

Evaluation of Glypican-3 Expression in Poorly Differentiated Carcinomas of Lung Origin

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Glypican-3 (GPC-3) is a glycoconjugate protein of heparan sulfate proteoglycan family and is important for embryogenesis but silenced in adult healthy tissue. GPC-3 protein is abnormally expressed in hepatocellular carcinoma (HCC) and has been used as a marker for pathological diagnosis of primary and metastatic HCC. However, GPC-3 expression has also been found in some tumors other than HCC. This study is to investigate the expression of GPC-3 expression immunohistochemically and the staining pattern in poorly differentiated carcinomas of lung primary in order to assess the value of GPC-3 as a marker for diagnosing metastatic HCC in lung. Lung tissue from 44 patients diagnosed with poorly differentiated carcinoma were evaluated, including 23 lung adenocarcinomas, 19 squamous cell carcinomas and 2 adenosquamous carcinomas. Immunohistochemical stains of GPC-3 was performed on tumor tissue samples. The expression pattern of GPC-3 was analyzed. Expression of GPC-3 was found in 45% of primary lung cancers, including 79% of squamous cell carcinomas, 18 % of adenocarcinomas, and 50% of adenosquamous carcinomas. The poorly differentiated carcinomas showed predominantly patchy positivity. The staining pattern ranged from weak granular cytoplasmic positivity to a strong membranous and cytoplasmic positivity, and both cytoplasmic and nuclear positivity. GPC-3 expression was not seen in non-neoplastic lung tissue. GPC-3 is a relatively specific marker for HCC. In this study, we demonstrated the expression of GPC-3 in a significant number of poorly differentiated carcinoma of lung. Therefore, in context of a possible metastatic HCC to the lung, caution should be made by using GPC-3 as a differential marker for HCC, and a panel of stains should be considered.

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INTRODUCTION

Glypican-3 (GPC-3) is one of six glycosylphosphatidylinositol-anchored, cell-surface heparan sulfate proteoglycans. It expresses primarily in embryonic tissue and plays an important role in cell division and regulation, but being silenced in adult tissue.^{1,2,3} GPC-3 has been reported to be associated with hepatocellular carcinoma (HCC).^{3,4,5} Immunohistochemistry using monoclonal antibodies against GPC-3 demonstrated strong membranous and cytoplasmic staining of GPC-3 in 72% of HCCs (21 out of 29), whereas absence of GPC-3 expression in normal and benign liver.⁵ GPC-3 immunohistochemistry was found to be helpful in detecting distant metastases of HCC in patients.^{6,7} Serological study showed that GPC-3 was undetectable in the serum of healthy individuals and patients with hepatitis. However,

patients with HCC exhibited increased level of GPC-3 in serum.^{6,8} These characteristics of GPC-3 make it a potential therapeutic target for treating liver cancer.^{9,10} Several therapeutic immunotoxins based on anti-GPC-3 antibodies have been developed.^{11,12} It has been demonstrated that GPC-3 promotes HCC growth by stimulating the canonical Wnt pathway.⁸ Overall, GPC-3 immunostaining has utility for differentiating hepatocellular carcinoma from dysplastic changes in cirrhotic livers and cholangiocarcinoma. Aberrant GPC-3 expression has been reported in various tumors other than HCC.¹³

Lung cancer is the leading cause of cancer death and the second most common cancer among both men and women in the United States. Poorly differentiated carcinomas were defined as tumors formed almost entirely of solid nests or cords of cells with very pleomorphic nuclei, frequently with prominent nucleoli, and variable amount of cytoplasm. It is a challenge to identify the primary origin of poorly differentiated cancer. IHC is very helpful to identify the

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primary origin, especially when distinct morphologic characteristics is absent. GPC-3 has been demonstrated to be a valuable immunohistochemistry marker for metastatic HCC. It is unclear whether GPC-3 can be used to differentiate metastatic HCC from primary lung cancer, because the presence and expression pattern of GPC-3 in poorly differentiated primary lung tumors has not been evaluated.

The present study aimed to assess the value of GPC-3 as a marker for diagnosing metastatic HCC in lung. We have tested the immunohistochemical expression of GPC-3 in poorly-differentiated non-small-cell lung carcinoma. Overall 45% of poorly-differentiated non-small-cell carcinoma of lung primary including adenocarcinoma and squamous cell carcinoma showed some degree of positive GPC-3 expression immunohistochemically. The GPC-3 expression is not noted in para-cancerous normal lung tissue.

METHODS

Case Selection and Histologic Assessment

Patients with a diagnosis of poorly-differentiated non-small-cell lung carcinoma between 2012 and 2014 were retrospectively identified from the institutional database. The clinical information was obtained from electronic medical records. The present study was approved by the Institutional Review Board of Baylor College of Medicine. A total of 44 patients who had tissue sample available for immunohistochemical (IHC) analysis were included in current study. All cases had been reviewed to confirm the pathologic diagnosis of poorly differentiated non-small-cell lung carcinoma.

IHC of GPC-3 was performed on tissue microarray (TMA) constructed from resected tumor samples (n=29) as well as routine slides from biopsy or frozen section samples (n=15). Three 1 mm cores from each sample were used for TMA with a duplicate. Tissue samples from 23 lung adenocarcinomas, 19 squamous cell carcinomas and 2 adenosquamous carcinomas were examined. The expression and expression pattern of GPC-3 were analyzed by two pathologists independently.

Immunohistochemical Analysis

Immunohistochemical stain on tissue was performed using Leica Bond III IHC autostainer. Paraffin sections were deparaffinized in xylenes, rehydrated through graded ethanol solutions to distilled water, and then treated with Diva Decloaker solution in Biocare's Decloaker Chamber for 20 minutes. After washed with dionized water, the slides were transferred to the BOND III autostainer and were incubated with the mouse monoclonal antibodies against GPC-3 (1G12, dilution 1:100, Biocare Medical). A positive reaction was visualized with diaminobenzidine solution, followed by counterstaining with hematoxylin. Appropriate negative controls for the immunostaining were prepared by using non-immune mouse IgG.

Sections were evaluated manually by two pathologists. Positive staining of GPC-3 was defined as membranous, cytoplasmic and/or nuclear expression in >10% tumor cells.

For manual quantitative analysis of GPC-3 expression levels, we utilized a scoring system for staining intensity: negative (0), weak (1+), moderate (2+), and strong (3+).

Immunohistochemistry on tissue was also performed utilizing mouse monoclonal antibodies against TTF-1 (8G7G3/1, dilution 1:100, Biocare Medical) and mouse monoclonal antibodies against p40 (BC28, dilution 1:100, Biocare Medical).

Statistical Analysis

The data were analysed using SPSS software. Differences in expression levels of GPC-3 between adenocarcinoma and squamous cell carcinoma tissues were assessed with student *t* test. Results were considered statistically significant only if $p < 0.05$.

RESULTS

Clinical Features

A total of 44 cases of poorly-differentiated primary lung carcinomas were investigated. All patients were adult males. None of the patients presented with distant metastasis at the time of diagnosis or had other malignancies. Nodal metastasis was found in two cases (5%). For squamous cell carcinoma, the mean age of patients at the diagnosis of squamous cell carcinoma was 67 years old, ranging from 57 to 73 years old. The mean age of patients with adenocarcinoma was 67 years old, ranging from 55 to 84 years old. The clinical information is summarized in **Table 1**.

Table 1. Clinical characteristics.

| | |
|--|------------|
| No. of patients | 44 |
| Age (years old), Range (median) | 55–84 (66) |
| Gender | |
| F | 0 (0%) |
| M | 44 (100%) |
| Histology | |
| Adenocarcinoma | 23 (52%) |
| Squamous cell carcinoma | 19 (43%) |
| Adenosquamous Carcinoma | 2(5%) |
| Stage | |
| 1 | 27 (61%) |
| 2 | 15 (34%) |
| 3 | 2 (5%) |
| 4 | 0 (0%) |

GPC-3 Immunostaining

Of 44 primary poorly-differentiated lung carcinoma cases, 20 cases (45%) were positive for GPC-3 IHC staining (**Figure 1. A & B**). Cases were furtherly subclassified as squamous cell carcinoma (n = 19), adenocarcinoma (n = 23) or adenosquamous carcinoma (n = 2), based on morphological features and IHC stain of TTF-1 and P40. Squamous cell carcinoma showed strong P40 expression and negative TTF-1 expression. Adenocarcinoma showed negative P40 expression and strong TTF-1 expression. Adenosquamous carcinoma contained glandular component with positive TTF-1 and negative P40 staining and a squamous component with negative TTF-1 and positive P40 staining.

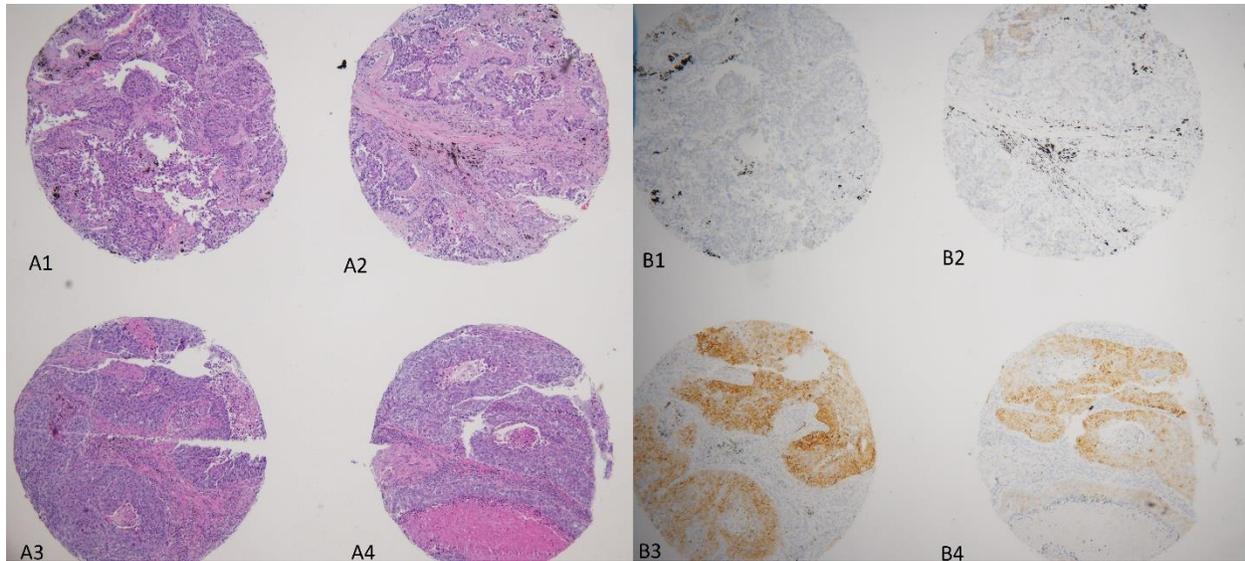


Figure 1. Tissue microarray of poorly-differentiated carcinomas of lung. **A.** H&E stained; **B.** glypican-3 Immunohistochemical stain. A1, A3, B1, B3: adenocarcinoma; A2, A4, B2, B4: squamous carcinoma. Magnification 40X.

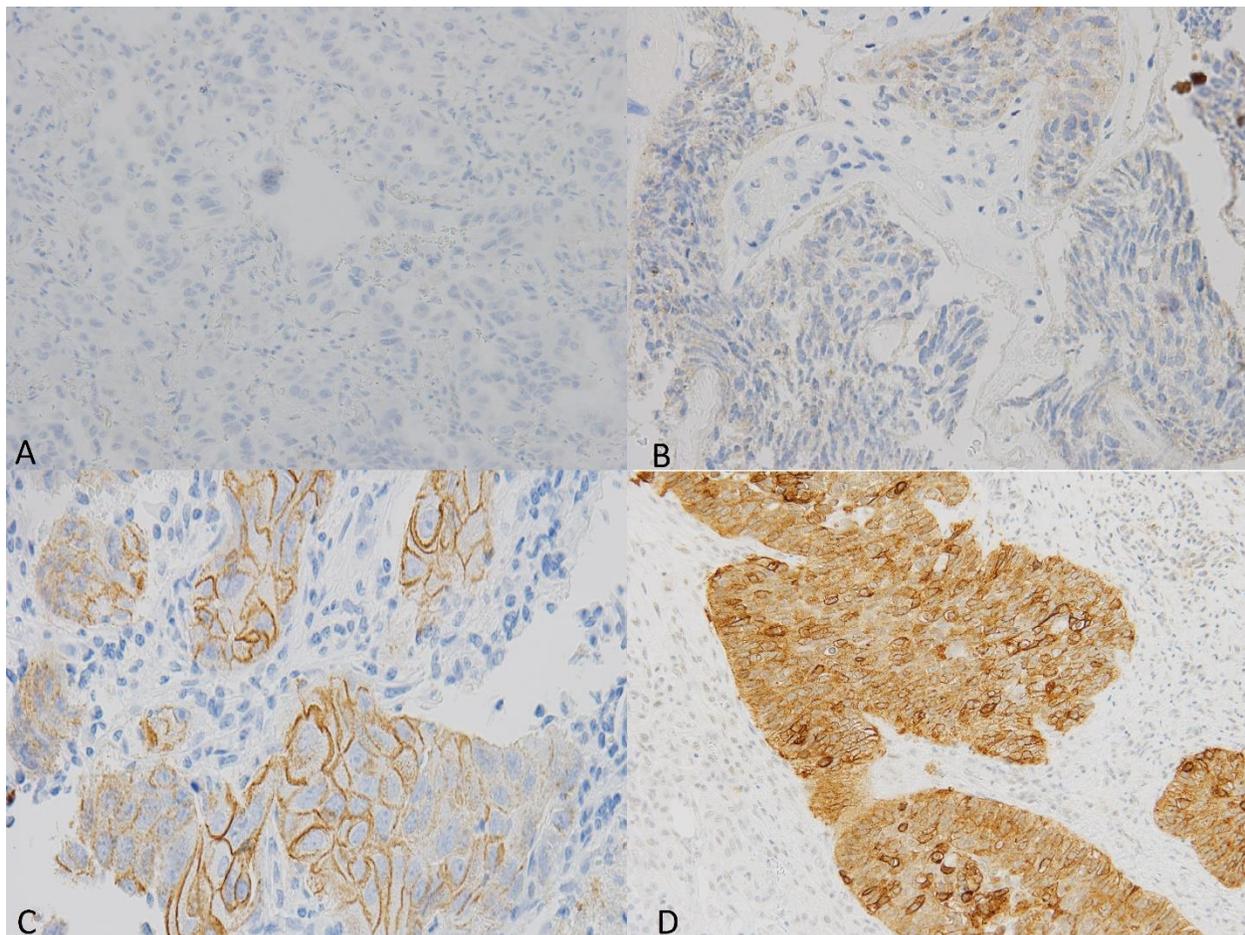


Figure 2. Intensity of glypican-3 Immunohistochemical stain. **A.** negative stain; **B.** 1+; **C.** 2+; **D.** 3+. Magnification 200X.

GPC-3 expression intensity

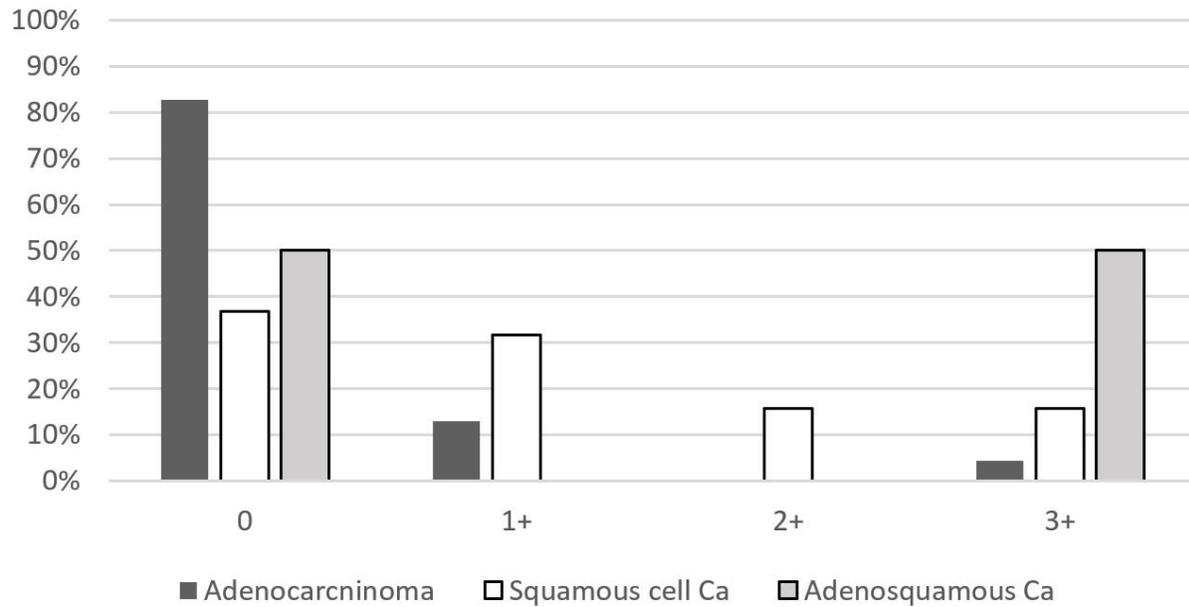


Figure 3. Intensity scores of GPC-3 expression in poorly-differentiated lung carcinoma.

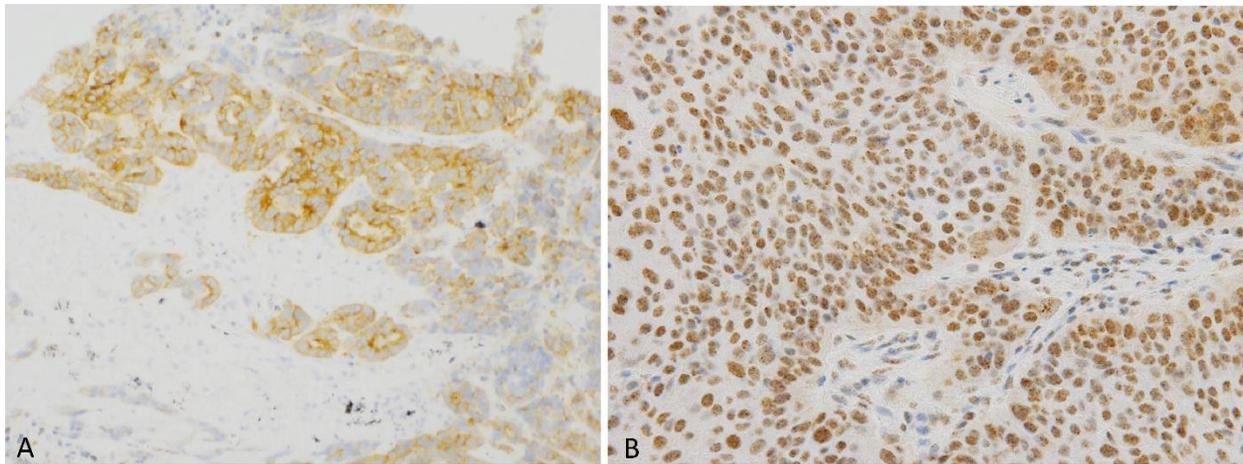


Figure 4. Cytoplasmic and nuclear staining of glypican-3. **A.** cytoplasmic staining of glypican-3 in a poorly differentiated squamous cell carcinoma; **B.** nuclear staining of glypican-3 in a poorly differentiated squamous cell carcinoma. Magnification 200X.

The positive staining of GPC-3 was found in 15 of 19 (78.94%) squamous cell carcinomas, 4 of 23 (17.98 %) adenocarcinomas, and 1 of 2 (50%) adenosquamous carcinomas. Positive expression in squamous cell carcinoma was significantly higher than that in adenocarcinoma (79% vs 18%, $p < 0.01$). The positivity of stain was defined as greater than 10% of tumor cells with GPC-3 staining. The poorly-differentiated carcinomas showed predominantly patchy positivity. The stain pattern ranged from weak granular

cytoplasmic positivity to a strong membranous and cytoplasmic positivity, and both cytoplasmic and nuclear positivity as well (**Figure 2. A-D**). Six cases (29%) had intensity score of 3+. 10 cases (47%) and 4 cases (19%) had intensity of 1+ and 2+, respectively (**Figure 3**). There were two squamous cell carcinoma cases that showed strong nuclear staining (**Figure 4. A-B**). The para-cancerous normal lung parenchyma was negative for GPC-3 expression.

DISCUSSION

GPC-3 is a member of heparan sulfate proteoglycan family which is linked to the cytoplasmic membrane by a glycosylphosphatidylinositol anchor. GPC-3 is composed of a core protein of 580 amino acids and two attached heparan sulfate glycosaminoglycan polysaccharide chains. These side chains bind specifically with different ligands, such as growth factors and chemokines, and trigger intracellular signaling pathways. GPC-3 is highly expressed in placenta and many fetal tissues. GPC-3 may bind growth factors such as insulin-like growth factors, bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), hedgehog (Hh) signaling pathway, or tissue factor pathway inhibitor to function primarily in cell growth and differentiation. GPC-3 is encoded by the GPC-3 gene which is located on human X chromosome (Xq26).¹⁴ Deletion mutations in this gene are associated with Simpson-Golabi-Behmel syndrome (SGBS) which is characterized by anomalies of postnatal overgrowth including coarse facies, macrostomia, macroglossia, and other abnormalities affecting the internal organs and bones.¹ In addition, patients with SGBS are at increased risk for certain tumors, most frequently hepatocellular carcinoma, hepatoblastoma and Wilms tumor.

GPC-3 has also been linked to various solid, hematologic, and pediatric tumors, particularly hepatocellular carcinoma. In 1997, Hsu et al first reported that mRNA and protein levels of GPC-3 were upregulated greatly in most HCCs than in normal liver, cholangiocarcinoma and metastatic carcinomas of the liver.¹⁵ Since then, extensive studies have demonstrated that GPC-3 is a reliable indicator for the diagnosis of HCC, and plays a significant role in the progression of HCC. GPC-3 functions as a coreceptor for some ligands, for instant, Wnt and FGF, via its heparan sulfate glycan side chains, and facilitates ligand and/or its receptors to stimulate the signaling pathways involved in HCC growth and invasion.^{16, 17} Moreover, studies found that GPC-3 promoted HCC progression and metastasis by inducing the epithelial-mesenchymal transition in tumor cells, and the ERK signaling pathway is involved in this GPC-3-induced process.¹⁸ Li et al. also showed that ectopic GPC-3 could increase the c-Myc expression which is a typical target of the canonical Wnt signaling pathway and c-Myc can also transcriptionally activate GPC-3 directly in HCC cells.¹⁶

The studies demonstrated that patients with poorly differentiated carcinoma had more lymph node metastases, worse 2-year overall survival, and more frequent local recurrences.¹⁹ It is a challenge to identify the primary origin of poorly differentiated carcinoma. GPC-3 may be used as a valuable immunohistochemistry marker for metastatic HCC. However, the present study found the elevated expression of GPC-3 in primary poorly-differentiated non-small-cell lung cancer including both adenocarcinoma and squamous cell carcinoma. And GPC-3 is absent in para-cancerous normal lung parenchyma. The overall positive expression of GPC-3 in lung cancer was 45%. Positive GPC-3 expression was significantly higher in poorly-differentiated squamous cell

carcinoma (79%) than that in poorly-differentiated adenocarcinoma (18%).

The findings in the present study are consistent with the result of a recent study which used functional genomic mRNA profiling to predict the GPC-3 protein expression.¹⁰ It was found that 45% of squamous cell lung cancers may over-express GPC-3 protein as determined by genomic mRNA profiling. Aviel-Ronen et al. also reported that Glypican-3 immunostaining was positive in 23% of lung carcinoma samples. A higher percentage (79%) of squamous cell carcinoma examples with GPC-3 overexpression was reported in present study. This may be due to the higher histological grade of tumors examined, since only cases of poorly differentiated carcinoma were included in the present study. Studies of HCC also described the higher percentage of GPC-3 expression in high grade HCC. The mechanism of GPC-3 overexpression in the development of lung cancer is barely studied. A recent study on non-small cell lung cancer cell line found that proliferative ability of cancer cells increased when GPC-3 was overexpressed.²¹

The staining pattern of GPC-3 in lung carcinoma is similar as that in HCC, including both membranous and cytoplasmic staining. This staining pattern is predictable as the GPC-3 protein expressed on cell surface as well as in the cytoplasm. Additionally, rare strong nuclear staining pattern was present in two cases of squamous cell carcinoma. Nuclear staining pattern of GPC-3 was previously reported in breast cancer.¹⁰ The antibody used in present study is a monoclonal antibody against last 70 amino acids of GPC-3 core protein. Cross reaction with other nuclear protein is unlikely. What mechanism resulted in this phenomenon is unclear, whether it is due to protein sequestration in nucleolus. More basic research of GPC-3 protein regulation and pathway may increase our understanding of the abnormal presence of GPC-3 protein in tumor cells and its role in tumor genesis and progression.

In summary, the present study found GPC-3 immunohistochemical staining positivity in 45% of poorly differentiated carcinoma of lung primary including adenocarcinoma and squamous cell carcinoma. GPC-3 has been used for demonstrating poorly differentiated hepatocellular carcinoma. Therefore, in context of a possible metastatic poorly-differentiated HCC to the lung, a panel and additional stains IHC stains are required.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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