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RESEARCH

Immunohistochemical Study of Glypican-3 and HepPar-1 in Differentiating Hepatocellular Carcinoma from Metastatic Carcinomas in FNA of the Liver

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Abstract Hepatocellular carcinoma (HCC) remains a common malignant cancer worldwide, it is considered the fifth most common malignant cancer. On the other hand, metastatic tumors are widespread in the liver , with metastatic adenocarcinoma (MA) constituting the greatest part, therefore differentiation of HCC from MA is a frequent problem facing the pathologist especially in liver fine-needle aspiration biopsies. Evaluating the diagnostic value of glypican-3 (GPC-3) and HepPar-1 immunostaining in differentiating hepatocellular carcinoma from metastatic tumors in liver cell block material. Fourty eight cell blocks prepared from FNA from the liver (30 cases HCC, 18 cases metastatic carcinoma in liver) stained by Glypican -3 and HepPar-1 immunohistochemical markers. Glypican-3 was immunoexpressed in 97 % of cases of HCC while all cases of metastatic carcinoma were negative . HepPar-1 was expressed in 93 % of cases of HCC and 11 % of metastatic carcinoma of the liver . In this study the sensitivity of GPC3 in the diagnosis of HCC in cytological material was 96.7% and the specificity was 100% while the sensitivity and specificity of HepPar-1 was 93.3 % and 88.9 % respectively. Immunohistochemical staining for GPC-3 in cell block material of the liver is highly sensitive and specific and it is a valuable tool capable of differentiating HCC from most of metastatic tumors of the liver.

Keywords HCC · MA · Fine needle aspiration · Glypican-3 · GPC-3 · HepPar-1 · Immunohistochemistry

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Introduction

Hepatocellullar carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer –related death worldwide, preceded only by lung and stomach cancers [1]. The annual number of new cases of HCC worldwide is over 1, 000,000 [2]. Egypt has high prevalence of hepatocellular carcinoma, it is the second most cancer site among males after cancer bladder and seventh among females [3]. The burden of hepatocellullar carcinoma has been increasing in Egypt with doubling in the incidence rate in the past 10 years [4]. The high incidence of HCC in Egypt is attributed to the high prevalance of hepatitis C virus (HCV). HCV is currently the most significant public health problem in Egypt with an overall prevalence of 17.4 % in males and 12.2 % in females [5].

Early detection is critically important because the most effective treatment for HCC is surgical resection or ablation therapy when the tumor is small. Continuing advances in technology have facilitated radiologic and imaging detection of small lesions in the liver, but the findings are frequently non specific and non-discriminating between small HCCs and benign conditions [6]. However, differentiating between HCC and benign or metastatic hepatic lesions is sometimes difficult. In such a circumstance, a marker that can assist in separating HCC from other hepatic lesions and from metastatic neoplasms to the liver would be useful [7]. A limited number of diagnostically useful immunohistochemical markers for identification of hepatocytes in routine surgical pathology practice are available including; polyclonal carcinoembryonic antigen (CEA), and CD10 with alfa-fetoprotein (AFP), however the utility of each of these markers is limited either by suboptimal sensitivity or difficulty in interpretation, particularly in fine-needle aspiration biopsies [8].

Glypican-3(GPC-3) a cell surface-linked heparan sulfate proteoglycan that is attached to the cell surface by a glycosylphosphatidylinositol anchor is an oncofetal protein that is highly expressed during embryogenesis and organogenesis. It has been found that glypican-3 interact with growth factors and modulate

their activities; hence they play an important role in cell growth, differentiation and migration [9]. Specifically, GPC-3 is expressed in fetal hepatoblasts and is absent (silenced) in most adult tissues including normal adult liver [10]. Its expression tends to reappear with malignant transformation [11].

Down-regulation of GPC-3 has also been observed in several human malignancies, including mesothelioma and ovarian, breast, and lung cancer, these observations indicate that GPC3 is an inhibitor of cell proliferation and a tumor suppressor in a tissue-specific manner [12].

There have been a number of studies showing that GPC-3 expression is frequently up-regulated in HCCs at the messenger RNA and protein levels when compared with normal livers and benign hepatic lesions [13].

HepPar-1 (hepatocyte paraffin-1), which is a positive marker for hepatocyte differentiation on paraffin-embedded tissue, it is mitochondrial urea cycle antigen links mitochondrial antigens from both malignant and non malignant hepatic cells, has been increasingly used as a positive marker for hepatic differentiation, does not discriminate benign from malignant hepatocyte and tends to be non immumoreactive in poorly differentiated HCCs, it is also occasionally expressed in non hepatocellular neoplasms [14].

Recently new immunohistochemical markers such as Arginase-1 (Arg-1) which is recognized as a potential marker for hepatocellullar differentiation [15], and enhancer of zeste homologue 2(EZH2) which is reliable marker for HCC compared to non-malignant hepatocellular lesions [16] .

The aim of this work is evaluating the diagnostic value of glypican-3 (GPC-3) and HepPar-1 immunostaining in differentiating hepatocellular carcinoma from metastatic carcinoma in liver cell block material.

Material and Methods

Fifty formalin – fixed FNAs of liver with their related data were collected from Tropical Medicine Department and Pathology Department, Faculty of Medicine, Zagazig University in the period 2011 to 2013. After review, 2 cases of pyogenic abscesses were excluded from this study The study was carried out with full local ethics approval.

All clinical data were reviewed, for all cases, at the time of immediate adequacy assessment, aspirated material was placed in saline to form a blood clot, which is then fixed in 10 % neutral buffered formalin and embedded in paraffin to form cell block, then stained with ordinary H&E stain to confirm the diagnosis. The diagnosis and grade of hepatocellular carcinoma was established based on the morphologic findings identified on the cell block sections using the World Health Organization criteria [17]. For grade I HCC, the nuclear features were similar to those of hepatocellular adenoma, and the diagnosis of hepatocellular carcinoma was based on focal atypical features like small cell change, widened cell plates. Then the tumor grade was increased, with increased nuclear/cytoplasmic ratio, prominent nucleoli, nuclear membrane irregularities and nuclear pleomorphism. Adequate cellularity for malignant cases was defined as at least 3 groups of atypical epithelial cells (more than 10 cells in each group) and single atypical cells [18].

Immunohistochemical Study

Immunostaining was performed using the avidin-biotin peroxidase technique for localization. Briefly 4 microns from formalin–fixed Paraffin embedded tissue blocks sections mounted were de-paraffinized with xylene, then sections were rehydrated through 100, 90, 70 and 50 % ethanol. The sections then treated with 0.1 mol/L citrate buffer (pH 6.0) in a microwave for 20 min to unmask antigens before further treatment. After a quick rinse in phosphate buffered saline (PBS).

Then sections were incubated in 3 % H_2O_2 for 15 min to abolish endogenous peroxidase activity (Dako ko411 kit) before blocking with 5 % horse serum for 2 hs at room temperature to inhibit the nonspecific immunoreactions.

Primary monoclonal antibodies were incubated overnight in a humidity chamber using the following dilutions: mouse monoclonal antibody specific for GPC3 (1: 100, clone 1G12, Biocare Medical, USA) and anti- HepPar -1(1: 30 OCH1E5, Dako, Denemark). After washing in PBS they were incubated with biotinylated secondary antibodies for 30 min, then followed by avidin-biotin peroxidase complex for another 30 min according to the instructions of manufacturer (Universal Detection Kit, Dako, Denemark). Finally immune reaction was visualized as a brown colour with 3,3 – diaminobenzidine tetra hydrochloride (DAB, Dako K0114 Kit) for 5 min, then washed in distilled water. Then the slides were counterstained with Mayer's hematoxylin for 1 min before mounting .

The entire procedures were performed at room temperature. Additionally, a negative control for both markers in which the primary antibody was omitted and replaced by phosphate buffered saline was used and positive controls (paraffin sections of HCC) were run in parallel.

The immunostaining was semiquantitatively evaluated by 2 pathologists (TI and SA) according to the following criteria; when less than 5 % of cells were stained positive classified as negative, 5–50 % considered as focal intensity, and more than 50 % positive for immunostaining classified as diffuse intensity [12]. Different staining patterns (cytoplasmic and/or membranous) were recorded for both glypican-3 and HepPar-1.

Statistical Analysis

The statistical analysis was performed using (SPSS 16.0 for windows; SPSS Inc,Chicago, Illinois, USA). Data were represented as number and percentage. The differences were

compared for statistical significance by chi-square (X^2) test, difference was considered significant at $P<0.05$. Validity of the markers was assessed by sensitivity, specificity, PPV (positive predictive value) and NPV (negative predictive value). With histologic diagnosis designated as the gold standard.

Results

This study was carried out in Pathology Department, Faculty of Medicine, Zagazig University, 48 cases were submitted for this study including 30 cases (62.5 %) primary HCC, 3 cases (10 %) were well differentiated (grade I) (Fig. 1a), 15 cases (50 %) were moderately differentiated (grade II) (Figs. 2a and 3a) and 12 cases (40 %) were poorly differentiated (grade II) (Fig. 4a).

And 18 cases (37.5 %) metastatic carcinoma (6 colorectal carcinomas, 3 pancreatic carcinomas, 4 gastric carcinomas, 2 pulmonary and 3 mammary carcinomas).

Demographic data included in the study are summarized in Table 1. Males are affected more than females (64 % for males, 32 % for females).

Sonographic studies obtained from the sheets of HCC cases revealed the presence of cirrhosis in 60 % of patients. While cirrhosis is not evident in 40 % of cases. The difference between prevalence of cirrhosis in both HCC and metastatic carcinoma is statistically significant (P value=0.003)

Results of Glypican-3 immunostaining (Figs. 1b, 2b, 3b, 4b and 5b), (Tables 2 and 3)

Among the studied cases of primary malignant nodule cytoplasmic staining for glypican-3 was observed in 29 HCCs (97 %) and in none of the metastatic carcinoma (0 %) (Fig. 5b). There was significant relation between malignant nodule and GPC-3 immunoexpression, P value <0.001.

Diffuse cytoplasmic staining for GPC-3 was observed in 15 HCCs (50 %), while 14 cases (46.7 %) showed focal GPC-3

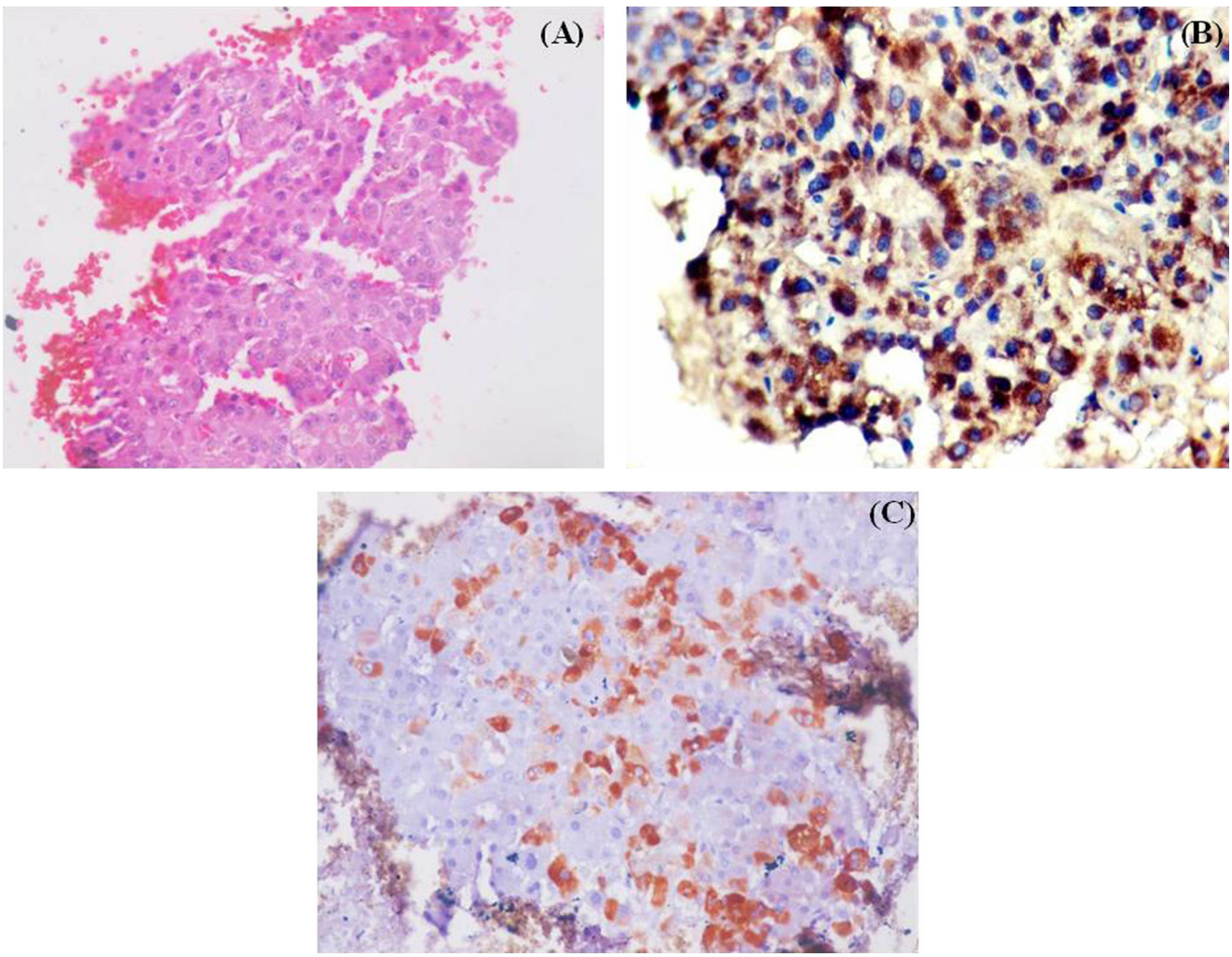

Fig. 1 A cell block section of grade I (GI) HCC, **a** shows clusters of malignant polygonal hepatocytes with characteristic granular eosinophilic cytoplasm (H & E, X200), **b** diffuse cytoplasmic immunostaining for glypican 3, with characteristic acinar pattern (ABC,DAB chromogen X400), **c** focal cytoplasmic immunostaining for HepPar-1 (ABC,DAB chromogen X200)

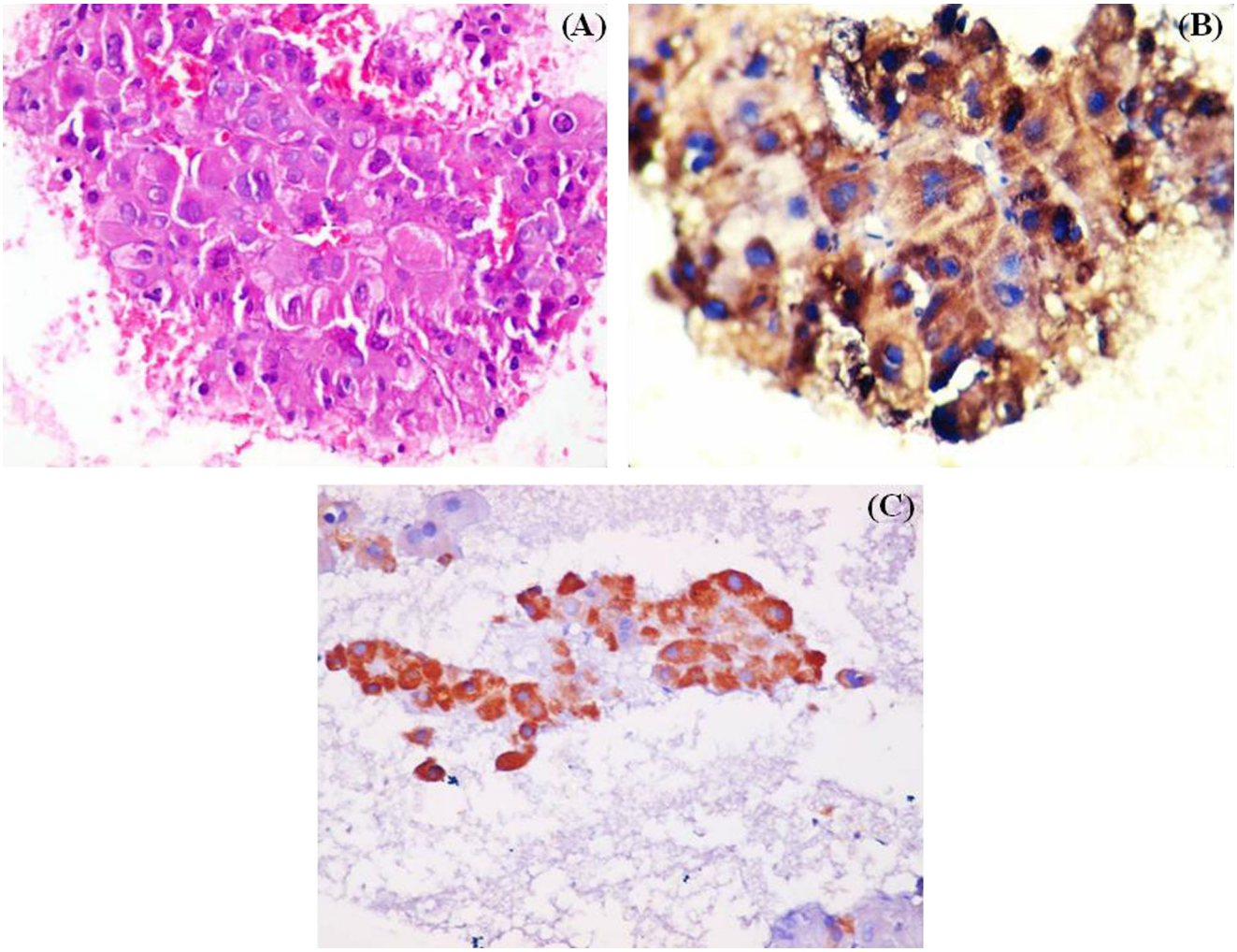

Fig. 2 A cell block section of grade II (GII) HCC, **a** shows clusters of malignant hepatocytes with increase in N/C ratio, prominent nucleoli and characteristic granular eosinophilic cytoplasm (H&EX400), **b** diffuse cytoplasmic immunostaining for glypican 3. (ABC,DAB chromogen X400), **c** diffuse characteristic granular cytoplasmic immunostaining for HepPar-1 (ABC,DAB chromogen X200)

expression and one case (3.3 %) showed negative GPC-3 expression. There was diffuse GPC-3 expression in 3 cases (100 %) of grade I HCC, 9 cases (60 %) of grade II and 3 cases (25 %) of grade III (Fig. 4b). There was statistically significant difference in GPC-3 expression among the different grades of primary HCCs, *P* value=0.03.

Results of HepPar-1 immunostaining (Figs. 1c, 2c, 3c, 4c and 5c), (Tables 2 and 3)

HepPar-1 was positive in 28 HCCs (93.3 %) and was positive in 2 cases of metastatic carcinoma from gastric adenocarcinomas (11 %) (Fig. 5c). There was significant relation between malignant nodule and HepPar-1 immunoexpression, P value <0.001.

Diffuse cytoplasmic staining for HepPar-1 was observed in 11 cases (36.7 %) while 17 cases (56.7 %) showed focal expression and 2 cases (6.6 %) were negative HepPar-1. There was diffuse expression of HepPar-1 in 1 case(33.3 %) of grade I, 7 cases(47 %) of grade II and 3 cases (25 %) cases of grade III. There was no statistically significant difference in HepPar-1 expression among the different grades of primary HCCs, P value=0.5 .

In this study, the sensitivity of GPC-3 in diagnosing HCC was 96.7 %, the specificity 100 %, the PPV 100 % and NPV was 94.7 % . While the sensitivity of HepPar-1 was 93.3 %, the specificity 88.9 %, the PPV 93.3 %, and NPV was 88.9 % (Table 4).

Discussion

HCC is the most common primary liver cancer and accounts for roughly 6 % of all human malignancies worldwide, with an estimated annual incidence between 500,000 and 1 million worldwide [1]. Although the cure rate for symptomatic HCC is very low, early stage tumors are often amenable to surgical resection or liver transplantation and are associated with favorable 5-year survival rates [19]. The surveillance of high risk patients with cirrhosis and chronic liver disease performed

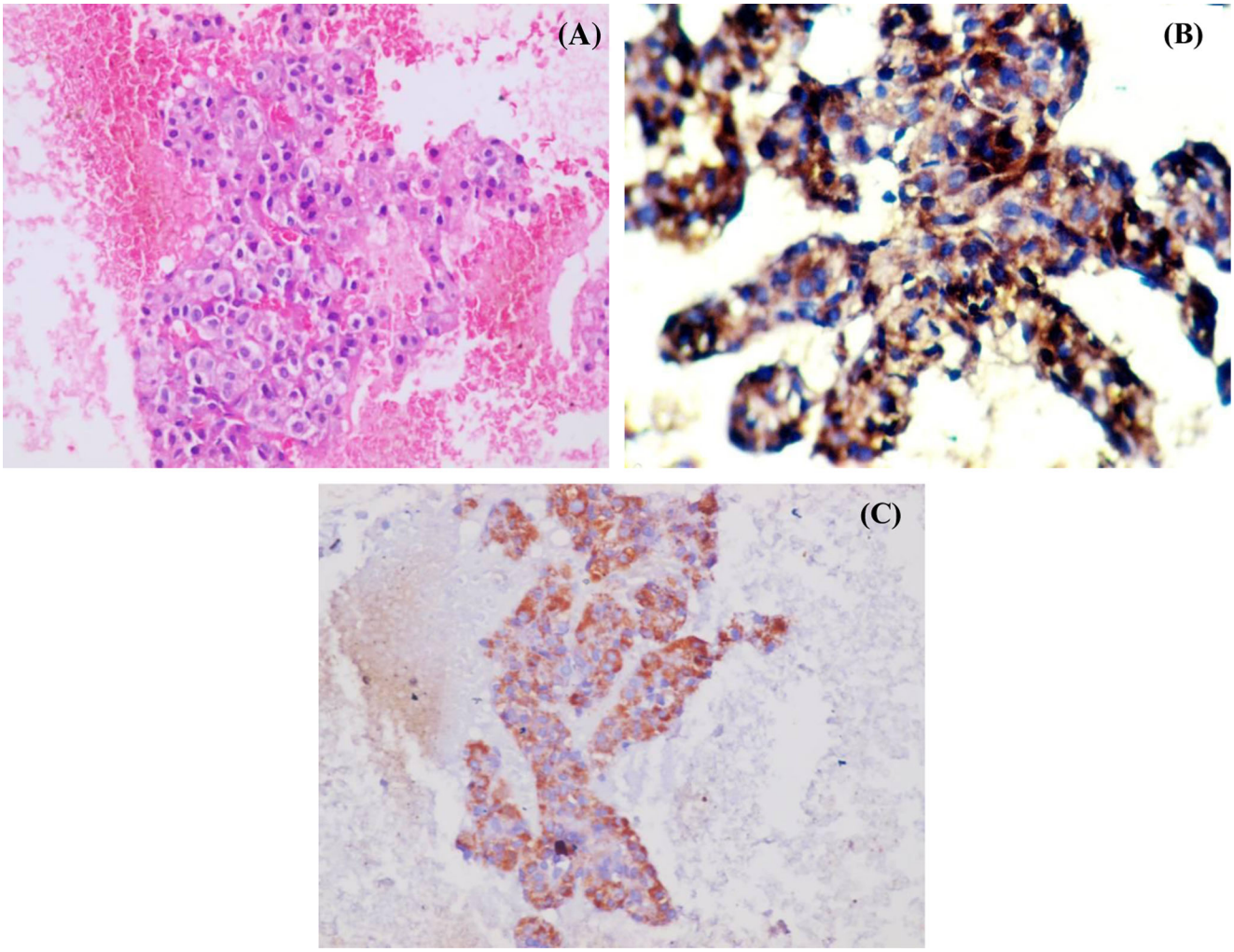

Fig. 3 Cell block section of moderatly differentiated (GII-III) HCC, **a** shows clusters of malignant hepatocytes with increase in N/C ratio and characteristic granular eosinophilic cytoplasm (H & E, X200), **b** diffuse cytoplasmic immunostaining for glypican 3 with trabecular (sinusoidal) pattern. (ABC, DAB chromogen X200), **c** diffuse granular cytoplasmic immunostaining for HepPar-1 (ABC,DAB chromogen X200)

using a combination of ultrasonographic imaging and serum alfa-fetoprotein levels. This enhanced screening method has contributed to an increased detection of asymptomatic focal liver lesions that often require histologic confirmation to aid in clinical decision making as well as treatment and prognostication [20].

Although core needle biopsy allows more accurate evaluation of benign hepatocellular lesions, the use of FNA biopsy with on-site cytopathologic evaluation and cell block preparation, is well recognized in the diagnosis of liver mass lesions, most notably the value of image guided FNA biopsy in the diagnosis of hepatic malignancies [21].

The most commonly encountered differential diagnostic challenge on liver needle biopsy specimens is HCC versus metastatic adenocarcinoma. Some of these diagnostic challenges can be attributed to: a) The liver represents one of the three most common sites of metastasis, b) HCCs may show a variety of histologic patterns, mimicking a wide variety of malignant tumors, in addition a number of metastatic tumors may mimic the trabecular, liver-like pattern of HCC, c)Complicating the diagnostic process is that the pathologists are frequently asked to handle and diagnose tiny liver needle core biopsies with various biopsy artifacts [22].

Glypican-3, a heparin sulphate proteoglycan expressed at high levels in HCC, has shown high specificity with suboptimal sensitivity in the diagnosis of HCC when used in isolation [23]. Abdelgawad et al. [24] reported that GPC-3 is a promising diagnostic marker with high sensitivity and specificity for HCC which can substitute AFP in early \diagnosis of HCC and in screening and follow up of patients with cirrhosis among the Egyptian population.

Over the past decade, HepPar-1 has been increasingly used as a positive marker for hepatic differentiation. However, HepPar-1 also suffers from relatively low sensitivity in poorly differentiated hepatocellullar carcinoma [22].

This study was performed on fourty eight cases of formalin-fixed paraffin-embedded cell blocks prepared from FNA of the liver (30 cases HCC,18 metastatic carcinoma).

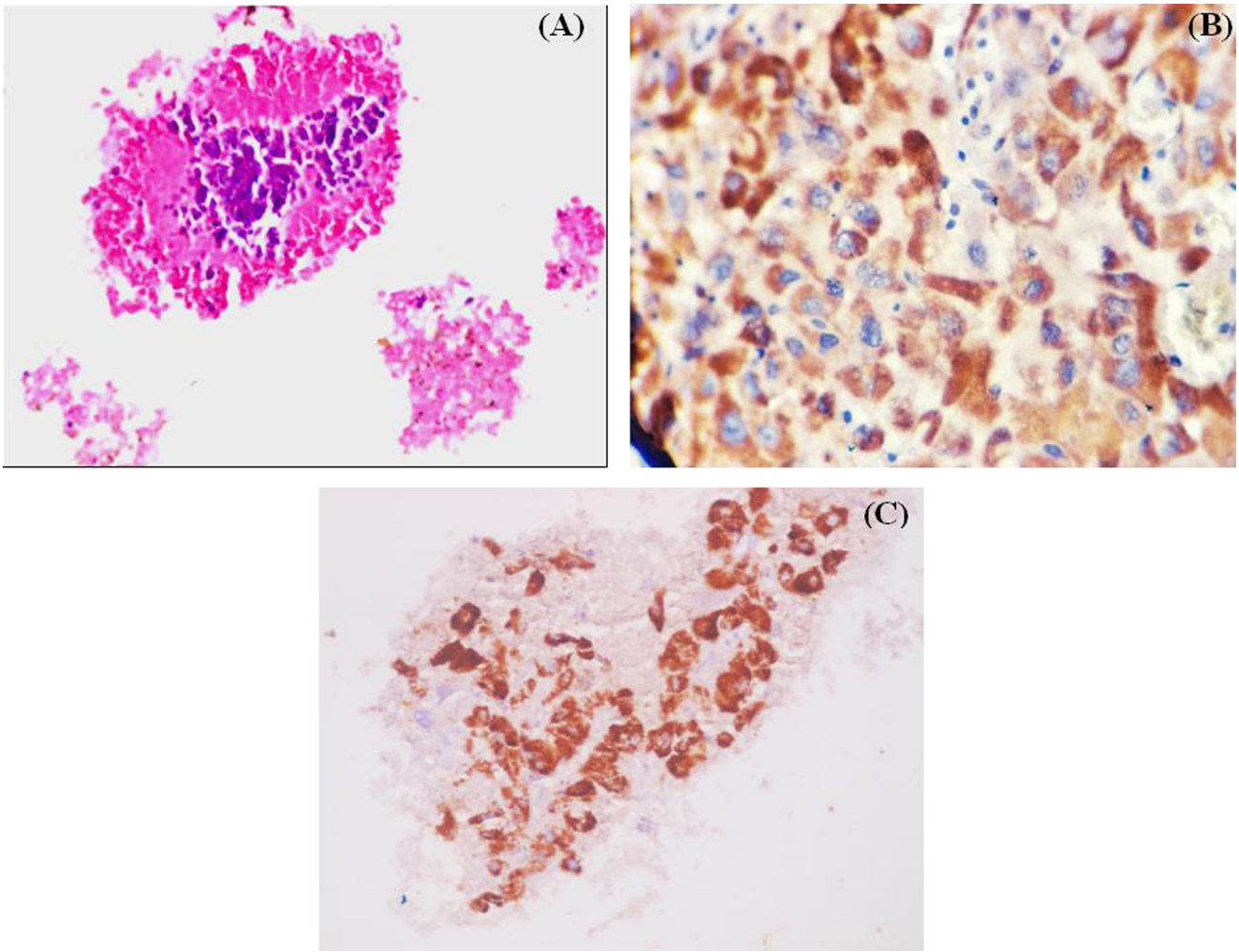

Fig. 4 A cell block section of grade III (GIII) HCC, **a** shows malignant polygonal hepatocytes with increase in N/C ratio. (H & E, X100), **b** diffuse cytoplasmic staining for glypican-3 antibody. (ABC,DAB chromogen X400), **c** focal cytoplasmic immunostaining for HepPar-1 (ABC,DAB chromogen X200)

In our study 62.5 % of cases diagnosed as HCC and 37.5 % as metastatic, this nearly similar to the study of Zaakook et al. [25] where in their study 70 % of cases were diagnosed as HCC and 30 % as metastatic. In our study 60 % of HCC were accompanied by cirrhosis, this in contrast to the study of Ligato et al. [7] where 75 % of their HCC cases were cirrhotic.

Table 1 Age and gender distribution in the studied cases

	Primary		Secondary		X^2	P
	N	%	N	%		
Age (years)						
<60	19	63.0	11	61.1	1.2	0.54
≥60	11	37.0	7	38.9		
Gender						
Male	19	63.0	13	72.0	0.63	0.72
Female	11	37.0	5	28.0		

We found that there was a highly significant differences between GPC-3 expression in HCC (97 %) compared to metastatic carcinoma (0 %) P value <0.001. This finding differed from the study of Ligato et al. [7] where 20 cases out of 24 cases (83.3 %) of HCC were positive for glypican-3 and the study of Anatelli et al. [12] that showed only one half of HCC needle biopsy specimens exhibited positive GPC-3 immunoreactivity. Nasser et al. [26] and McKnight et al. [27] found that GPC-3 was immunoexpressed in (56.8 %) of HCC but not expressed in benign or metastatic carcinomas. In the study of Zaakook et al. [25] 95.2 % of HCC cases expressed GPC-3 and 83.3 % of metastatic carcinomas were negative for GPC-3.

In our study, HepPar-1 was immunoexpressed in 28/30 HCC (93.3 %) and expressed in 11 % of metastatic carcinomas, there was statistically significant differances between HepPar-1 expression in HCC and metastatic carcinomas. Nasser et al. [26] and McKnight et al. [27] found that HepPar-1 was immunoexpressed in (72.7 %) of HCC, (100 %) of benign hepatic lesions and (2.9 %) of metastatic carcinomas. Fujiwara

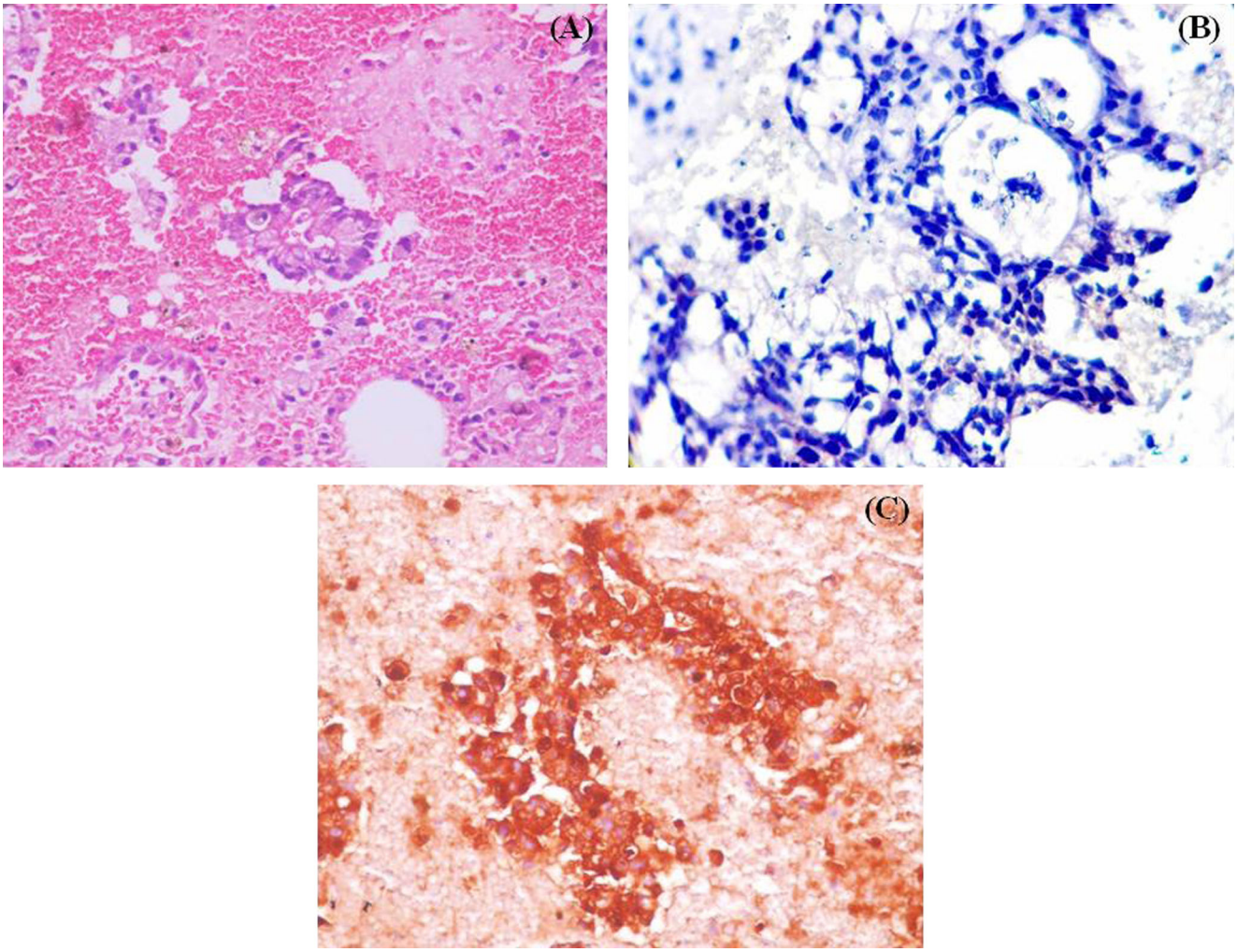

Fig. 5 A cell block section of metastatic malignant adenocarcinoma with (**a**) large hyperchromatic nuclei and increase in N/C ratio (H & E, X200), **b** lack of glypican-3 immunoexpression (ABC,DAB chromogen X200), **c** diffuse cytoplasmic immunostaining for HepPar-1 (ABC,DAB chromogen X200)

et al. [28] demonstrated immunoreactivity for HepPar-1 in 26 of 37(70 %) hepatocellular carcinoma, of them 81 % showed diffuse staining while 19 % showed weak immunoreactivity . In contrast, HepPar-1 exhibited staining only in 5 % of metastatic adenocarcinoma. Timek et al. [29] demonstrated that HepPar-1 immunoreactivity was detected in 83 % of HCC, 0 % of metastatic carcinoma and 100 % of benign liver lesions

We noticed that as the nuclear grade of HCC increases, the expression of GPC-3 decreases. GPC-3 was diffusely expressed in all 3 cases (100 %) of HCC with grade I, (60 %) of cases of HCC with grade II and (25 %) of cases of HCC with grade III suggesting that GPC-3 was a useful marker for early diagnosis. Our results were similar to the

Table 2 Results of Glypican-3 and HepPar-1 expression in different malignant nodules

	Glypican -3		HepPar-1	
	N	%	N	%
HCC n=30	29/30	97	28/30	93.3
Metastatic n=18	0	0.0	2/18	11 %
	X^2=43.96, P<0.001**		X^2=32.45, P<0.001**	

Table 3 Correlation of glypican-3 and HepPar-1expression with tumor grade in primary HCCs(n=30)

Grade	I		II		III		P
	N	%	N	%	N	%	
Glypican -3							
-ve	0	0.0	0	0.0	1	8.0	
Focal	0	0.0	6	40.0	8	67.0	0.03* sig
Diffuse	3	100.0	9	60.0	3	25.0	
HepPar-1							
-ve	0	0.0	0	0.0	2	17.0	
Focal	2	66.7	8	53.0	7	58.0	0.5 NS
Diffuse	1	33.3	7	47.0	3	25.0	

Table 4 Results of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of glypican-3 and HepPar-1 in HCC

IH marker	Sensitivity	Specificity	PPV	NPV
Glypican-3	96.7 %	100.0 %	100.0 %	94.7 %
HepPar-1	93.3 %	88.9 %	93.3 %	88.9 %

findings reported in the study of Ligato et al. [7] that GPC-3 was expressed in all seven cases (100 %) of HCC with nuclear grade I, eight of nine cases (88.9 %) with grade II and five of eight cases (62.5 %) with grade III. Zaakook et al. [25] could not agree with the present study, they found grade III (poorly differentiated) HCC cases showed the highest GPC-3 expresssion (100 %), followed by grade II (moderately differentiated) cases (96.5 %), while grade I (well differentiated) cases expressed GPC-3 in 90 % of cases . Also McKnight et al. [27] found in their study GPC-3 expression was only in 23 % of well differentiated HCC while immunostaining was observed in 81 and 20 % of moderatly differentiated and poorly differentiated HCC, respectively.

The present work showed overall expression of HepPar-1 for diagnosis HCC was 93 %. It was 100 % in well differentiated and moderately differentiated HCC and 83 % in poorly differentiated HCC. This is not in accordance with Radwan and Ahmed [30], they demonstrated HepPar-1 sensitivities of 100, 73.3 and 22.2 % for well, moderately and poorly differentiated HCC respectively. McKnight et al. [27] found in their study HepPar-1 expression was noted in all cases (100 %) of well differentiated HCC; however HepPar-1 was only noted in (69 %) of moderately differentiated HCC and (20 %) of poorly differentiated HCC. This difference may be attributed to antibody-antigen interaction may be altered in some fashion (such as with inadequate fixation) in cytologic preparation.

Arginase-1(arg-1), an enzyme involved in the hydrolysis of arginine to orthinine and urea,was recently recognized as both sensitive and specific marker for benign and malignant hepatocytes [29].

In our study the sensitivity of GPC-3 in diagnosing HCC was 96.7 %, the specificity 100 %, PPV 100 % and NPV of 94.7 %. This findings is nearly similar to the findings of Zaakook et al. [25], in their study, the sensitivity of GPC-3 in HCC was 95.2 %, specificity was 83.3 %, PPV 93 % and NPV of 88.2 %. But in the study of Fujiwara et al. [28] the sensitivity of GPC-3 was 54 %, specificity 92 %, PPV 80 % and NPV 77 %. In the study of Ligato et al. [7] the sensitivity of GPC3 in HCC was 83.3 %, the specificity 96 %, the PPV 95 % and NPV of 85.7 %. McKnight et al. [27] found that the sensitivity of GPC-3 56.8 %, specificity 100 %, PPV 100 %, NPV 71.6 %. In the study of Nasser et al. [26] the sensitivity and the specificity of GPC3 in HCC 56.8 % and 100 % respectively. Timek et al. [29] showed lower sensitivity of GPC-3 39 %, specificity 41 %, PPV 83 % and NPV 96.7 %.

In this study the sensitivity of HepPar-1 immunohistochemical marker in diagnosing HCC was 93.3 %, the specificity was 88.9 %, PPV 93.3 % and NPV 88.9 %. Timek et al. [29] found the sensitivity of HepPar-1 87 % and the specificity 97.4 %. This findings is different to the findings of Nasser et al. [26] where the sensitivity and the specificity of HepPar-1 in HCC was 72.7 % and 70.8 % respectively. McKnight et al. [27] found that sensitivity of HepPar-1 in distinguishing HCC from other malignant non –HCC lesions 72.7 %, specificity 70.8 %, PPV 69.6 % and NPV 73.9 %. Radwan and Ahmed [30] found that sensitivity of HepPar-1 was 70 %, the specificity 84 %, PPV 81.4 % and NPV 73.7 %. In the study of Fujiwara et al. [28] the sensitivity of HepPar-1 was 70 %, specificity 95 %, PPV 90 % and NPV 84 %.

Conclusion

We have demonstrated that immunohistochemical staining for GPC-3 has better sensitivity and specificity than HepPar-1 in cytological material. It is a valuable tool capable of differentiating HCC from most of the metastatic carcinomas to the liver. Since GPC-3 immunohistochemical stain is simple to interpret and can be performed in most laboratories on cytological specimens, the routine use of this marker in clinical practice may be a valuable diagnostic tool in the cytological diagnosis of HCC.

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