, when treatments are of limited effectiveness.[4] Although serum α-fetoprotein (AFP) and ultrasonography are routinely used in diagnosis at present,

complementing clinical assessments, they have a relatively low sensitivity and specificity, and are not able to identify small and early stage HCCs.[5 6] To diagnose small and early stage HCCs clinically, a biopsy of hepatic nodules is usually recommended as a check on diagnoses deriving from serological and radiological tests.[4] However, it is difficult to make a histological distinction between early well-differentiated (WD) HCC and certain benign hepatocellular lesions, such as dysplastic nodule (DN) in liver biopsy specimens, even for experienced pathologists; while immunomarkers commonly used by clinicians, such as AFP, CD34 and carcinoembryonic antigen (CEA), have significant diagnostic limitations.[7–9] There is therefore an urgent need to discover and identify new biomarkers that can distinguish between HCCs and other benign liver lesions in liver needle biopsy tissues.

Enhancer of zeste homologue 2 (EZH2), the catalytic subunit of polycomb repressive complex 2 (PRC2), has been identified as the sole histone methyltransferase that methylates histone H3 lysine 27 (H3K27) and mediates transcriptional silencing.[10] EZH2 has been found to contribute to the maintenance of cell identity, cell cycle regulation and oncogenesis.[11 12] A previous study by our group showed that knockdown of EZH2 expression in HCC cells was sufficient to significantly reverse tumorigenicity in a nude mouse model, and demonstrated the potential therapeutic value of EZH2 inhibition in HCC.[13] Recent studies have reported that increased expression of EZH2 was frequently detected in HCC tissues and it was correlated with the aggressiveness and/or poor prognosis of HCCs.[14–16] However, these results were evaluated from a small cohort of liver tissue samples, and the criteria for evaluation of EZH2 positive expression were ambiguous. The potential utility of EZH2 as a diagnostic biomarker of HCC in liver resection and needle biopsy tissues still remains to be demonstrated.

The aim of the present study was to investigate the potential diagnostic utility of EZH2 in HCCs. We first analysed the protein levels of EZH2 in a panel of HCC and hepatic cell lines and tissues by western blotting. We then performed immunohistochemistry (IHC) to examine the expression pattern of EZH2 in a large surgical cohort of HCCs and corresponding non-neoplastic liver tissues. Two other potential molecules, heat-shock protein 70 (HSP70) and glypican 3 (GPC3), have previously been proposed for use in the differential diagnosis of hepatocallular nodules. These molecules provided a predictive power for the diagnosis of early HCC,[17–20] and have been recently identified as two useful biomarkers in the diagnosis of HCC.[21 22] We therefore further validated the diagnostic value of EZH2 both alone and in combination with HSP70 and GPC3 by IHC in another independent surgical cohort of HCCs and benign hepatic lesions. Finally, we re-evaluated the diagnostic performances of EZH2, together with HSP70 and GPC3, in HCCs by using an additional cohort of liver needle biopsy samples. We now report for the first time that the expression of EZH2 protein, as examined by IHC, may serve as a promising diagnostic biomarker of HCC, and that the use of EZH2 in combination with HSP70 and GPC3 improves the rate of detection of HCCs in liver needle biopsy tissues.

MATERIALS AND METHODS

Cell line and cell cultures

Six HCC cell lines (Huh7, HepG2, 7402, 7721, Hep3B and Lm3) and one normal hepatic cell line (Lo2) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C with 5% CO_2, in accordance with a previously described protocol.[13]

Cohorts

Formalin-fixed, paraffin-embedded, primary HCCs from 212 patients, who underwent initial surgical resection between March 2003 and August 2006, were randomly selected from the archives of the Department of Pathology of the First Affiliated Hospital, Sun Yat-Sen University (Guangzhou, China). We used these patients as a testing cohort. This cohort of patients with HCC included 174 (82%) men and 38 (18%) women, with a median age of 48 years, and their clinico-pathological characteristics are summarised in table 1. Average follow-up time was 28.79 months (median, 22.5 months; range, 1.0–81.0 months).

In parallel, we assessed another randomly collected, independent validation cohort of 163 patients diagnosed with liver disease between July 2005 and May 2008. These patients, whose diseases encompassed 126 HCCs and 37 DNs, underwent initial surgical treatment at Sun Yat-Sen University Cancer Center (Guangzhou, China). The 126 patient with HCC included 95 (75.4%) men and 31 (24.6%) women, with a median age of 49.5 years (table 1). Average duration of follow-up was 23.69 months (median, 23.5 months; range, 1.0–53.0 months). In this cohort, the 37 cases of DNs included 12 (32.4%) women and 25 (67.6%) men. The ages of the patients ranged from 19 to 72 years, and the mean age was 42.3 years. Patients with HCC were selected for both the testing and validation cohorts only if they had been given a distinctive pathological diagnosis, were undergoing primary and curative resection, and had not received preoperative anticancer treatment. Availability of patients' resection tissues and follow-up data were also criteria for selection.

We collected an additional cohort of 129 cases of 18-gauge needle biopsy specimens with hepatic nodules (<3 cm) from March 2007 to May 2010 in our institutes and from the Department of Pathology, Guangdong Provincial People's Hospital, (Guangzhou, China). To ensure that the pathological diagnosis of this cohort was accurate, only cases in which the patients subsequently underwent surgical resection were selected. The diagnoses of these biopsy tissues were confirmed as identical to those of the resultant surgically resected specimens. Of the 129 cases of liver biopsy, 72 were diagnosed as HCC, 25 as DN, 20 as focal nodular hyperplasia (FNH), six as hepatocellular adenoma (HA) and six as large regenerative nodule (LRN).

In this study, all diagnoses were formulated by expert pathologists (H-LR and Q-LW), according to the criteria for terminology established by the International Working Party.[23] Tumour differentiation was based on the criteria of the World Health Organization Classification of Tumours (2000). Tumour stage was defined according to the 2002 American Joint Committee on Cancer/International Union Against Cancer tumour–node–metastasis (TNM) classification system.[24] In addition, for EZH2 western blotting analysis, fresh tissue specimens from 10 patients with HCC who underwent surgical resection were collected in 2009.

Western blotting analysis

Equal amounts of whole cell and tissue lysates were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and electrotransferred on a polyvinylidene difluoride (PVDF) membrane (Pall, Port Washington, New York, USA). The tissues were then incubated with primary mouse monoclonal antibodies against human EZH2 (1:1000 dilution; BD Transduction Laboratories, Franklin Lakes, New Jersey, USA). The immunoreactive signals were detected with enhanced chemiluminescence kit (Amersham Biosciences, Uppsala, Sweden). The procedures followed were conducted in accordance with the manufacturer's instructions.

Table 1 Association of enhancer of zeste homolog 2 (EZH2) expression with patients' clinicopathological features in primary hepatocellular carcinomas

	EZH2 protein							
	Testing cohort				Validation cohort			
Variable	All cases	Negative expression	Positive expression	p Value*	All cases	Negative expression	Positive expression	p Value*
Age (years)				0.104				0.892
≤47.9†	105	9 (8.6%)	96 (91.4%)		61	8 (13.1%)	53 (86.9%)	
>47.9	107	17 (15.9%)	90 (84.1%)		65	8 (12.3%)	57 (87.7%)	
Sex				0.718				0.510
Male	174	22 (12.6%)	152 (87.4%)		109	13 (11.9%)	96 (88.1%)	
Female	38	4 (10.5%)	34 (89.5%)		17	3 (17.6%)	14 (82.4%)	
Hepatitis history				0.578				0.661
Yes	164	19 (11.6%)	145 (88.4%)		107	13 (12.1%)	94 (87.9%)	
No	48	7 (14.6%)	41 (85.4%)		19	3 (15.8%)	16 (84.2%)	
α-Fetoprotein (ng/ml)				0.210				0.309
≤20	67	11 (16.4%)	56 (83.6%)		56	9 (16.1%)	47 (83.9%)	
>20	145	15 (10.3%)	130 (89.7%)		70	7 (10.0%)	63 (90.0%)	
Liver cirrhosis				0.608				0.064
Yes	132	15 (11.4%)	117 (88.6%)		88	8 (9.1%)	80 (90.9%)	
No	80	11 (13.8%)	69 (86.3%)		38	8 (21.1%)	30 (78.9%)	
Tumour size (cm)				0.000				0.017
≤5	59	15 (25.4%)	44 (74.6%)		76	14 (18.4%)	62 (81.6%)	
>5	153	11 (7.2%)	142 (92.8%)		50	2 (4.0%)	48 (96%)	
Tumour multiplicity				0.326				0.408
Single	128	18 (14.1%)	110 (85.9%)		87	12 (13.8%)	75 (86.2%)	
Multiple	84	8 (9.5%)	76 (90.5%)		39	4 (10.3%)	35 (89.8%)	
Differentiation				0.250				0.362
Well	24	6 (25.0%)	18 (75.0%)		16	4 (25%)	12 (75%)	
Moderate	129	14 (10.9%)	115 (89.1%)		71	8 (11.3%)	63 (88.7%)	
Poor	50	5 (10.0%)	45 (90.0%)		33	4 (12.1%)	29 (87.9%)	
Undifferentiated	9	1 (11.1%)	8 (88.9%)		6	0 (0%)	6 (100%)	
Stage				0.000				0.001
I	28	11 (39.3%)	17 (60.7%)		12	6 (50.0%)	6 (50.0%)	
II	60	8 (13.3%)	52 (86.7%)		50	6 (12.0%)	44 (88.0%)	
III	97	6 (6.2%)	91 (93.8%)		50	3 (6.0%)	47 (94.0%)	
IV	27	1 (3.7%)	26 (96.3%)		14	1 (7.1%)	13 (92.9%)	
Vascular invasion				0.075				0.023
Yes	108	9 (8.3%)	99 (91.7%)		57	3 (5.3%)	54 (94.7%)	
No	104	17 (16.3%)	87 (83.7%)		69	13 (18.8%)	56 (81.2%)	
Relapse				0.104				0.410
Yes	105	9 (8.6%)	96 (91.4%)		43	4 (9.3%)	39 (90.7%)	
No	107	17 (15.9%)	90 (84.1%)		83	12 (14.5%)	71 (85.5%)	

*χ^2 test.
†Mean age.

Tissue microarray and immunohistochemistry

Tissue microarrays (TMAs) were constructed in accordance with a previously described method.[25] Triplicate 0.6 mm diameter cylinders (two identical cylinders taken from intra-tumoural tissue and one cylinder from peritumoural tissue) were punched from representative areas of an individual donor tissue block, and re-embedded into a recipient paraffin block in a defined position, using a tissue arraying instrument (Beecher Instruments, Silver Spring, Maryland, USA).

The TMA blocks were cut into 5-μm sections and processed for IHC in accordance with a previously described protocol.[26] TMA slides were incubated respectively with anti-EZH2 (1:100 dilution; BD Transduction Laboratories, Franklin Lakes, New Jersey, USA), anti-HSP70 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, California, USA) and anti-GPC3 (1:100 dilution; BioMosaics, Burlington, Vermont, USA), and stored overnight at 4°C. Immunostaining was performed using the Envision System with diaminobenzidine (Dako, Glostrup, Denmark). A negative control was obtained by replacing the primary antibody with a normal murine or rabbit IgG. In the case of non-informative TMA samples (ie, samples with <500 tumour cells per case and lost samples), IHC staining was performed by using whole tissue slides.

Evaluation by immunohistochemistry

Immunoreactivity for EZH2, HSP70 and GPC3 proteins was scored using a semi-quantitative method by evaluating the number of positive tumour cells over the total number of tumour cells. Scores were assigned by using 5% increments (0%, 5%, 10% … 100%), as in our previous study.[26] Expression for the markers was assessed by three independent pathologists (M-YC, H-LR and DX), who were blinded to the clinicopathological data. Their conclusions agreed in approximately 81% of the cases, indicating that this scoring method is highly reproducible. If two or three assessments were consistent with the results they scored, that value was selected. In cases where completely different results occurred, the three assessors discussed their assessments and agreed on the appropriate score to award.

Statistical analysis

Statistical analyses were performed using the SPSS software program (SPSS Standard version 13.0). Receiver operating characteristic (ROC) curve analysis was applied to determine the cut-point for marker positivity by the 0,1-criterion, and sensitivity, specificity and the areas under the ROC curves (AUC) were calculated. To combine the three markers (EZH2, HSP70 and GPC3), we found the linear coefficient to maximise AUC for the combination. The associations between EZH2 expression and other variables were analysed by using the Spearman rank test. The statistical significance of the correlation between biomarker expression and disease-specific survival was estimated by the log-rank test. Multiple Cox proportional hazards regression was carried out to identify the independent factors which had a significant impact on patient survival. A difference was considered significant if the p value from a two-tailed test was less than 0.05.

RESULTS

Protein expression levels of EZH2 in liver cell lines and tissues by western blotting analysis

Western blotting analysis revealed an obviously higher level of EZH2 expression in all six HCC cell lines than in normal liver cell line Lo2, which was used for purposes of comparison (figure 1A, left). In liver tissues, upregulated expression of EZH2 was noticed in all cases of primary HCC tissues, while the level of expression of EZH2 was negligible in the adjacent non-neoplastic liver tissues (figure 1A, right).

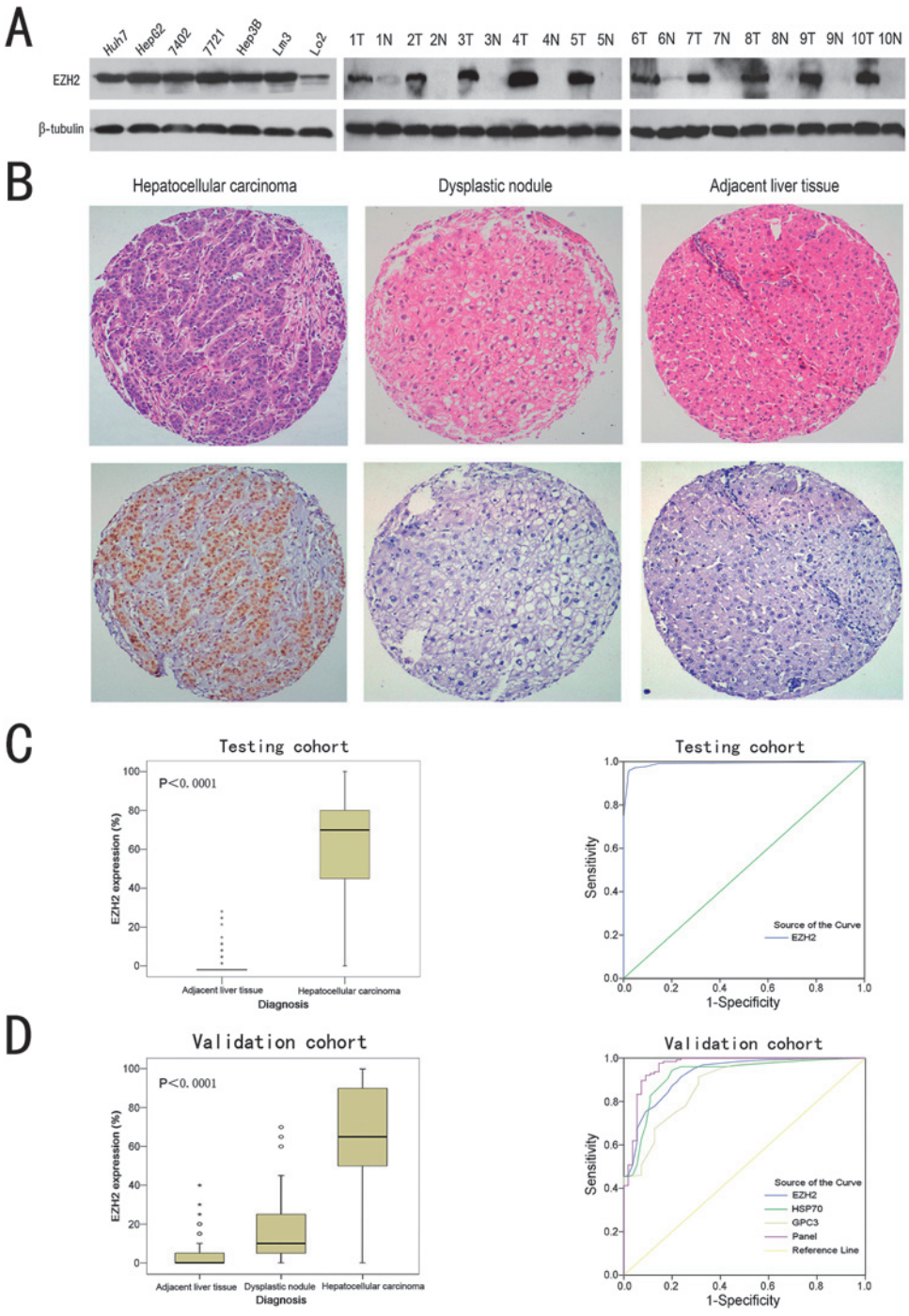

Figure 1 The expression of enhancer of zeste homolog 2 (EZH2) in human hepatocellular cells and tissues. (A) The six hepatocellular carcinoma (HCC) cell lines, Huh7, HepG2, 7402, 7721, Hep3B and Lm3, exhibited higher levels of EZH2 expression, as determined by western blotting, than normal liver cell line Lo2. Upregulated expression of EZH2 was detected in all 10 cases of primary HCC tissues compared to adjacent non-neoplastic liver tissues. T, hepatocellular carcinoma tissue; N, non-neoplastic liver tissue. (B) Positive expression of EZH2 was examined by immunohistochemistry in HCC case No. 69. The adjacent non-neoplastic liver tissues (case 69) and a dysplastic nodule (DN) (case 9) negatively expressed EZH2 (upper panels, H&E staining; lower panels, immunohistochemical staining; ×100). (C) Left: the box plot shows the mean staining intensity of EZH2 from HCC and non-neoplastic liver in the testing cohort ($p<0.0001$). Right: ROCs curves of EZH2 (area under curve (AUC)=0.990, $p<0.0001$) in HCCs compared to adjacent non-neoplastic liver tissues in the testing cohort. (D) Left: the box plots demonstrate the range of EZH2 expression within each group (HCC, $n=126$; DN, $n=37$; non-neoplastic liver tissues, $n=126$) in the validation cohort ($p<0.0001$). Right: ROC curves comparing EZH2 (AUC=0.935, $p<0.0001$), HSP70 (AUC=0.936, $p<0.0001$), GPC3 (AUC=0.890, $p<0.0001$) and the combination of the three markers (AUC=0.957, $p<0.0001$) in patients with HCC versus adjacent non-neoplastic liver tissues and DN. ROC, receiver operating characteristics.

Identification of expression pattern of EZH2 in HCC tissue by IHC

The degree of immunoreactivity of EZH2, which was observed primarily in the hepatocellular cell nuclei, ranged from 0% to 100% (figure 1B). Semi-quantification of primary HCC (n=212) and adjacent non-neoplastic liver tissue (n=212) in our testing cohort demonstrated a mean EZH2 staining intensity of 65.94% (SE, 1.652%; 95% CI 62.69% to 69.20%) and 2.41% (SE, 0.492%; 95% CI 1.44% to 3.38%), respectively (Wilcoxon exact test, p<0.0001, figure 1C, left). Overall, the sensitivity, specificity and AUC values of EZH2 expression levels for HCC detection versus non-neoplastic liver tissues were 95.8%, 97.8% and 0.990, respectively (p<0.0001, figure 1C, right). These values indicate that EZH2 is a potential diagnostic immunomarker for HCC.

Validation of EZH2 as an IHC diagnostic marker for HCC

To determine the reproducibility of these findings, the expression dynamics of EZH2 in HCC were next evaluated in our validation cohort, which included 126 HCCs and 37 DNs, by IHC. In this cohort, the mean staining intensity of EZH2 in HCCs was 63.73% (SE, 2.259%; 95% CI 59.26% to 68.20%), which was significantly higher than those in the adjacent non-neoplastic liver tissues (mean, 4.01%; SE, 0.647%; 95% CI 2.73% to 5.29%) and in DNs (mean, 17.16%; SE, 3.139%; 95% CI 10.80% to 23.53%; Wilcoxon exact test, p<0.0001, figure 1D, left). Further analysis also showed a significant difference in the expression percentages of EZH2 between the WD HCCs (mean, 60.81%) and the DNs (mean, 17.16%, Wilcoxon exact test, p<0.0001). Overall, the sensitivity, specificity and AUC for the diagnosis of HCC versus DN/non-neoplastic liver tissue using the expression levels of EZH2 above were 83.3%, 90.9% and 0.935, respectively (p<0.0001, figure 1D, right).

Since HSP70 and GPC3 have recently been suggested as promising biomarkers for distinguishing between malignant and non-malignant hepatocellular lesions,[17 20] we also compared the diagnostic performance of EZH2 for HCC with that of HSP70 and GPC3. We further investigated whether a combination of these three potential markers improved their ability to diagnose HCC. The results demonstrated that HSP70 and GPC3 immunoreactivity were primarily examined in the nucleocytoplasm and cytoplasm, respectively (figure 2A). The sensitivity, specificity and AUC values for HCC diagnosis were 82.8%, 92.7% and 0.936 when HSP70 was used, and 77.0%, 90.4% and 0.890 when GPC3 was used (p<0.0001, figure 1D, right). Strikingly, the sensitivity, specificity and AUC values increased to 89.7%, 94.5% and 0.957, when EZH2 was combined with HSP70 and GPC3 (p<0.0001, figure 1D, right). Furthermore, for the detection of WD HCCs, the sensitivity and specificity of individual markers or a combination were 68.9% and 91.5% for EZH2, 62.5% and 98.5% for HSP70, 50.0% and 92.1% for GPC3, and 75.0% and 100% for the three-marker panel (ie, a combination of EZH2, HSP70 and GPC3). A comparison for ROC curves showed that the AUC for the three-marker combination (EZH2, HSP70 and GPC3) was significantly larger than that for GPC3 alone (p=0.010), while there were no significant differences of AUC between the three-marker combination and EZH2 or HSP70 alone and between each of the three markers (p>0.05). These findings indicate (a) that EZH2 alone is more sensitive for the diagnosis of WD HCC than either HPS70 or GPC3, and (b) that the combination of the three markers could greatly improve the diagnostic accuracy of HCC, particularly for WD HCC detection (figure 2B).

Potential diagnostic utility of EZH2, HSP70 and GPC3 for HCC detection in liver needle biopsies

In clinical settings, a needle biopsy is normally recommended to identify the features of malignancy when small hepatic nodules are detected. Additional biomarkers for the discrimination of WD HCC and DN are needed.[27] To evaluate the diagnostic values of EZH2 and the three-marker combination (EZH2,

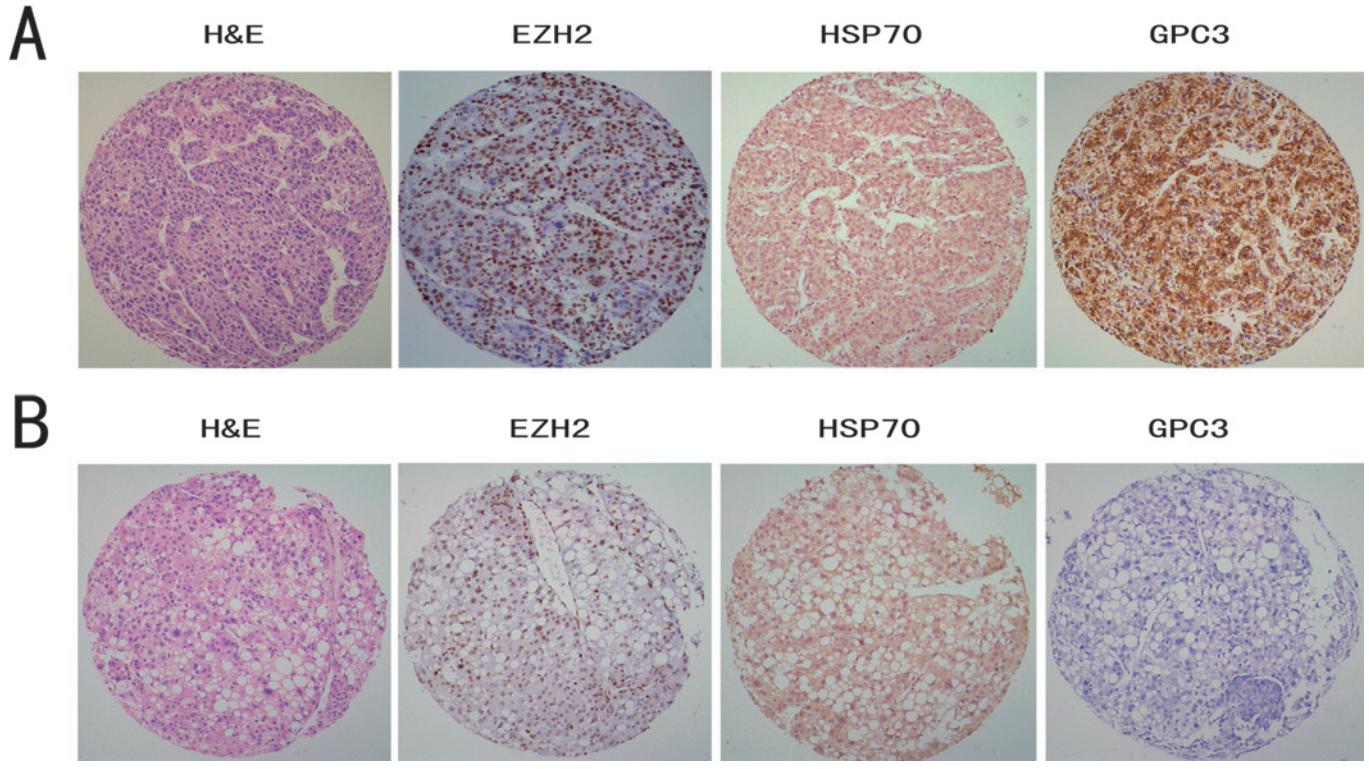

Figure 2 The expression dynamics of enhancer of zeste homolog 2 (EZH2), heat shock protein 70 (HSP70) and glypican 3 (GPC3) examined by immunohistochemistry in hepatocellular carcinoma (HCC) resection tissues. (A) A moderately differentiated HCC case positively expresses EZH2, HSP70 and GPC3. (B) A well-differentiated HCC case positively expresses EZH2 and HSP70, but negatively expresses GPC3.

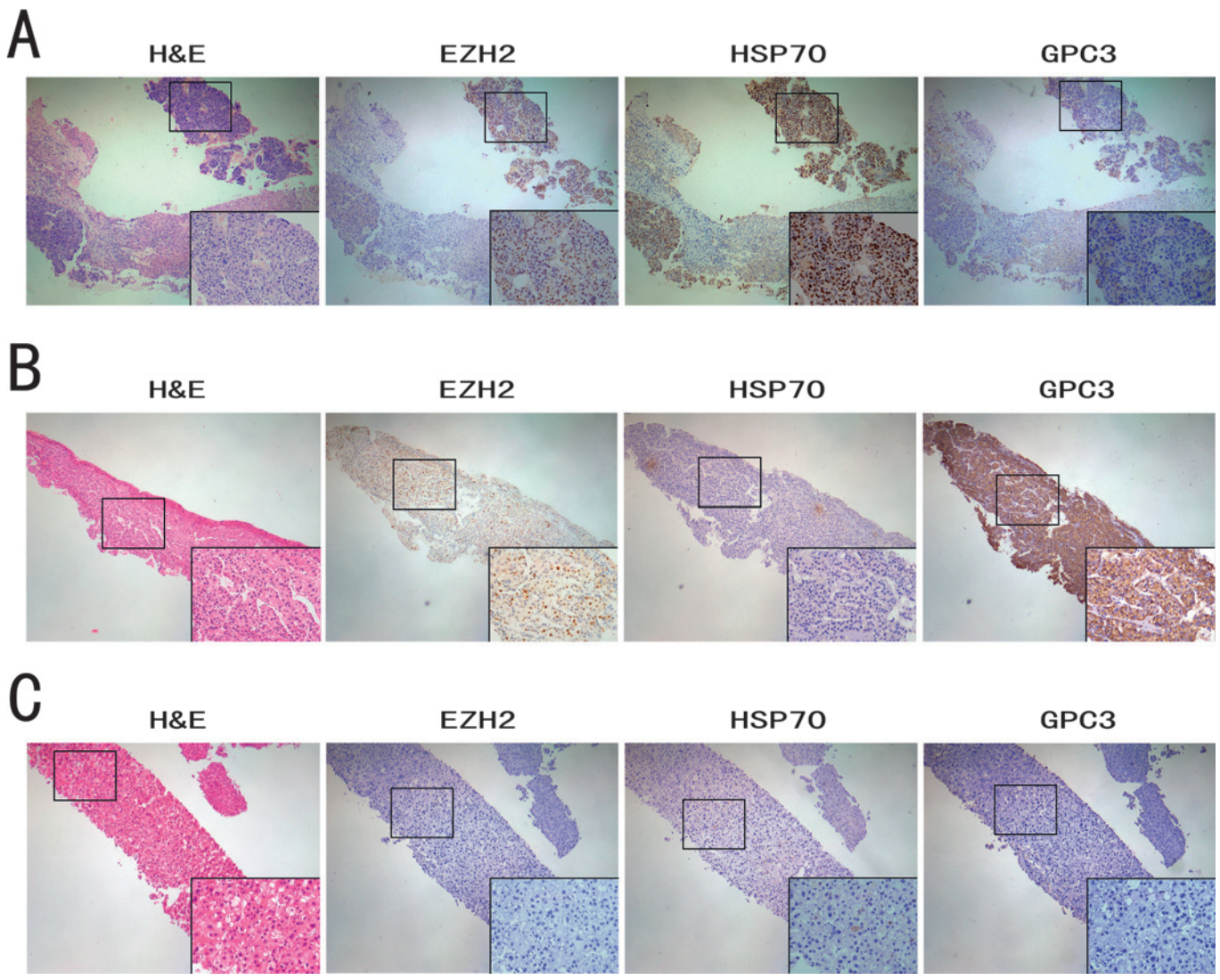

Figure 3 The expression patterns enhancer of zeste homolog 2 (EZH2), heat shock protein 70 (HSP70) and glypican 3 (GPC3) examined by immunohistochemistry in liver needle biopsy tissues. (A) A poorly differentiated hepatocellular carcinoma (HCC) biopsy tissue positively expresses EZH2, HSP70 and GPC3, while the adjacent non-neoplastic liver tissues show negative staining of the three markers. (B) A moderately differentiated HCC biopsy case shows positive immunostaining of EZH2 and GPC3, but only weak immunostaining by HSP70. (C) A dysplastic nodule biopsy case negatively expresses EZH2, HSP70 and GPC3. Representative sites in liver needle biopsy tissue at low (×100) and high (inset, ×400) magnification are shown.

HSP70 and GPC3) in liver needle biopsy samples, we further performed IHC examinations of EZH2, HSP70 and GPC3 in an additional cohort of 129 biopsy specimens with hepatic nodules. To identify a single, optimal cut-point for positivity, ROC curve analysis was applied to the cohort of surgical resection tissues to determine the cut-off score for positive expressions of the three markers concerned. The score closest to the point with both maximum sensitivity and specificity, that is, the point (0.0, 1.0) on the curve, was selected as the cut-off score, leading to the greatest number of patients correctly classified as HCC or non-

Table 2 Immunohistochemical analysis of the different lesions in liver needle biopsies

	LRN n=6	HA n=6	FNH n=20	DN n=25	WD HCC n=18	MD HCC n=37	PD HCC n=17
All three positive	0	0	0	0	4	16	6
At least two positive	0	0	0	0	11	32	15
At least one positive	0	0	2	7	16	37	17
EZH2+/HSP70+	0	0	0	0	7	22	8
EZH2+/GPC3+	0	0	0	0	6	21	10
HSP70+/GPC3+	0	0	0	0	6	21	9
EZH2+	0	0	1	3	12	31	13
HSP70+	0	0	1	2	10	28	11
GPC3+	0	0	0	2	9	26	14

DN, dysplastic nodule; FNH, focal nodular hyperplasia; HA, hepatocellular adenoma; LRN, large regenerative nodule; MD HCC, moderately differentiated hepatocellular carcinoma; PD HCC, poorly differentiated hepatocellular carcinoma; WD HCC, well-differentiated hepatocellular carcinoma.

Table 3 Degree of diagnostic accuracy in HCC and non-malignant (NM) nodules (whole series)

	HCC (n=72)	NM (n=57)	Sensitivity	Specificity	PPV	NPV	Accuracy	Youden index
All three positive	26	0	36.1%	100%	100%	55.3%	64.3%	0.36
At least two positive	58	0	80.6%	100%	100%	80.3%	89.2%	0.81
At least one positive	70	9	97.2%	84.2%	88.6%	96.0%	91.5%	0.81
EZH2+/HSP70+	37	0	51.4%	100%	100%	62.0%	72.9%	0.51
EZH2+/GPC3+	37	0	51.4%	100%	100%	62.0%	72.9%	0.51
HSP70+/GPC3+	36	0	50.0%	100%	100%	61.3%	72.1%	0.50
EZH2+	56	4	77.8%	93.0%	93.3%	76.8%	84.5%	0.71
HSP70+	49	3	68.1%	94.7%	94.2%	70.1%	79.8%	0.63
GPC3+	49	2	68.1%	96.5%	94.2%	70.5%	80.6%	0.65

NPV, negative predictive value; PPV, positive predictive value.

HCC. Thus, tumours designated positive for EZH2, HSP70 and GPC3 were those with scores above the value of 30%, 10% and 10%, respectively. By using these criteria, in our biopsy tissues, all 12 cases of LRN/HA were negatively stained by each of the three markers, whereas the number of immuno-positive cases for which there was at least one marker increased from 2/20 (10.0%) in the case of FNHs to 7/25 (28.0%) for DNs and to 16/18 (88.9%) for WD HCCs; and to 54/54 (100.0% in the case of moderately differentiated (MD) + poorly differentiated (PD) HCCs (figure 3A,B, table 2). Immuno-positive cases for which there were at least two markers (regardless of their identity) were observed in 0/6 LRNs, in 0/6 HAs, in 0/20 FNHs, in 0/25 DNs (figure 3C), in 11/18 (61.1%) WD HCCs, in 32/37 (86.5%) MD HCCs, and in 15/17 (88.2%) PD HCCs (table 2). Further statistical analysis showed that when at least one positive marker was considered, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy and Youden index for differentiating HCCs from non-malignant nodules were 97.2%, 84.2%, 88.6%, 96.0%, 91.5% and 0.81, respectively. When at least two positive markers were used, the sensitivity, specificity, PPV, NPV, accuracy and Youden index were 80.6%, 100%, 100%, 80.3%, 89.2% and 0.81%, respectively (table 3).

Association between EZH2 expression and clinico-pathological features and survival of patients with HCC

Using the ROC curve of EZH2 described before, the positive expression of EZH2 was examined in 186/212 (87.7%) of HCCs in the testing cohort and in 110/126 (87.3%) of HCCs in the validation cohort, respectively. Further correlation analysis in both cohorts showed that the positive expression of EZH2 in HCCs was significantly associated with a more aggressive phenotype of the tumour ($p<0.05$, table 1). Kaplan–Meier analysis established that, in the testing cohort, the median disease-specific survival time for patients with HCC who positively expressed EZH2 was 34.8 months, compared to 66.5 months for patients with HCC who negatively expressed EZH2 ($p=0.001$, log-rank test, figure 4A, table 4). In the validation cohort, patients with HCC who positively expressed EZH2 also exhibited a shorter survival time (median survival time, 33.5 months) than patients with HCC who negatively expressed EZH2 (median, 49.2 months; $p=0.009$, log-rank test, figure 4B, table 4). Further multivariate Cox regression analysis indicated that EZH2 expression is an independent prognostic factor for poor survival of HCC patients in both cohorts (table 5).

DISSCUSSION

The polycomb group protein EZH2 has recently been suggested to play a crucial role in the tumourigenesis of several types of human cancer, including HCC.[10–12] We previously found that the knocking down of EZH2 by shRNA can significantly inhibit the growth of HCC cells in vitro and their tumourigenicity in vivo.[13] Several reports noted that EZH2 was over-expressed in most of the HCC resection tissues by IHC, whereas it was negatively expressed in nearly all the corresponding non-tumour tissues and DNs.[14 16] These observations indicate that it may be possible to use the expression levels of EZH2 as a diagnostic tool to distinguish HCC tissues from non-malignant liver tissues. To date, however, studies of the use of EZH2 in HCC cases have defined EZH2 protein expression using a predetermined and arbitrary cut-off score. Furthermore, no investigation and validation studies are available.

In the present study, our initial western blotting established that EZH2 was expressed at a higher level in a panel of HCC cell lines and tissues than in a hepatic cell line and adjacent liver tissues. We next used IHC staining to examine the expression dynamics of EZH2 in two large independent (testing and validation) cohorts of HCCs and non-malignant liver tissues. According to our results, the mean staining intensity of EZH2 in HCCs in both cohorts was significantly greater than in non-

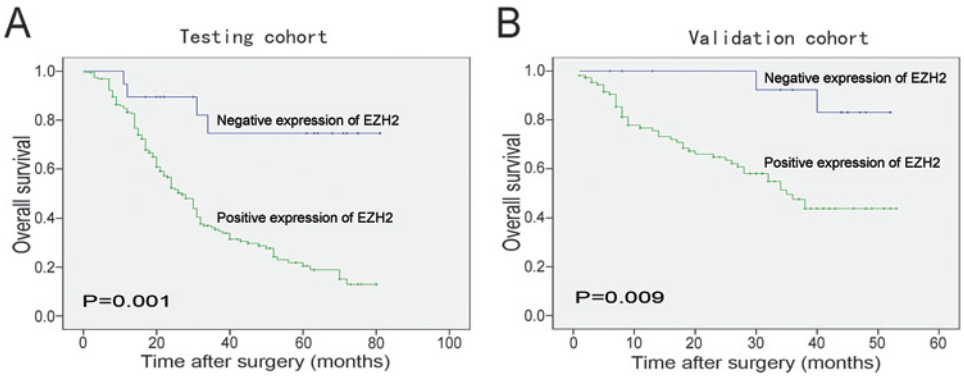

Figure 4 Kaplan–Meier survival analysis according to enhancer of zeste homolog 2 (EZH2) protein expression in 338 patients with hepatocellular carcinoma (log-rank test). (A) Probability of survival of patients in testing cohort: negative expression of EZH2, $n=26$; positive expression of EZH2, $n=186$ ($p=0.001$). (B) Probability of survival of patients in validation cohort: negative expression of EZH2, $n=16$; positive expression of EZH2, $n=110$ ($p=0.009$).

Table 4 Univariate analysis of enhancer of zeste homolog 2 (EZH2) expression and various clinicopathological parameters in 338 patients with primary hepatocellular carcinoma

	Testing cohort			Validation cohort		
Variable	**All cases**	**HR (95% CI)**	**p value***	**All cases**	**HR (95% CI)**	**p Value***
Age (years)			0.627			0.721
≤47.9†	105	1.0		61	1.0	
>47.9	107	1.090 (0.770 to 1.542)		65	1.109 (0.628 to 1.959)	
Sex			0.045			0.558
Male	174	1.650 (1.012 to 2.688)		109	1.293 (0.548 to 3.052)	
Female	38	1.0		17	1.0	
Hepatitis history			0.810			0.991
Yes	164	1.053 (0.692 to 1.602)		107	1.004 (0.450 to 2.240)	
No	48	1.0		19	1.0	
AFP (ng/ml)			0.000			0.000
≤20	67	1.0		56	1.0	
>20	145	2.262 (1.490 to 3.434)		70	5.305 (2.826 to 9.958)	
Liver cirrhosis			0.891			0.983
Yes	132	0.975 (0.682 to 1.395)		88	1.0	
No	80	1.0		38	0.993 (0.539 to 1.831)	
Tumour size (cm)			0.000			0.000
≤5	59	1.0		76	1.0	
>5	153	6.295 (3.688 to 10.747)		50	2.839 (1.586 to 5.082)	
Tumour multiplicity			0.000			0.000
Single	128	1.0		87	1.0	
Multiple	84	3.480 (2.429 to 4.987)		39	3.672 (2.065 to 6.529)	
Differentiation			0.021			0.102
Well-moderate	153	1.0		87	1.0	
Poor-undifferentiated	59	1.534 (1.068 to 2.203)		39	1.630 (0.908 to 2.927)	
Stage			0.000			0.000
I–II	88	1.0		62	1.0	
III–IV	124	5.393 (3.525 to 8.252)		64	5.400 (2.526 to 11.542)	
Vascular invasion			0.000			0.000
Yes	108	4.923 (3.310 to 7.322)		57	5.362 (2.724 to 10.553)	
No	104	1.0		69	1.0	
Relapse			0.000			0.000
Yes	105	1.907 (1.330 to 2.734)		43	2.860 (1.600 to 5.112)	
No	107	1.0		83	1.0	
EZH2			0.001			0.009
Negative expression	26	1.0		16	1.0	
Positive expression	186	3.506 (1.703 to 7.219)		110	5.451 (1.318 to 22.543)	

*χ^2 test.
†Mean age.

malignant liver tissues. The expression levels of EZH2 were able to distinguish HCCs from non-malignant liver tissues with very high sensitivity, specificity and AUC. Furthermore, the staining intensity of EZH2 in WD HCCs was significantly greater than that in DNs. These observations strongly suggest that the evaluation of EZH2 expression by IHC can enable us not only to discriminate HCCs and non-neoplastic liver tissues, but also to distinguish WD HCCs from DNs with a high degree of accuracy.

In clinical practice, an increasing number of small hepatocellular nodules (<3 cm) are detected by imaging during the follow-up of patients with liver cirrhosis, but the sensitivity of this imaging for the detection of small HCCs is only around 33%.[28 29] Recently, liver needle biopsies have been recommended as a check on diagnoses of small hepatic nodules which were not satisfactorily addressed by imaging. However, since histological diagnosis by needle liver biopsy is based solely on the analysis of tiny fragments of the tissue, it is very difficult to distinguish between early WD HCCs and certain benign hepatocellular diseases such as DNs. This often results in diagnostic delays.[30–33] A number of biomarkers have been suggested that can potentially distinguish HCCs from DNs in cirrhosis cases. The diagnostic yield of a panel of putative HCC markers, such as HSP70, GPC3 and glutamine synthetase has been examined, and they have been proposed as useful immunomarkers for HCC.[21 22] In this study, the diagnostic performance of HSP70 or GPC3 and their combination with EZH2 was first analysed in our surgical resection liver specimens. As expected, both HSP70 and GPC3 alone showed a high sensitivity, specificity and AUC for HCC detection. Interestingly, the sensitivity, specificity and AUC for HCC diagnosis increased when EZH2 was used in combination with HSP70 and GPC3. In addition, for the diagnosis of WD HCCs, the sensitivity and specificity of individual markers or a combination were 68.9% and 91.5% for EZH2, 62.5% and 98.5% for HSP70, 50.0% and 92.1% for GPC3, and 75.0% and 100% for the 3-marker panel.

We wondered whether the expression status of EZH2 would be retained in liver needle biopsies, thereby also serving as a useful diagnostic immunomarker to distinguish between HCCs and benign hepatocellular tissues. We therefore re-evaluated the diagnostic values of EZH2 and the three-marker combination (EZH2, HSP70 and GPC3) in an additional cohort of liver needle biopsy samples. By using the cut-off score established in ROC curve analysis before, we found that in liver biopsies, LRNs and HAs were all stained negatively by each of the three markers, whereas

Table 5 Cox multivariate analyses of prognostic factors on overall survival

Characteristic	HR (95% CI)	p Value
The First Affiliated Hospital cohort		
Sex (female vs male)	1.303 (0.793 to 2.140)	0.297
α-Fetoprotein, ng/ml (≤20 vs >20)	1.398 (0.912 to 2.141)	0.124
Tumour size, cm (≤5 vs >5)	0.768 (0.437 to 1.348)	0.358
Tumour multiplicity (single vs multiple)	1.711 (1.123 to 2.605)	0.012
Differentiation (well—moderate vs poor—undifferentiated)	0.964 (0.518 to 1.792)	0.907
Stage (I—II vs III—V)	1.818 (1.313 to 2.516)	0.000
Vascular invasion (no vs yes)	2.609 (1.716 to 3.966)	0.000
Relapse (no vs yes)	1.159 (0.806 to 1.667)	0.426
EZH2 (positive vs negative)	2.305 (1.178 to 4.530)	0.032
The Cancer Center cohort		
α-Fetoprotein, ng/ml (≤20 vs >20)	2.083 (1.076 to 4.033)	0.029
Tumour size, cm (≤5 vs >5)	1.627 (0.836 to 3.165)	0.152
Tumour multiplicity (single vs multiple)	1.628 (0.843 to 3.144)	0.147
Stage (I—II vs III—IV)	1.616 (0.862 to 3.031)	0.134
Vascular invasion (no vs yes)	5.228 (1.486 to 18.390)	0.000
Relapse (no vs yes)	1.163 (0.606 to 2.232)	0.650
EZH2 (positive vs negative)	4.522 (1.020 to 20.054)	0.047

immuno-positive cases for at least one marker increased from FNH (2/20) and DN (7/25) to WD HCC (16/18), and to MD+PD HCC (54/54). Using the three-marker panel, immuno-positive cases (ie, cases where any two markers showed positive) were observed in none of the benign liver lesions, but were frequently examined in 11/18 WD HCCs, in 32/37 MD HCCs, and in 15/17 PD HCCs. Further statistical analysis determined that sensitivity was 97.2% when at least one marker was used, but only 80.6% when at least two markers were used. Specificity increased from 84.2% when at least one marker was used, to 100% when at least two markers were used; while the PPV increased from 88.6% to 100%. The accuracy and Youden index remained nearly or exactly identical. These findings were intriguing, since PPV is highly useful in both marker situations as well as for NPV. Collectively, our findings not only confirm the value of EZH2 for the detection of HCC, but also demonstrate that the use of a three-marker panel (EZH2, HSP70 and GPC3) can improve the rate of detection of HCCs in liver biopsy tissues.

To sum up, in this study we describe the protein expression patterns of EZH2 in several cohorts of human HCCs and non-malignant liver tissues, and demonstrate the potential utility of EZH2 as an immunomarker of HCCs during the diagnosis of liver lesions. We show, for the first time, that a three-marker panel (ie, EZH2, HSP70 and GPC3) is better able to identify HCCs in liver biopsy tissues than EZH2 alone. Our results also provide evidence that the positive expression of EZH2 in HCCs may be important for the detection of an aggressive phenotype or a phenotype with poor prognosis. We believe that the use of EZH2 protein, as examined by IHC, as a diagnostic biomarker of HCCs could improve the prospects of the early detection of HCCs in liver needle biopsies; and that an improved rate of detection would have important prognostic implications for patients with HCC. It is necessary to point out that our current study is of the retrospective nature. The main limitations of this study are that (a) patients with lesions confidently classified as HCC by biopsy and explant analysis were included; and (b) a high proportion of large lesions (>5 cm) was included in our surgically resected HCC cohorts to establish the diagnostic criteria of biomarkers, in which the diagnosis by haematoxylin&eosin is usually not difficult. Clearly, further prospective studies designed to include HCC lesions smaller than 2 cm are needed to validate the conclusions of this study.

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Competing interests None.

Ethics approval This study was conducted with the approval of The Institute Research Medical Ethics Committee of Sun Yat-Sen University Cancer Center.

Contributors MYC, ZTT and FZ contributed equally to this study. Each author who contributed to this work has been listed as an author on the paper.

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Editor's quiz: GI snapshot

ANSWER

From the question on page 922

The diagnosis was acute pancreatitis complicated with pseudo-cyst and pseudo-aneurysm of splenic artery.

The colour Doppler and three-dimensional sonogram revealed the nodule to be hyper-vascular and communicating with the splenic artery (figures 1 and 2). A CT scan (figure 3) and its volume rendering reformatted angiography (figure 4) showed a pseudo-cyst and a pseudo-aneurysm from the splenic artery. Therefore, acute pancreatitis complicated with a pseudo-cyst and pseudo-aneurysm was diagnosed. The pseudo-aneurysm was removed by surgery uneventfully and minimal rupture was disclosed during the operation.

Rupture of a pseudo-aneurysm is a rare complication of pancreatitis, and is associated with poor prognosis. The mortality correlates with the severity of the pseudo-aneurysm and the overall mortality of acute pancreatitis, around 7.8%. The pseudo-aneurysm caused by acute pancreatitis usually develops

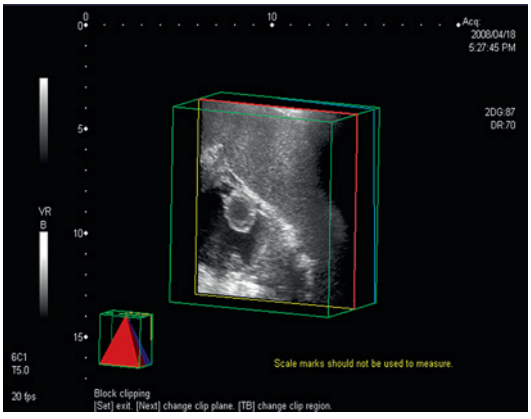

Figure 1 Three-dimensional sonogram.

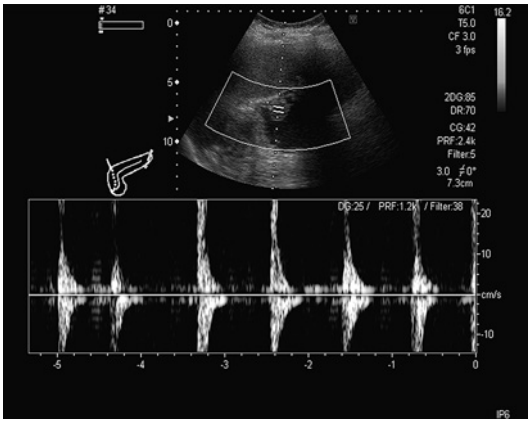

Figure 2 Color Doppler sonogram.

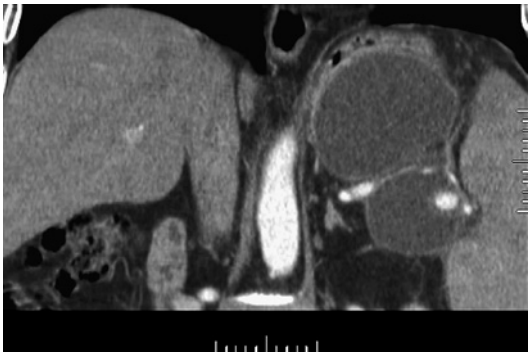

Figure 3 Pseudo-aneurysm from the splenic artery by CT scan.

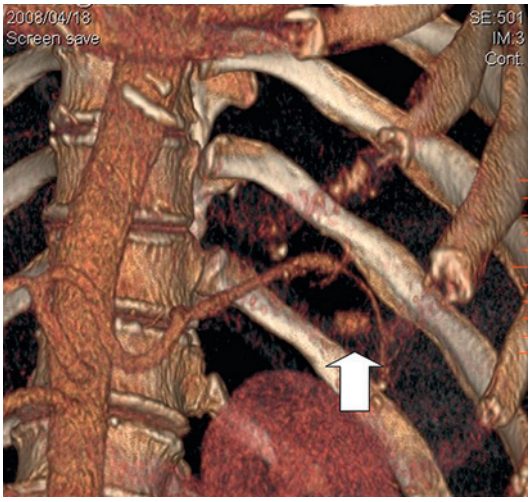

Figure 4 Reformatted angiography. The arrow indicates the pseudo-aneurysm.

at superior mesentery artery, splenic artery or gastro-duodenal artery with manifestation of gastro-intestine bleeding, haemobilia, obstructive jaundice, or internal bleeding. Angiography is usually required for confirmation of the diagnosis. Non-invasive imaging techniques such as colour Doppler ultrasound and contrast-enhanced CT scan are also useful in diagnosing pancreatic pseudo-aneurysm associated with acute pancreatitis.[1—3]

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EZH2 protein: a promising immunomarker for the detection of hepatocellular carcinomas in liver needle biopsies

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