Diagnostic Discrimination of Fine Needle Aspiration Specimens of Hepatic Nodules using Immunohistochemical Expression of GPC3 and EZH2

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Abstract

The goal of the present study was to examine the immunohistochemical expression of Glypican-3 (GPC3) and Enhancer of zeste homologue 2 (EZH2) in various histological types of hepatic nodules in order to clarify their discriminatory diagnostic value. We correlated biomarkers' expressions with the clinicopathological variables of primary liver malignancy. Biomarkers' expression was investigated in 64 liver needle biopsies. The specimens included primary liver malignancy (57.81%), metastatic carcinomas (15.62%) and non-malignant nodules 26.56%. The expression of GPC3 was detected in 83.33% and 15.38% of hepatocellular carcinoma (HCC) and cholangiocarcinoma (CC) respectively, but not expressed in any of metastatic nodules. In HCC, GPC3 was more expressed in cases with cirrhosis, large masses of tumor and high HCCs grades with statistically significant differences with P value of 0.01, 0.035 and 0.03 respectively. The EZH2 expression was detected in 91.66% of HCC, in all cases of CC and metastatic nodules and in 5.88% of non-malignant nodules. The sensitivity, specificity and diagnostic accuracy of differentiating HCCs from non-malignant nodules were 80.95%, 100% and 90.24% respectively for GPC3; and 85.71%, 95.65% and 91.89% respectively for EZH2. The sensitivity, specificity and diagnostic accuracy for differentiating HCCs from CCs were 73.33%, 90.91% and 83.78% respectively for GPC3; and 0.0%, 62.86% and 59.46% respectively for EZH2. The sensitivity, specificity and diagnostic accuracy for differentiating HCCs from metastatic nodules were 71.43%, 100% and 88.24% respectively for GPC3; and 0.0%, 68.75% and 64.71% respectively for EZH2. In conclusion, GPC3 might be used as a good biomarker for differential diagnosis of HCC from non-malignant nodules, CC and metastasis. Its overexpression might be an indication of poor HCC prognosis. On the other hand, EZH2 is not specific for HCC, but could be a reliable biomarker for discrimination of hepatic cancers compared to non-malignant nodules.

Keywords: Liver; Immunohistochemistry; EZH2; GPC3; Needle biopsies

Introduction

Liver cancer is one of the common malignancies that are rapidly increasing throughout the world [1]. Most malignant liver lesions are metastatic and few are primary tumors [2]. On the basis of morphological and cytogenetic characteristics, primary liver cancers are classified into three types; hepatocellular carcinoma (HCC) originating from hepatocytes, intrahepatic cholangiocarcinoma (ICC) arising from the epithelium of the intrahepatic bile ducts and combined hepatocellular-cholangiocarcinoma (CHC). HCC is the most common type of primary malignant liver tumor [3]. However, CHC is a rare type of liver cancer with features of both hepatocellular and biliary differentiation [1].

Patients with chronic diseases of the liver and virus infection-based cirrhosis are at a great risk of developing HCC. Dysplastic nodules (DN) that are usually detected in cirrhotic livers are considered pre-cancerous lesions of HCC of high-grade DN (HGDN) [4]. Distinguishing between low-grade dysplastic nodules (LGDN) and HGDN and between HGDN and well-differentiated HCC is sometimes difficult [5]. Cholangiocarcinomas (CC) also can be challenging as they are usually adenocarcinomas. Therefore, they can be difficult to differentiate them from metastatic tumors or sometimes from less differentiated HCC [6].

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% [7]. Liver needle biopsies have been recommended as a check on diagnoses of such small nodules which are not satisfactorily addressed by imaging. However, since histological diagnosis by needle liver biopsy is based solely on the analysis of tiny fragments of tissue, it is very difficult to distinguish between early well differentiated HCCs and certain benign hepatocellular diseases such as dysplastic nodules. This often results in diagnostic delays [8]. Thus, development of reliable tumor biomarkers that can help to differentiate HCC from other hepatic lesions and from metastatic neoplasms of the liver is urgently needed [9,10].

Glypican-3 (GPC3) belongs to the family of heparin sulfate proteoglycans [11]. Specifically, GPC3 is expressed in the fetal hepatoblasts; and is absent (silenced) in most adult tissues including liver [12]. Its expression tends to reappear with malignant transformation [13]. Overexpression of this protein has been observed in HCC cells [14]. Immunohistochemical studies detected high expression of GPC3 in HCC, but not in adjacent normal liver or in benign liver lesions [15,16]. It has been suggested that GPC3 can substitute alpha-feto
protein (AFP) in early diagnosis of HCC and in screening and follow-up of cirrhotic Egyptian patients [17].

Enhancer of zeste homologue 2 (EZH2) has been suggested to play a crucial role in the tumourigenesis of human cancers, including HCC. It is the catalytic portion of polycomb repressive complex 2 (PRC2), being identified as the sole histone methyl transferase that methylates histone H3 lysine 27 (H3K27) and mediates transcriptional silencing [18]. EZH2 has been found to contribute to the maintenance of cell identity, cell cycle regulation and oncogenesis [19]. It has been found that EZH2 is linked to the aggressiveness of different human cancers, including lymphomas [20], breast cancer [21], prostate cancer [22], HCC [23] and astrocytic gliomas [24].

The goal of the present study was to examine the immunohistochemical expression of GPC3 and EZH2 in hepatic nodules in a trial to clarify their discriminatory diagnostic value between primary malignant, metastatic and non malignant liver nodules. We also aimed to correlate the expression of these biomarkers with the clinicopathological variables of primary liver malignancy (HCC and CC).

Materials and Methods

Patients and clinical data

A prospective and retrospective study was performed at the Departments of Pathology and Tropical Medicine, Zagazig University Hospital, Egypt. We collected 20 cases of 18-gauge needle biopsy specimens with hepatic nodules from August 2014 to September 2015 in tropical medicine department. Moreover, 44 formalin-fixed paraffin-embedded liver specimens from patients who underwent a needle biopsy between March 2011 and August 2014 were randomly selected from the archive of pathology department. We re-examined the corresponding hematoxylin-eosin (Hx and E) slides to confirm the diagnosis of the cases achieved from pathology department; and to determine the grade of primary liver cancers. At the same time, the specimens collected from cases of tropical department are processed for histological examinations [25]. The clinicopathological characteristics of all cases were obtained. Grading of HCCs was done following the criteria of the World Health Organization Classification of Tumors [26]. The CCs were also classified into 3 grades [27]. The study was carried out with full local ethics approval.

Immunohistochemical procedure

The sections (4–5 μm) obtained from tissue sample blocks were deparaffinised with xylene, rehydrated in graded alcohols and placed in 0.5% hydrogen peroxide in methanol for 10 min to block endogeneous peroxidase activity. Antigen retrieval was carried out by incubation in 0.01 M citrate buffer (pH 6.0) for 5 min in a pressure cooker. The sections were exposed to the primary antibody for 60 min at room temperature. The standard streptavidin-biotin-peroxidase complex method was used for GPC3 (mouse monoclonal antibody, 1:100, clone 1G12, Biocare Medical, USA) and EZH2 (mouse monoclonal antibody, 1:100, clone 11/EZH2, BD Biosciences, San Jose CA, USA) by employing diaminobenzidine (DAB) as the chromogen. The whole procedures were performed at room temperature. Additionally, a negative control for both biomarkers in which the primary antibody was removed and replaced by phosphate buffered saline was used and positive controls (paraffin sections of HCC) were run in parallel. Cells were considered GPC3-positive when a distinct plasma or membrane staining was identified and considered EZH2-positive when a distinct nuclear staining was identified.

Evaluation of immunohistochemical staining

The expressions for the two biomarkers were scored using a semi-quantitative method by two independent pathologists (SB, TI), who were blinded to the clinicopathological data. For analysis of GPC3 expression, the results of immunohistochemical staining were classified according to the density of GPC3-positive staining cells as follows; negative (<10%), weakly positive (10–30%) and positive (>30%). Finally, for statistical analysis, the expression of GPC3 was grouped into GPC3-negative (<10%) and GPC3-positive (>10%) [28].

Regarding EZH2 expressions, scores were assigned based on the density of nuclear positivity. Specimens were scored as positive for expression of EZH2 when >21% cells were positive and negative for expression of EZH2 when 0–20% of the cells were positive [29].

Statistics

Analysis of categorical data was performed using the chi-square (x²) or Fisher’s exact test. The statistical analyses were performed using SPSS software (version 19.0; SPSS, Chicago, IL) and P ≤ 0.05 was considered to indicate a statistically significant difference. Data were represented as number and percentage. The validity of the biomarkers was assessed by sensitivity, specificity, PPV (positive predictive value), NPV (negative predictive value) and diagnostic accuracy. With a histologic diagnosis designated as the gold standard.

Results

Patients’ characteristics and histopathological classification

Among the studied 64 liver needle biopsies that were obtained from patients with hepatic nodules, 57.81% (37 cases) were primary liver malignancy (24 HCC and 13 CC) while 15.62% (10 cases) were metastatic (4 colorectal, 2 gastric, one pulmonary and 3 mammary carcinomas). 26.56% (17 cases) were non-malignant (5 cirrhosis, 5 LGDN and 7 HGDN) (Figures 1 and 2). The clinicopathologic characteristics of patients with primary liver malignancy (HCC and CC) like age, sex, history of hepatitis, presence of cirrhosis and serum alpha fetoprotein and their association with GPC3 and EZH2 expression are summarized in Table 1.

Immunohistochemical expression of GPC3

Among the studied cases of primary malignant nodules, GPC3 expression was detected in 83.33% (20 out 24) and 15.38% (2 out 13) of HCC and CC respectively, but not expressed in any of the metastatic or non-malignant (benign) nodules (0%) (Table 2). The neoplastic cells showed cytoplasmic and membranous GPC3 immunoreactivity (Figure 3). In HCC, GPC3 was more expressed in patients with cirrhosis and with large masses of tumor with a statistically significant difference (p=0.01 and p=0.035 respectively). The GPC3 expression also showed a statistically significant difference (p=0.03) with high HCCs grades. The GPC3 tended to be more expressed in patients with high AFP, with borderline significance relationship (p=0.059). In CC patients, all cases were non-cirrhotic; and GPC3 expression was found to be significantly more expressed in higher grades CC, but the relationship did not reach a significance level (p=0.076) (Table 1).

Immunohistochemical expression of EZH2

Among the studied cases of primary malignant nodules, EZH2 expression was detected in 91.66% (22 out 24) of HCC, in all cases of the CC and in all metastatic nodules. EZH2 expressed only in 5.88% (1 out 17) of non-malignant nodules (Table 2). The neoplastic cells showed nuclear EZH2 immunoreactivity (Figures 3-5). In HCC, EZH2 was
more expressed in patients with large masses of tumor with borderline significance relationship (p=0.054). In CC, P values were not computed because all cases of CC were stained positively by EZH2 (Table 1).

EZH2 and GPC3 expressions for detection and distinguishing HCCs

In this study, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy of differentiating HCCs from non-malignant nodules were 80.95%, 100%, 100%, 83.33% and 90.24% respectively for GPC3 and 85.71%, 95.65%, 92.31%, 91.67% and 91.89% respectively for EZH2 (Table 3).

The sensitivity, specificity, PPV, NPV and diagnostic accuracy for differentiating HCCs from CCs were 73.33%, 90.91%, 84.62%, 83.33%...
HCC cases. This is similar to our result; but in contrast to ours, it was undetectable in CC. Such inconsistent results may be due to different methodology and small numbers of the studied CC (Current study: 13; Man et al. [30]: 21; Yu et al. [31]: 7 patients).

In our study, serum AFP was elevated in 70.83% (17/24) and 15.38% (2/13) of the studied HCC and CC respectively. This in contrast to the study of Yu et al. [31], where the serum AFP was elevated in 55% (22/40) and 28.5% (2/7) of HCC and ICC respectively. Our analysis revealed that GPC3 immunoreactivity in HCC tended to increase with increased serum AFP with borderline significance relationship (p=0.059); a similar but significant relationship was found by Yorita et al. [32]. This is in contrast to the study of Ning et al. [33], who founded that this relationship was non-significance.

The sensitivity, specificity, PPV, NPV and diagnostic accuracy for differentiating HCCs from Metastatic nodules were 71.43%, 100%, 100%, 83.33% and 88.24% respectively for GPC3 and 0.0%, 68.75%, 0.0%, 91.67% and 64.71% respectively for EZH2 (Table 5).

### Table 1: Association of clinicopathological parameters of the studied cases of primary liver malignancy (HCC, CC) with GPC3 and EZH2 expressions.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HCC (n=24)</th>
<th>GPC3</th>
<th>EZH2</th>
<th>Cholangiocarcinoma (n=13)</th>
<th>GPC3</th>
<th>EZH2</th>
<th>Non-malignant nodules (n=17)</th>
<th>Cirrhosis (n=5)</th>
<th>LGDN (n=5)</th>
<th>HGDN (n=7)</th>
<th>Non-malignant nodules (n=17)</th>
<th>Cirrhosis (n=5)</th>
<th>LGDN (n=5)</th>
<th>HGDN (n=7)</th>
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<td>3</td>
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<tr>
<td>Total</td>
<td>20</td>
<td>4</td>
<td>22</td>
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<td>2</td>
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<td>13</td>
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*P value was not computed because all cases of CC were not cirrhotic
** P values were not computed because all cases of CC were stained positively by EZH2

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

and 83.78% respectively for GPC3 and 0.0%, 62.86%, 0.0%, 91.67% and 59.46% respectively for EZH2 (Table 4).

The sensitivity, specificity, PPV, NPV and diagnostic accuracy for differentiating HCCs from Metastatic nodules were 71.43%, 100%, 100%, 83.33% and 88.24% respectively for GPC3 and 0.0%, 68.75%, 0.0%, 91.67% and 64.71% respectively for EZH2 (Table 4).

### Discussion

In the present study, the GPC3 was expressed in 83.33% and 15.38% of the studied HCC and CC respectively. This finding is nearly similar to the study of Man et al. [30] where in their study GPC3 was expressed in 90% and 19% of the studied HCC and CC respectively. On the other hand, Yu et al. [31] reported GPC3 expression in 85% of HCC cases. This is similar to our result; but in contrast to ours, it was undetectable in CC. Such inconsistent results may be due to different methodology and small numbers of the studied CC (Current study: 13; Man et al. [30]: 21; Yu et al. [31]: 7 patients).

In our study, serum AFP was elevated in 70.83% (17/24) and 15.38% (2/13) of the studied HCC and CC respectively. This in contrast to the study of Yu et al. [31], where the serum AFP was elevated in 55% (22/40) and 28.5% (2/7) of HCC and ICC respectively. Our analysis revealed that GPC3 immunoreactivity in HCC tended to increase with increased serum AFP with borderline significance relationship (p=0.059); a similar but significant relationship was found by Yorita et al. [32]. This is in contrast to the study of Ning et al. [33], who founded that this relationship was non-significance.
Figure 3: Immunohistochemical expression of GPC3 and EZH2 in HCC: (A) Diffuse GPC3 cytoplasmic immunostaining in GI HCC; (B) Diffuse GPC3 cytoplasmic immunostaining in GII HCC, the surrounding liver is negative; (C) EZH2 nuclear immunostaining in GIII HCC; (D) EZH2 nuclear immunostaining in GIII-IV HCC. (All the figures are AB, DAB chromogen X200).

Figure 4: Liver needle biopsy of cholangiocarcinoma GII: (A) Malignant epithelial cells with hyperchromatic nuclei and increase in N/C ratio surrounded by dense fibrosis (Hx and E X200); (B) Positive EZH2 nuclear immunostaining (ABC, DAB chromogen X200); (C) Lack of GPC3 immunostaining (ABC, DAB chromogen X200).
In our study, 62.5% of HCCs were accompanied by cirrhosis while all CCs were non-cirrhotic. This is similar to the studies of Ning et al. [33] and Cai et al. [34] where 62.2% and 60.9% of HCC were respectively cirrhotic. On the other hand, Yu et al. [31] detected cirrhosis only in 50% of HCC cases; but similar to our finding, none of CC was cirrhotic. Our analysis showed that HCC patients with hepatic cirrhosis found to have a higher GPC3 over-expression rate than non-hepatic cirrhosis with significant relationship (p=0.011). Our analysis also showed that GPC3 expression was significantly associated with histological differentiation. More GPC3 expression was observed in the higher grades of tumors (p=0.031). Both previous observations were consistent with other reports [33,35]; but Yu et al. [31] found no correlation of GPC3 expression with the size of the tumor, pathological grade, hepatitis and cirrhosis. Our analysis also showed significant association between GPC3 expression and larger tumor size (p=0.035). This is consistent with the study of Guanghui et al. [36], but in contrast to other related studies [31,33]. Our analysis found a significant correlation between GPC3 expression in HCC and parameters of tumor size and tumor grade, both of which are conventional poor prognostic factors.

In the present study, EZH2 was expressed in 91.66% of HCC and in all CC. This finding is nearly similar to the study of Kalcakosz et al. [37] where in their study; EZH2 was expressed in 90.9% of HCC and in 96% of CC. Such slight difference in results of CC staining may be

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**Table 3:** Degree of diagnostic accuracy in HCC and Non-malignant nodules.

<table>
<thead>
<tr>
<th></th>
<th>HCC (n=24)</th>
<th>Non-malignant (n=17)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Diagnostic Accuracy</th>
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<td>0</td>
<td>80.95%</td>
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<td>100%</td>
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<td>EZH2+</td>
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<td>95.65%</td>
<td>92.31%</td>
<td>91.67%</td>
<td>91.89%</td>
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NPV: Negative Predictive Value; PPV: Positive Predictive Value

**Table 4:** Degree of diagnostic accuracy in HCC, CC.

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<tr>
<th></th>
<th>HCC (n=24)</th>
<th>CC (n=13)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Diagnostic Accuracy</th>
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<tbody>
<tr>
<td>GPC3+</td>
<td>20</td>
<td>2</td>
<td>73.33%</td>
<td>90.91%</td>
<td>84.62%</td>
<td>83.33%</td>
<td>83.78%</td>
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<tr>
<td>EZH2+</td>
<td>22</td>
<td>13</td>
<td>0.0%</td>
<td>62.86%</td>
<td>0.0%</td>
<td>91.67%</td>
<td>59.46%</td>
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</table>

NPV: Negative Predictive Value; PPV: Positive Predictive Value

**Table 5:** Degree of diagnostic accuracy in HCC and Metastatic nodules.

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<tr>
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<th>HCC (n=24)</th>
<th>Metastatic (n=10)</th>
<th>Sensitivity</th>
<th>Specificity</th>
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<tr>
<td>EZH2+</td>
<td>22</td>
<td>10</td>
<td>0</td>
<td>68.75%</td>
<td>0.0%</td>
<td>91.67%</td>
<td>64.71%</td>
</tr>
</tbody>
</table>

NPV: Negative Predictive Value; PPV: Positive Predictive Value

Figure 5: Liver needle biopsy of metastatic adenocarcinoma: (A) Scattered group malignant epithelial cells with large hyperchromatic nuclei and prominent nucleoli (Hx and E X400); (B) Lack of GPC3 immunostaining (ABC, DAB chromogen X200); (C) Positive EZH2 nuclear immunostaining (ABC, DAB chromogen X200).
due to different methodology and different numbers of the studied CC (Current study: 13; Kalcakosz et al. [37]; 23 patients).

In our study, analysis of EZH2 expression in HCCs revealed that EZH2 immunoreactivity tends to increase with larger tumor size with borderline significance relationship (p = 0.054). A similar but significant relationship was found by Cai et al. [34] who also reported in their study that the positive expression of EZH2 in HCCs was significantly associated with a more aggressive phenotype of the tumor, such as higher stage tumor and vascular invasion. Similar to our analysis, Cai et al. [34] found that EZH2 expression did not correlate with age, sex, hepatitis history, AFP, cirrhosis, tumor multiplicity, or histological differentiation. Kalcakosz et al. [37] also found no correlation between EZH2 expression in HCC or CC and tumor grade.

In our study the diagnostic performance of GPC3 and EZH2 in diagnosing of HCCs and their differentiation from non malignant nodules was high (the sensitivity 80.95% and 85.71%, the specificity 100% and 95.65% and the accuracy 90.24% and 91.89% for both GPC3 and EZH2 respectively). These findings are nearly similar to the findings of Cai et al. [34]. In their study, the diagnostic performance of heat shock protein 70 (HSP70) or GPC3 and their combination with EZH2 for HCCs detection and their differentiation from non malignant nodules was analyzed. As expected, both HSP70 and GPC3 alone showed a high sensitivity, specificity and accuracy. Interestingly, the sensitivity, specificity and accuracy for HCC diagnosis increased when EZH2 was used in combination with HSP70 and GPC3. Cai et al. [34] concluded that EZH2 was able to differentiate HCCs with high accuracy from hepatocellular adenomas, focal nodular hyperplasia (FNH) and dysplastic nodules. However, no malignant liver tumors other than HCCs were analyzed in their study.

In our study, we also analyzed the diagnostic performance of GPC3 and EZH2 in differentiating between the two major kinds of primary liver malignancies "HCC and CC". There was higher diagnostic accuracy for GPC3 than EZH2 in differentiating between the two types of primary liver malignancy (the sensitivity 73.33% and 0.0%, the specificity 90.91% and 62.86%, and the accuracy 83.78% and 59.46% for both GPC3 and EZH2 respectively). Consistently with our findings, Ryu et al. [38] concluded that GPC3 can be used as a first line marker for differential diagnoses of HCC and ICC (accuracy rate: 73.5%). Previous studies, also reported the same conclusion [30,31].

Finally, we analyzed the diagnostic performance of GPC3 and EZH2 in differentiating between HCCs and metastatic nodules (the sensitivity 71.43% and 0.0%, the specificity 100% and 68.75%, and the accuracy 88.24% and 64.71% for both GPC3 and EZH2 respectively). We found that GPC3 is highly specific for HCC, but not expressed in any of the metastatic nodules, with relatively high accuracy in differentiation between the two kinds of liver malignancy. This is consistent with the results obtained by previous related studies [39]. On the other hand, EZH2 was expressed in most of HCCs and in all metastasis. Consequently, this biomarker does not provide help in differentiating HCC from metastasis. Similar finding was previously detected by Kalcakosz et al. [37].

**Conclusion**

Based on our results, GPC3 can be used as a good biomarker for differential diagnosis of HCC from non-malignant liver disease, CC and metastasis. In HCC, overexpression of GPC3 is associated with poor prognostic factors such as large tumor size and high tumor grade. We concluded that EZH2 is a reliable immune marker for HCCs, compared to non-malignant nodules (accuracy rate, 91.89%). However EZH2 is not specific for HCC since all other examined hepatic malignancies were positive as well. Further studies of GPC3 and EZH2 functions and expressions with a much larger number of patients are recommended to get better clinicopathological correlations.

**References**


