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GLUTAMINE SYNTHETASE, GLYPICAN-3 AND ARGINASE-1 EXPRESSION IN THE DIFFERENTIAL DIAGNOSIS OF HEPATOCELLULAR CARCINOMA *VS.* METASTATIC CARCINOMAS OF THE LIVER

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Abstract – *Objective:* *Diagnosis of metastatic liver carcinomas (MLCs) vs. hepatocellular carcinomas (HCCs) may be problematic especially in the non-cirrhotic liver. Glutamine Synthetase (GS), Glypican3 (GPC3, and Arginase-1 (Arg-1) immunohistochemistry can demonstrate the hepatocellular origin of a given tumor. This study aims to investigate the characteristics of GS, GPC3, and Arg-1 expression and the value of their combination in MLCs and in HCCs.*

Patients and Methods: *Tissue samples were obtained from 86 patients with liver tumors, (16 HCCs, 70 MLCs) who underwent liver transplantation or resection. Immunohistochemical staining for GS, GPC3, and Arg-1 was performed on formalin fixed paraffin embedded sections. Demographic, laboratory, and clinical data obtained from patient files were analyzed for the confirmation of the primary origin of the tumors. Statistical analyses were made using the SPSS version 19.0 (IBM, Armonk, NY, USA).*

Results: *Staining pattern of GS and GPC3 was cytoplasmic. Arg-1 staining was cytoplasmic in MLCs vs. cytoplasmic and nuclear in HCCs. Among HCCs, 100%, 38%, and 94% showed positive staining with GS, GPC3, and Arg-1, respectively. Overall, MLCs showed positive staining with GS, GPC3, and Arg-1 in 73%, 4%, and 4% of the cases, respectively. The combination of GS+/GPC3+/Arg-1+ was detectable in 38% of HCCs, but in 0% of MCTs. The specificity of GS, GPC3, and Arg-1 for HCC was 27%, 96%, 96%, and sensitivity was 100%, 38%, and 94%, respectively.*

Conclusions: *Expression of GS in MLCs is high, therefore GS/GPC3/Arg-1 should be used as a panel in addition to other markers when the differential diagnosis of MLC vs HCC is challenging. Cytoplasmic and nuclear Arg-1positivity should be a prerequisite for the diagnosis of HCC.*

KEYWORDS: *HCC, Liver metastasis, Glutamine Synthetase, Glypican-3, Arginase-1, Nuclear positivity.*

INTRODUCTION

Although hepatocellular carcinoma (HCC) is the most common primary hepatic malignancy, metastatic liver carcinomas (MLCs) are more frequently encountered in routine surgical pathology[1]. Liver biopsy may be essential in the diagnostic workup of malignant liver masses, especially in the non-cirrhotic background. Although most metastatic liver tumors are carcinomas with characteristic histological features that enable them to be recognized and distinguished easily from HCC on hematoxylin and eosin (H&E) stain, the differential diagnosis may be important in certain conditions. In the presence of a single mass lesion in the non-cirrhotic liver, when the clinical information is not available or when there is no prior known history of malignancy, the distinction between primary malignant liver tumor and metastasis relies on the biopsy findings. In the absence of typical morphological features, the demonstration of the cell origin using immunohistochemistry is necessary: first, for the distinction of HCC from tumors with large polygonal eosinophilic cells; second, for the distinction of poorly differentiated HCC from other poorly differentiated carcinomas. Various immunochemical panels are suggested to contribute to the differ-

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ential diagnosis[2-5]. The immunohistochemical panel should include markers, which help the distinction of HCC from primary and secondary hepatic tumors. It is known that Glutamine Synthetase (GS), Glypican3 (GPC3), and Arginase-1 (Arg-1) are useful markers for distinguishing hepatocellular tumors[2,6-8]. However, the expression of these markers in non-hepatocellular tumors is controversial[6,7,9-13]. Conversely, well known intestinal differentiation markers can also be positive in a small amount of HCCs[14]. Thus, appropriate immunohistochemical panels are necessary for proper differential diagnosis between metastatic and primary liver tumors. The aims of the present study were to investigate the expression characteristics of GS, GPC3, and Arg-1 in MLCs, search the value of the combination of GS, GPC3, and Arg-1 in the differential diagnosis between HCC and metastatic tumors, as well as determining the sensitivity and spe
cificity of these markers.

PATIENTS AND METHODS

Tumor sections from 86 liver tumor biopsies and/or resection specimens between 2016 October and 2017 July were selected for this study from archival material. The tumor tissues were obtained from diagnostic and/or therapeutic operation materials in a single institute. The study was approved by the local Ethics Committee. Written informed consents were obtained from patients before the operations or liver biopsy procedures.

Demographic, laboratory, radiological and clinical data obtained from the patients' files in the hospital information system were analyzed for the confirmation of the primary origin of the tumors. A total of 86 cases with liver tumors, including 16 HCCs and 70 MLCs were included in the study. Metastatic liver carcinomas were selected from cases with moderate/ poor differentiation. Well-differentiated cases that do not cause a differential diagnosis problem were not included. Combined hepatocellular-cholangiocarcinomas were also excluded.

All tissue samples were fixed in formalin, processed with conventional methods and embedded in paraffin. All haematoxylin and eosin (H&E) stained sections were reevaluated for the confirmation of the diagnosis, and selection of the most appropriate tissue block for immunohistochemistry. Determination of the type and differentiation of the tumors were made according to the 2010 classification of World Health Organisation[1,15-17]. Tumors were classified as HCC, MLC, or neuroendocrine carcinoma (NEC). The primary site of each metastatic tumor was noted separately.

Immunohistochemical analyses were performed on 5-µm-thick sections taken on lysine-coated slides. Sections were deparaffinized in xylene and then rehydrated. Immunohistochemical staining for anti-GS (Polyclonal, Biocare, 1/200 dilution), anti- GPC3 (Clone 1G12, Ventana, prediluted), and anti- Arg-1 (Clone SP156, Ventana, prediluted) antibodies were performed using an automated immunohistochemical stainer according to the manufacturer's guidelines (streptavidin-peroxidase protocol; BenchMark, Ventana, PA, USA). The sections were then stained with 3, 3-diaminobenzidine tetrahydrochloride (DAB), a chromogen stain (brown in color), and counterstained with hematoxylin.

Evaluation of staining: staining at least 5 high-power fields (HPF) were evaluated semi-quantitatively for each marker. Cytoplasmic staining for GS and GPC3, and cytoplasmic and/or nuclear staining for Arg-1 were considered as positive. The intensity of the staining was divided into two groups as weak and strong according to the staining intensity of the positive control cells. For each marker the expression patterns were classified as negative: <1% positive staining of tumor; 1+positive: 1% -50% weak staining of tumor; 2+ positive: ≥ 50% weak staining or ≤ 50% strong staining of tumor; 3+ positive: > 50% strong staining of tumor. Total of 2+ and 3+ staining was accepted as true positivity. Sensitivity and specificity analyses included 2+ and 3+ staining for true positives and false positives, negative and 1+ staining for true negatives and false negatives, respectively. Statistical analysis was performed in SPSS software 19.0 program (IBM, Armonk, NY, USA).

RESULTS

Clinical Data

There were 41 female and 45 male cases (age range, 39-85 years; median age, 63 years) Of the 86 tumors, 16 were HCCs, 66 were metastatic carcinomas, and 4 were NECs (poorly differentiated). Hepatocellular carcinoma differentiation was moderate/poor in all cases. The primary site of the metastatic tumors was pancreas in 20, stomach in 3, colon in 19, prostate in 4, lung in 9, and breast in 11 cases. All neuroendocrine carcinoma metastases were from the lung (n=4).

Immunohistochemistry results

The details of immunohistochemical expression and staining intensity of GS, GPC-3, and Arg-1 in all cases and the relation between the histological type of the tumors are given in Table 1 and Table 2.

TABLE 1. Expression and staining intensity of GS, GPC3, and Arg-1 in HCC and MLC cases.

	Negative n (%)	*1+ n (%)*	*2+ n (%)*	*3+ n (%)*	*Total (+) n (%)*	*2+ and 3+ n (%)*
Hepatocellular Carcinoma (n:16)						
Glutamine Synthetase	0 (0)	0 (0)	0 (0)	16 (100)	16 (100)	16 (100)
Glypican-3	9 (56.3)	1 (6.3)	3 (18.8)	3 (18.8)	7 (43.8)	6 (37.5)
Arginase-1	1 (6.2)	0 (0)	0 (0)	15 (93.8)	15 (93.8)	15 (93.8)
Metastatic Carcinoma (n:66)						
Glutamine Synthetase	7 (10.6)	11 (16.6)	20 (30.3)	28 (42.4)	59 (89.4)	48 (72.7)
Glypican-3	60 (90.9)	5 (7.6)	1 (1.5)	0 (0)	6 (9.1)	1 (1.5)
Arginase-1	53 (80.3)	10 (15.2)	3 (4.5)	0 (0)	13 (19.7)	3 (4.5)
Metastatic NEC* (n:4)						
Glutamine Synthetase	1	0	0	3	3 (75)	3 (75)
Glypican-3	2 (50)	0 (0)	1 (25)	1 (25)	2 (50)	2 (50)
Arginase-1	4 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

HCC: Hepatocellular carcinoma, NEC: Neuroendocrine carcinoma, *Primary lung in all cases.

Glutamine Synthetase Staining

The staining pattern of GS was cytoplasmic in all positive cases (Figure 1). Glutamine Synthetase was, at least 1+ positive in 16 (100%) HCC cases, in 59 (89.4%) MLCs, and in 3 (75%) NECs. When 2+ and 3+ staining was evaluated together, 48 (72.7%) of MLCs and 3 (75%) NECs were positive for GS. For the diagnosis of HCC, the specificity and sensitivity of GS was 27.1% and 100%, respectively. When 2+ and 3+ cases were regarded as positive, GS was negative in only prostate adenocarcinomas. The remaining MLCs showed positive staining with GS as follows: 16 out of 20 out of (80%) pancreatic, 3 out of 3 (100%) gastric, 17 out of 19 (89.5%) colorectal, 7 out of 9 (63.6%) breast, and 5 out of (55.6) lung carcinomas.

Glypican-3 Staining

The staining pattern of GPC3was cytoplasmic in all positive cases (Figure 2). At least 1+ positive staining with GPC3 was detected in 7 (43.8%) HCCs, 6 (9.1%) MLCs, and 2 (50%) NECs. When

TABLE 2. Expression and staining intensity of GS, GPC3, andArg-1 in MLCs according to the primary site.

	Negative n (%)	*1+ n (%)*	*2+ n (%)*	*3+ n (%)*	*Total (+) n (%)*	*2+ and 3+ n (%)*
Pancreas (n:20)						
Glutamine Synthetase	0	4	6	10	20 (100)	16 (80)
Glypican-3	20	0	0	0	0 (0)	0 (0)
Arginase-1	10	7	3	0	10 (50)	3 (15)
Stomach (n:3)						
Glutamine Synthetase	0	0	2	1	3 (100)	3 (100)
Glypican-3	2	1	0	0	1 (33.3)	0 (0)
Arginase-1	3	0	0	0	0 (0)	0 (0)
Colon (n:19)						
Glutamine Synthetase	1	1	9	8	18 (94.7)	17 (89.5)
Glypican-3	17	2	0	0	2 (10.5)	0 (0)
Arginase-1	19	0	0	0	0 (0)	0 (0)
Prostate (n:4)						
Glutamine Synthetase	2	2	0	0	2 (50)	0 (0)
Glypican-3	2	1	1	0	1 (11.1)	1 (11.1)
Arginase-1	4	0	0	0	0 (0)	0 (0)
Lung (n:9)						
Glutamine Synthetase	3	1	2	3	6 (66.7)	5 (55.6)
Glypican-3	8	1	0	0	1 (11.1)	0 (0)
Arginase-1	8	1	0	0	1 (11.1)	0 (0)
Breast (n:11)						
Glutamine Synthetase	1	3	1	6	10 (90.9)	7 (63.6)
Glypican-3	11	0	0	0	0 (0)	0 (0)
Arginase-1	9	2	0	0	2 (18.2)	0 (0)

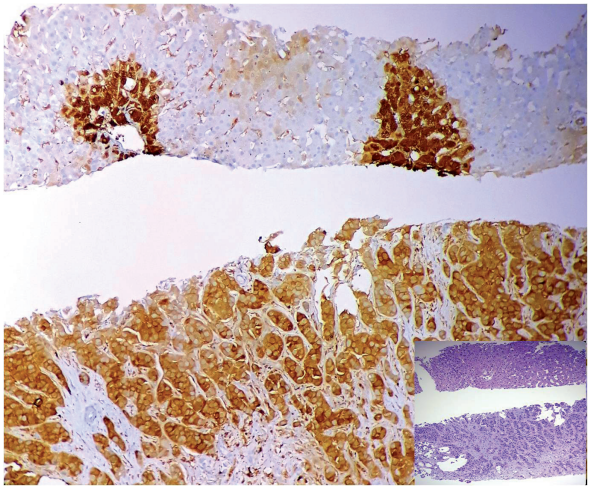

Fig. 1. Glutamine Synthetase positivity was limited in the perivenular area of the normal liver parenchyma, while it was diffuse and strongly stained in the metastatic tumor. The H&E stained slide is shown in the right bottom corner (Magnification 10x).

2+ and 3+ staining was considered together, 1 (1.5%) of metastatic carcinomas and 2 (50%) NECs were positive for GPC3. Among the MLCs, 2+ and 3+ staining was detected only in 1 out of 4 (11.1%) prostate carcinomas.

Among the HCC cases, 6 (37.5%) were 2+/ 3+ positive and for the diagnosis of HCC, the specificity and sensitivity of GPC3was 95.7%, and 37.5%, respectively.

Arginase-1 Staining

The Arg-1 staining pattern was cytoplasmic and nuclear in HCCs and only cytoplasmic in all metastatic tumors (Figure 3). 15 (93.8%) HCCs showed positive staining with Arg-1. The specificity and sensitivity of Arg-1 for the diagnosis of HCC were 95.7%, and 93.8%, respectively. Globally, MLCs showed any degree of positive staining with Arg-1 in 13 (19.7%) of the cases. When 2+ and 3+ staining was evaluated together, only 3 (4.5%) of MLCs were positive. All these 3 cases were pancreatic adenocarcinomas. Moreover, 3 out of 20 (15%) pancreatic carcinomas in this series were Arg-1 positive and there was no such strong positivity in other MLCs.

Staining characteristics of GS, GPC3, Arg-1 as a panel

The documentation of the GS + GPC3+ Arg-1 staining as a panel is given in Table 3 and the potential combinations of the panel and relative rates for detection are illustrated. The combination GS-/GPC3/Arg-1-(all negative) was detectable in 24.3% of MLCs as opposed to 0% of HCCs. The combination GS+ /GPC3 + /Arg-1+ (all positive) was detected only in HCCs but in only 6 (37.5%) cases. The positivity of 2 concurrent markers was detected in 2 out of 4 (50%) NECs (namely GS and GPC3), and in 2 out of 20 (10%) pancreas carcinomas (namely GS and Arg-1) and 9 out of 16 (%56,25) HCCs (namely GS and Arg-1).

The remaining metastatic carcinomas were all 2+/3+ GS positive, and also showed focal staining (defined as 1+ in this study) with either GPC3 (prostate, stomach, and colon) or Arg-1 (breast). Metastatic lung carcinomas showed 1+ positivity with GPC3 and Arg-1 in 11% of cases besides GS positivity.

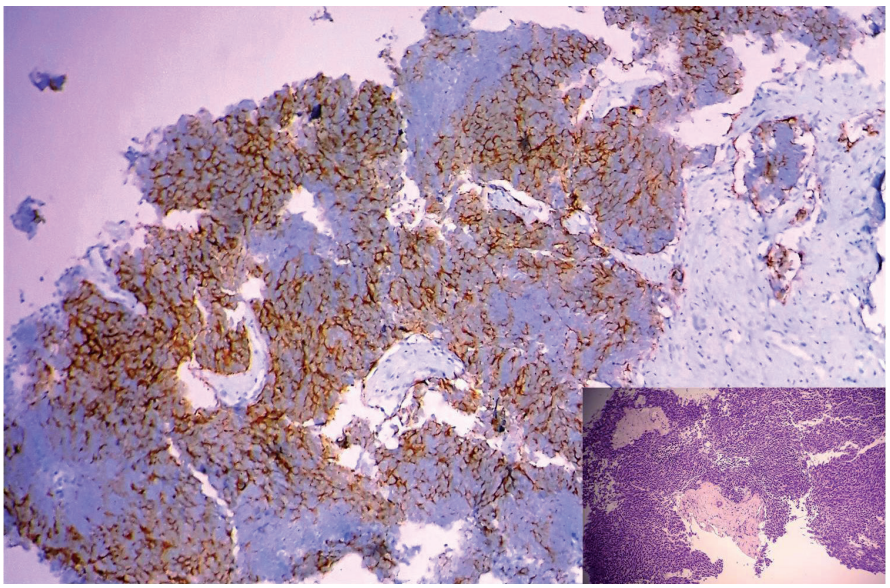

Fig. 2. Diffuse, strong, and granular staining of Gylpican-3 in one of the NECs. The H&E stained slide is shown in the right bottom corner (Magnification 10x).

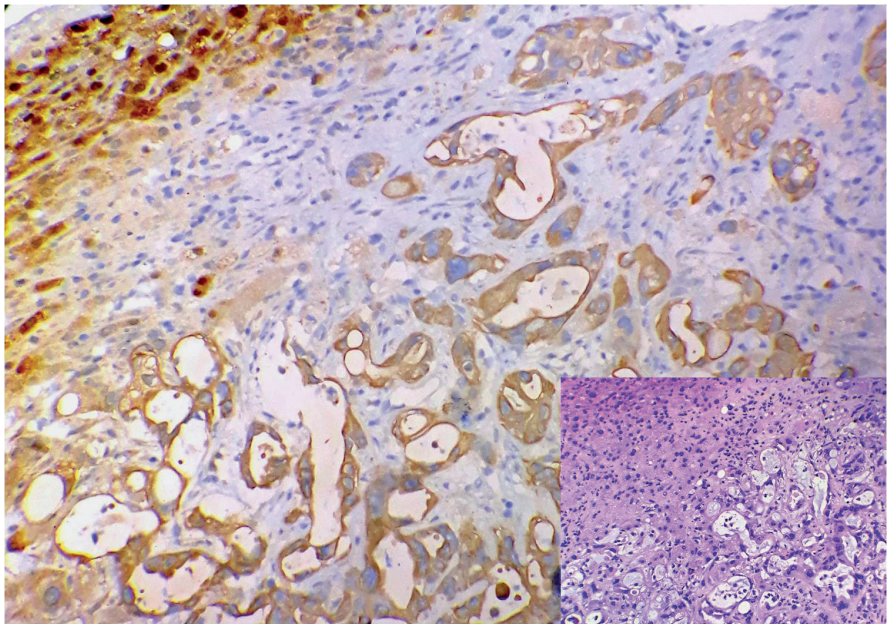

Fig. 3. Although Arg-1 showed cytoplasmic and nuclear staining in normal liver parenchyma, only cytoplasmic staining was observed in metastatic tumors. The H&E stained slide is shown in the right bottom corner (Magnification 10x).

DISCUSSION

Differential diagnosis of MLCs and hepatocellular carcinoma may be problematic especially in the non-cirrhotic liver. In cases with poor tumor differentiation establishing the diagnosis on a small biopsy specimen may be additionally challenging. In such cases, the algorithm of the immunohistochemical markers should be selected carefully. Immunohistochemical demonstration of the hepatocellular origin of the tumor is one of the main steps in the differential diagnosis. Glutamine Synthetase, GPC3, and Arg-1 are well-known markers for distinguishing the hepatocellular origin of a given tumor; however, these proteins can also be expressed in tumors of non-hepatocellular origin[2,4,6,13]. In the current study, we attempted to search the expression of these markers in a selected series of a relatively poorly differentiated group of HCCs, MLCs, and metastatic NECs.

Glutamine synthetase, which is a well-recognized target of the Wnt/β- catenin pathway, is an enzyme of nitrogen metabolism and it catalyzes the conversion of glutamine to glutamate[18]. This reaction also takes place in the control of many important cellular processes such as autophagia, activation of the mTOR pathway, and the release of inflammatory mediators[19,20]. The pattern of GS expression has an important place in the diagnosis of primary hepatocellular masses[6,13,21,22]. In routine practice, GS may find a place in the immunohistochemical panels to differentiate between MLCs and HCCs, since diffuse GS positivity is reported between 43.9% and 7% in HCCs from different series[8,23,24]. In this study we found GS positivity in 100% of HCC cases, but the specificity and sensitivity of GS for the diagnosis of HCC were 27.1% and 100%, respectively. The reason for this low sensitivity was the high expression rate of GS in MLCs ranging between 56% and 100% of pancre-

TABLE 3. The prevalence of the 8 potential combinations of the Markers Under Study

	MLC (n=66) n (%)	*HCC (n=16) n (%)*	*NEC (n=4)*
GS-/GPC3-/Arg-1-	16 (24.25%)	0 (0%)	1 (25%)
GS-/GPC3-/Arg-1+	1 (1.51%) (Pancreas)*	0 (0%)	0 (0%)
GS-/GPC3+/Arg-1-	1 (1.51%) (Prostate)*	0 (0%)	0 (0%)
GS-/GPC3+/Arg-1+	0 (0%)	0 (0%)	0 (0%)
GS+/GPC3-/Arg-1-	46 (69.70%)	1 (6.2%)	1 (25%)
GS+/GPC3-/Arg-1+	2 (3.03%) (Pancreas)*	9 (56.2%)	0 (0%)
GS+/GPC3+/Arg-1-	0 (0%)	0 (0%)	2 (50%)
GS+/GPC3+/Arg-1+	0 (0%)	6 (37.5%)	0 (0%)

Arg-1: Arginase-1, **GPC3**: Glypican-3, **GS**: Glutamine Synthetase; **MLC**: Metastatic Liver Carcinomas.

atic, gastric, colorectal, breast, and lung carcinomas. Our data and other results from the literature indicate that GS should not be a choice of priority as a marker in the differential diagnosis of HCC and MLCs[4,8,10,11]. In this context, GS positivity is meaningful only with concurrent GPC3 and Arg-1 positivity, as all positive profiles were detected only in HCCs.

On the other hand, positive staining of GS in MLCs may have a different meaning. Since GS is involved in the control of many important cellular events (autophagy, activation of the mTOR pathway, the release of inflammatory mediators) this may be related to metastatic potential[19,20,25-27]. If the GS expression determines the metastatic potential of a given tumor needs further investigation in different series.

Glypican-3 is a heparin sulfate proteoglycan oncofetal protein expressed in 63-91% of hepatocellular carcinomas, as well as many other tumors, especially in melanomas, extragonadal germ cell tumors, squamous cell carcinoma of the lung, squamous and adenocarcinoma of the esophagus, ovarian tumors, and in 14% of the liver metastasis of tumors from gastrointestinal tract and pancreas[28-30]. In this study, GPC3 was positive in 37.5% of HCCs, 2.3% of MLCs (one case metastatic from prostate, and 50% of NECs (from the lung). We also detected focal positivity (defined as 1+ in this study) with GPC3 in prostate, stomach, and colon carcinomas. In our series, the results indicate that GPC3 is a highly specific (95.7%) but not very sensitive (37.5%) marker for HCC. Although the number of NECs in this series was limited, it is of note that 50% of these tumors expressed GS and GPC3 concurrently.

Arginase-1, which is involved in the hydrolysis of arginine to ornithine and urea in the urea cycle, is highly expressed in the liver at cytoplasmic and/or nuclear level[31]. The overall sensitivity of Arg-1 in the diagnosis of HCC is reported between 84 and 96%, but it may also be expressed in colon, gastric, lung, and pancreatic cancers, with specificity for the diagnosis of HCC of 96%[31-32]. In the current study, Arg-1 was positive in 93.8% HCC cases, with high specificity (95.7%) and sensitivity (93.8%). Among MLCs, we detected 4.5% positivity with Arg-1, and these cases were exclusively pancreatic adenocarcinomas. On the other hand, focal positive staining (defined as 1+ in this study) was present in 19.7% of the metastatic cases (pancreas, colon, lung, and breast). In our series, HCCs expressed Arg-1 both in the cytoplasm and nucleus, whereas its expression in MLCs was exclusively cytoplasmic. This is also reported in other series[4,12].

CONCLUSIONS

In conclusion, when making the differential diagnosis between MLC *vs.* HCC 1) expression of GS can be confusing, since its positivity in MLCs is high, therefore GS/GPC3/Arg-1 should be used as a panel besides other markers, 2) cytoplasmic and nuclear Arg-1positivity should be a prerequisite for the diagnosis of HCC, and 3) poorly differentiated NEC may be an important diagnostic pitfall in HCC differential diagnosis, since the co-expression of GS and GPC3 in this series.

Compliance with Ethical Standards

The study was approved by the Ethics Committee of Ege University Medical School.

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Contributions

Author Argon A, Author Nart D and Author Yilmaz F conceived and designed the study, and wrote, edited and reviewed the manuscript. Author Argon A, Author Erbil s, Author Yilmaz F researched and analyzed data, and wrote, edited and reviewed the manuscript. All authors gave final approval for publication. Author Argon A takes full responsibility for the work as a whole, including the study design, access to data and the decision to submit and publish the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interests.

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