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Role of CD34, SMA & Ki-67 Immunohistochemistry– An Important Diagnostic Tool in Differentiating Early Well Differentiated Hepatocellular Carcinoma from Benign Hepatic Mimickers: A Retrospective Study from North East India

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Abstract **Original Research Article**

North East India has higher incidence of cancers than rest of the country& with advent of early screening, Hepatocellular carcinoma is getting detected at an early stage. Core needle biopsies have become indispensible tool for diagnosis of HCC and it is difficult to identify cell thickness in early well-differentiated HCC from benign mimickers on histomorphology. To overcome this difficulty, present study was designed to see the utility of CD 34, SMA & Ki-67 in this regard and also to see role of these markers in differentiating Early well differentiated hepatocellular carcinoma from benign hepatic mimickers. 48 FFPE blocks of various lesions of liver were retrieved from Oncopathology Department, BBCI Guwahati in last one year. Cases were divided into Group A (n=28-HCC) Group B (n=20-benign mimickers). IHC CD34, SMA & Ki-67 were done on all cases. Demographic &clinical details with histomorphological findings were correlated. In Group A- 26 out of 28 HCC cases (92.8%), CD34 was strongly, completely & diffusely expressed by sinusoidal vessels with more than 3 cell plate thickness. Group B- In normal liver (10 out of 20), hepatic sinusoids are negative to weak positive for CD34.In 7 out of 20 (35.1%) benign conditions, it is sparsely expressed in capillarized sinusoids at periportal & perinodular area& high-grade dysplastic nodules showed peripheral and focal staining for CD34. Stromal cells are strongly Positive for SMA in 85.7% (group A 24 out of 28). Ki-67 was slightly increased in Group A (6-10%) than in Group B (1-2 %). Our data demonstrates that CD34, SMA & Ki-67 has significant increased expression in early WD HCC as compared to benign mimickers. Hence, CD34, SMA & ki-67 are significant helpful IHC tool for reaching to a definite diagnosis of Early WD HCC when disease is yet at early stage & and ideal for setup where specific markers of malignant hepatic tumors are not available. Thus, immunohistochemical markers essentially expressed in HCC in a specific pattern of immune-expression, but not in benign mimickers, would be helpful.

Keywords: Well differentiated hepatocellular carcinoma, immunohistochemistry, CD34, SMA, Ki-67.

INTRODUCTION AND BACKGROUND

- Primary liver cancer primarily includes Hepatocellular carcinoma. It is the most prevalent primary liver malignancy globally, making up over 80% of cases, and ranks as the sixth most common cancer and the fourth leading cause of cancer-related deaths worldwide [1]. The incidence and age of onset vary by region. Asia has the highest incidence at 72.5%, followed by Europe at 9.8%, Africa at 7.7%, North America at 5%, Latin America and the Caribbean at 4.6%, and Oceania at 0.4% [2]. In North America and Europe, the median age of onset is typically over 60, while in Asia and Africa, it ranges from the 30s to the 60s.It is the second most common primary liver malignancy among children, accounting for 20-30% of cases. Approximately three quarters of all new cases occur in low- and middle-income countries, with the highest incidence rates observed in Africa, China, and south-eastern Asia.
- North East India has higher incidence of cancers than rest of the country and with advent of early screening, Hepatocellular carcinoma are getting detected at an early stage.
- Most of cases of liver mass lesions are benign, and include entities such as cirrhosis, regenerative nodules, adenoma, and focal

nodular hyperplasia (FNH), but may mimic malignant liver lesions (benign mimickers) both clinically and radiologically. Most malignant liver lesions are metastatic tumors and only few are hepatocellular carcinoma (HCC). Therefore, for a liver mass, the differential diagnosis between HCC and benign mimickers frequently confronts surgical pathologists.

- Histologically, HCC resembles normal liver cells to a variable extent. Thus, traditionally, the diagnosis of HCC requires the demonstration of evidence of hepatocellular differentiation by tumor cells. This exemplified morphologically by polygonal cells with eosinophilic cytoplasm forming plates separated by sinusoids and sometimes the presence of intracytoplasmic bile pigment.
- Histologic grading is done by (3 tiered system) WHO grading system.
- Well differentiated: tumor cells resemble mature hepatocytes; minimal to mild nuclear atypia.
- Moderately differentiated: tumor cells appear malignant on H&E and morphology suggests hepatocellular differentiation; moderate nuclear atypia.
- Poorly differentiated: tumor cells appear malignant on H&E and often cannot be distinguished from other poorly differentiated neoplasms; marked nuclear atypia.
- However, both benign mimickers and HCC often share these particular histologic features, thus making the differential diagnosis difficult. The matter is further complicated by the fact that most liver mass lesions are evaluated through transcutaneous or transvenous needle core biopsies, and pathologists frequently must deal with small pieces of tissue. Core needle biopsies have become indispensible tool for diagnosis of HCC and it is difficult to identify cell thickness in early well-differentiated HCC from benign lesions on histomorphology. Below Figure 1 depicts a Chart from WHO Tumours of the liver and intrahepatic bile ducts, which also highlights the use of CD34 for the same.
- To overcome this difficulty, present study was designed to see the utility of IHC CD 34, SMA & Ki-67 in this regard.
- Thus, immunohistochemical markers essentially expressed in HCC in a specific pattern of immune-expression, but not in benign mimickers, would be helpful.

Table 8.09 Histological features and diagnostic tools in dysplastic nodules and early hepatocellular carcinoma (HCC)

Histological features / diagnostic tools	LGDN	HGDN	Early HCC
Cytology			
Small-cell change	–	+	+
Large-cell change	±	±	–
Foci with clonal appearance	±	+	+
Growth patterns			
Increased cell density over surrounding liver parenchyma	< 1.3 times	1.3–2 times	> 2 times
Pseudoglandular/acinar changes	–	±	+
Architectural changes			
Portal tracts	Present	Present	Often absent
Reticulin framework[a]	Intact	Intact	Usually at least focal loss
Unpaired (non-triadal) arteries and sinusoidal capillarization (CD34)	±	±	+
Additional diagnostic tools			
Stromal invasion and loss of ductular reaction (CK7/CK19)[a]	–	–	±
Overexpression[a] (of ≥ 2 among HSP70[b], glypican-3 [GPC3][b], and GS[b]) {776,2919A}	–	–	+ (most)
Nodule-in-nodule growth[a]	–	–	±

Figure 1: Shows a chart depicting histological features and diagnostic tools in dysplastic nodules and early hepatocellular carcinoma

AIMS AND OBJECTIVES

Aim:

- To evaluate Role of CD34, SMA & Ki-67 Immunohisochemistry in differentiating Early Well differentiated Hepatocellular carcinomas from benign hepatic mimickers.

Objectives:

- **Primary Objective:** To Perform comprehensive evaluation of CD34, SMA, Ki-67 IHC expression in

Primary hepatic malignancies with Hepatic differentiation with a focus on its role in differentiating between WD-HCC and benign hepatic mimickers.

- **Secondary Objective:** To study the different CD34 staining patterns in various hepatic lesions and evaluate that certain CD34 expression patterns may be useful in differentiating WD-HCC and benign hepatic mimickers in Set ups where Ideal Hepatic markers are not available

MATERIALS AND METHOD

- Study Site: Department of Oncopathology, Dr B Borooah Cancer Institute Guwahati.
- Study Design: Retrospective and observational Study, was carried out in one year (2021-2022).
- Study Population/size: 48 FFPE blocks of various lesions of hepatic cases were retrieved from the departmental laboratory and were divided after histopathological examination of each cases into:
- Group A-Well Differentiated Hepatocellular carcinomas (n=28).
- Group B– Benign Hepatic mimickers such as regenerative nodule, focal nodular hyperplasia, low grade dysplastic nodule, high grade dysplastic nodule, cirrhosis (n=20).
- Demographic & clinical details with Age, Sex, Habits, associated medical conditions, BMI, Serum AFP, histomorphological findings were correlated.
- The main approach to identifying lesional tissue on biopsies is to;
- Look for a subpopulation of cells that form a contiguous group of cells with a distinctly different morphology from that of the background liver along with loss of normal portal tracts.
- Immunohistochemistry CD34, SMA & Ki-67 were done on all cases.
- Formalin-fixed paraffin-embedded tissue blocks of HA cases were submitted for immunohistochemical studies utilizing antibodies directed against the following antigens: CD34 (QBEen10, sc52312, 1:100 dilution; Santa Cruz Biotechnology), Ki-67 (monoclonal mouse, MIB-1 clone, Dako, Denmark), After blocking of the endogenous peroxidase reaction and non-specific reaction, each primary anti-body (1: 100 dilution) was allowed to react on serial frozen sections overnight at 4°C. From paraffin blocks, in some cases, 4-pm-thick serial sections were cut, dewaxed, rehydrated in water, and pretreated either with 0.4% pepsin in 0.01 N HCl for 30 min at 37°C prior to CD34 immunohistochemistry or with autoclave heating for antigen retrieval before Ki-67 immunohistochemistry [3-8]. Tris-buffered saline without primary antibody was applied for the negative control. The tissue localization of CD34 and Ki-67 was then visualized by the streptavidin-biotin-peroxidase complex method with 3,3'-diaminobenzidine (DAB) as the chromo-gen. Several fields were assessed randomly under a medium power view (X200) and the Ki-67 positive index (PI.) was determined by scoring at least 1000tumor cell nuclei. There were no significant differences be-tween the sets of results obtained by the two investigators.
- Demographic and clinical observations were recorded in the study proforma and then entered in MS excel sheet. Prior to analysis all the entries were double checked for any error.
- Data was expressed as mean ± S.D. as percentage.
- Statistical analyses between well, moderately, and poorly differentiated hepatocellular carcinoma, dysplastic nodule and focal nodular hyperplasia and CD34, SMA and Ki-67 IHC expression were performed using the $\chi 2$ test for comparing proportions.
- Students t test was used to assess significance of difference in ordinal data.
- p Value < 0.05 was considered to be statistically significant.
- MS Excel® (Microsoft Corp. New Mexico USA) were used for other statistical calculations.

RESULTS

Group A

- In 26 out of 28 HCC cases (92.8%), expression of CD34 was strongly, completely & diffusely expressed by sinusoidal vessels with more than 3 cell plate thickness.
  - Three immunoreaction patterns of CD34 were identified in hepatic lesions.
  - The first pattern was a negative CD34 immunoreaction, where only blood vessels and bile ducts in portal tracts, along with a few sinusoidal spaces near portal tracts, showed positivity. This pattern was present in all control patients, some non-malignant neoplastic nodules, and in all non-tumor cells surrounding the hepatic mass, but was not observed in HCC cells.
  - The second pattern exhibited complete, homogeneous diffuse CD34 expression, with most sinusoidal spaces throughout the mass showing positivity. This pattern was found in 95.4% of HCC tissues and 37.9% of non neoplastic benign mimickers (p=0.001) which showed statistical significance.
  - The third pattern was characterized by focal CD34 immunoreaction, where only certain sinusoidal spaces exhibited immunoreactivity

to CD34. This pattern was noted in 4.6% of HCC tissues and 13.8% of cases with non neoplastic benign mimickers.

- In 24 out of 28 HCC cases (85.7%), Expression of SMA was Strongly and diffusely Positive for Hepatic stromal cells (p value 0.78), which was not statistically significant.
- Ki-67 was slightly increased in Group A (index-6-10%) than in Group B (index<1-2%).
- Serum Alpha fetoprotein levels – were Mild to moderately higher in Group A than in Group B (p value -0.43).
- Mean Serum AFP levels in Group A was 468.02 ng/ml.

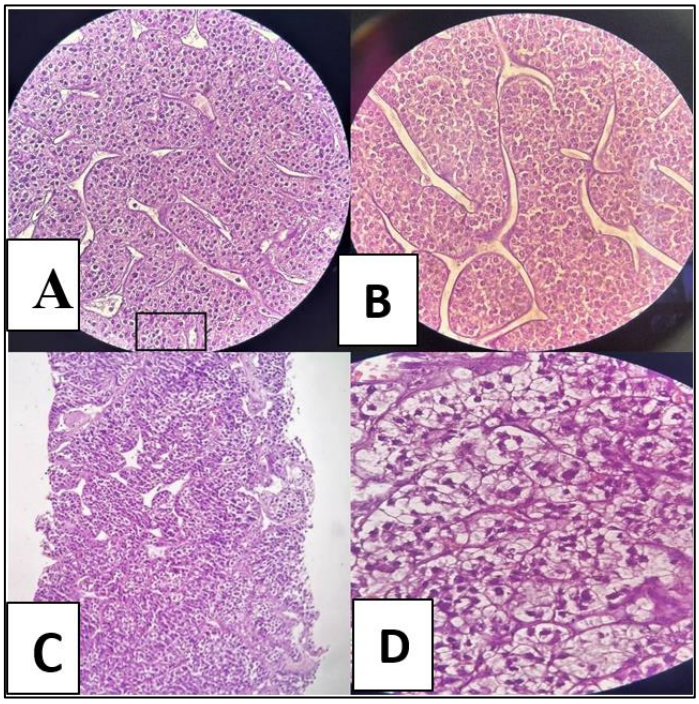

Figure 2: Depicts various histomorphology of early well differentiated hepatocellular carcinomas (A, B, C) with increased cell plate thickness and hepatocellular differentiation. D shows clear cell variant of hepatocellular carcinoma

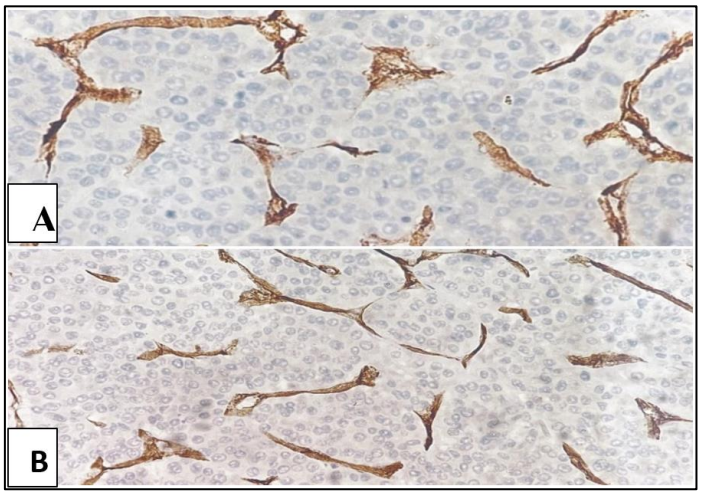

Figure 3: Shows pattern two of IHC CD34 staining (A+B) showing CD34 was strongly and diffusely expressed by the endothelial lining of sinusoid-like tumor vessels

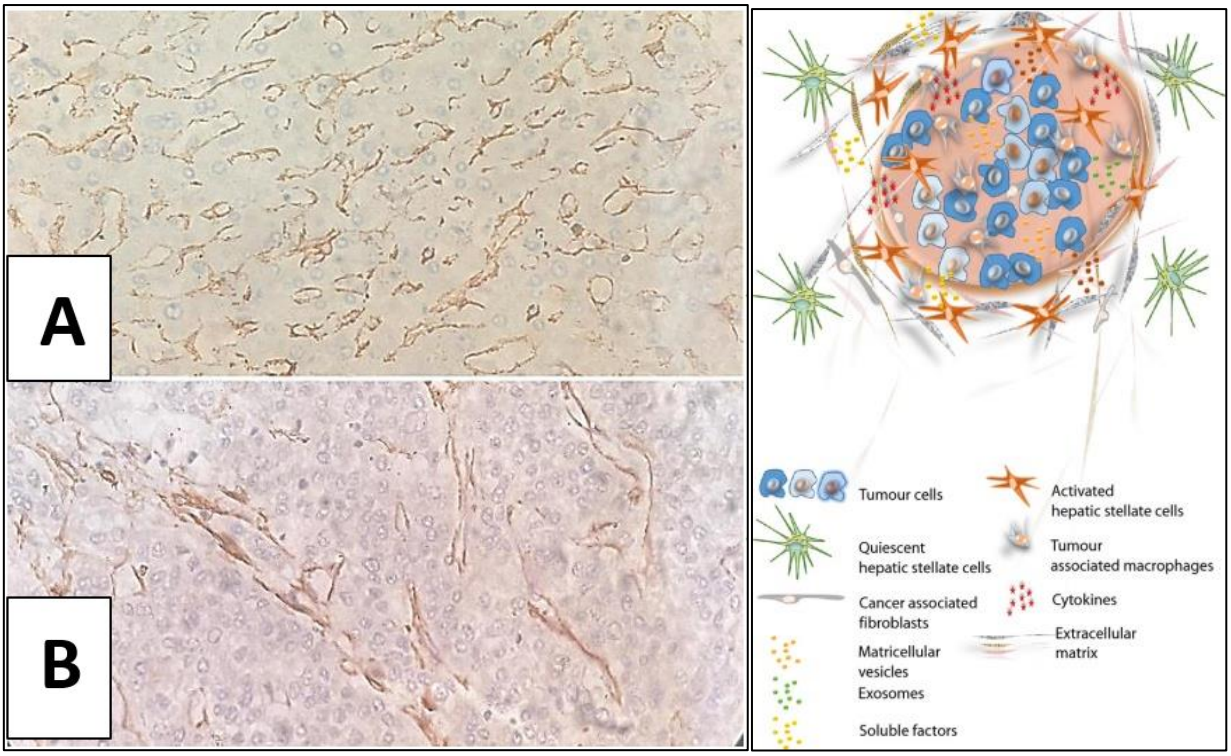

Figure 4 (A+B): Shows various expression of IHC SMA in group A: Long cytoplasmic process can be observed by immunoreaction with SMA

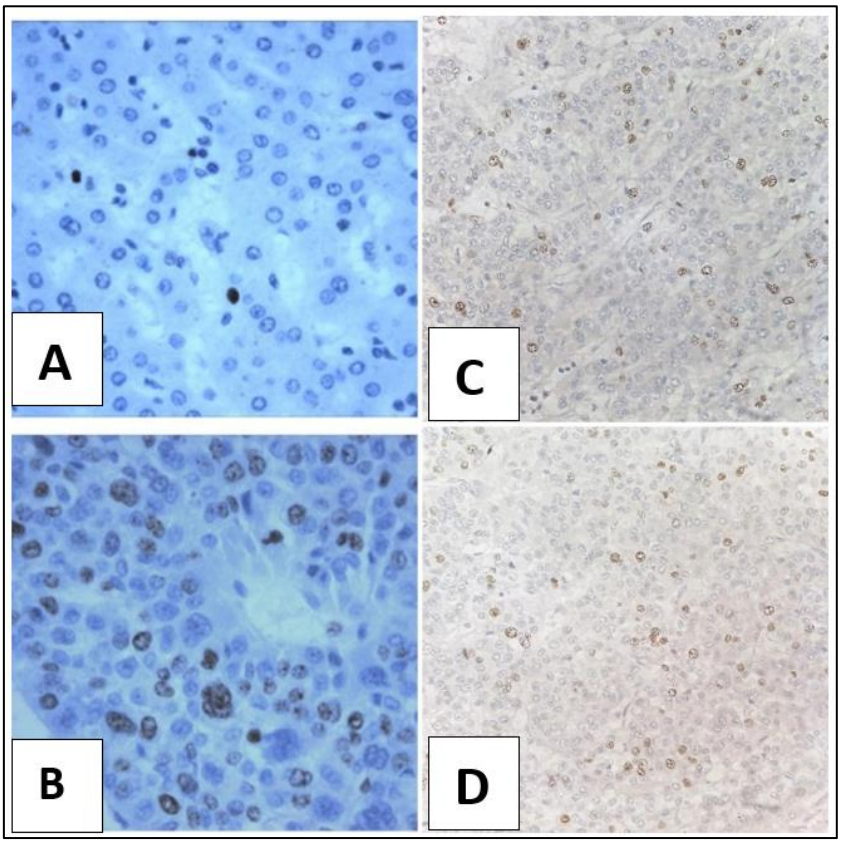

Figure 5: Shows various proliferative index of IHC Ki-67
(A: Ki67-1% in group b of benign mimickers, B: Ki67>10% in group A of hepatocellular carcinoma, C and D: Ki67-(6-10%) in group A)

Group B (Benign mimickers)

- In non neoplastic liver (10 cases out of 20), CD34 was negative to weak focal positive in Hepatic sinusoids.
- In 7 out of 20 (37.4%) benign Mimickers (Cirrhosis, Focal nodular hyperplasia, low or high dysplastic nodule), CD34 is sparsely expressed in capillarized sinusoids at periportal & perinodular area.
- High-grade dysplastic nodules showed peripheral and focal staining for CD34.
- Expression of SMA was variable, group B, whereas Ki-67 index was less than Group A.

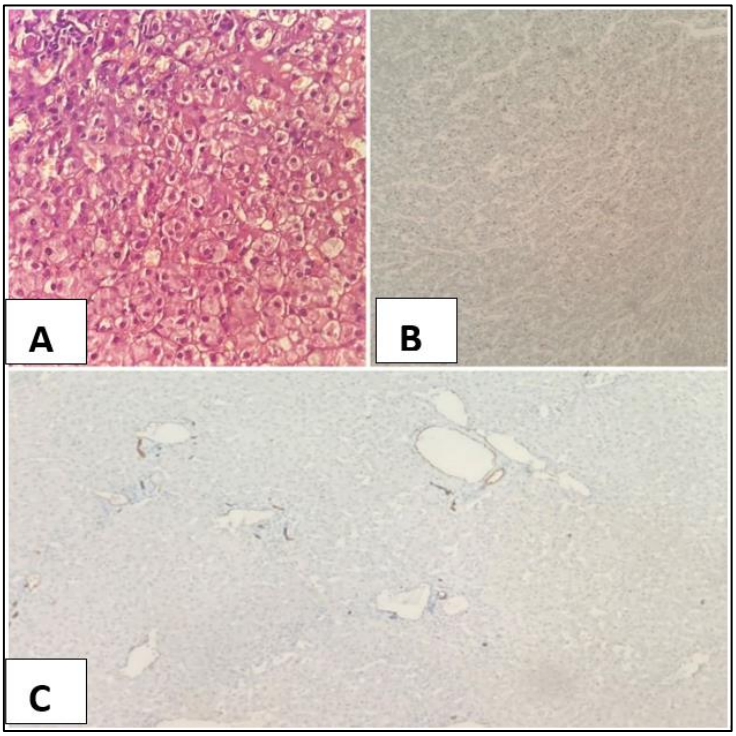

Figure 6: Shows results Group B, (A=HPE of Focal nodular hyperplasia B=Ki67 <1%, C=IHC CD34-Negative)

Table 1: Highlights comparative analysis of Group A & B

Groups (A+B=48)	CD34	p value	SMA	p value	Ki-67 index	p value
Group A(n=28)	Strong Diffuse + 26/28 (92.8 %)	0.001	Strong diffuse + 24/28 (85.7%)	0.78	10%	0.43
Group B (n=20)	Diffuse + 7/20 (37.9%)		variable (18.6%)		1-2%	

DISCUSSION

In liver disease, the differential diagnosis between HCC and benign conditions frequently confounds surgical pathologists. Several histological studies have examined the utility of CD34 in making a distinction between benign hepatic lesions and HCC (Heukamp *et al.*, 2006; Li WC *et al.*, 2006; Coston *et al.*, 2008; Tatrai *et al.*, 2009) [12].

In our study, we determined that CD34 remains the most sensitive immunostaining marker for diagnosing HCC. 95.4% HCC cells exhibited a diffuse CD34 staining pattern, contrasting with the negative expression observed in surrounding non-tumor liver cells, including those affected by hepatitis, cirrhosis, or normal liver conditions. In our study, CD34 was found expressed diffusely in the sinusoids of HCC, even in well-differentiated HCC, focally in some sinusoids of high grade dysplastic nodule and in no sinusoid of cirrhotic nodule and nodular hyperplasia

.

While 10 cases (20.8%) of non neoplastic benign mimickers were negative for CD34 immunoreaction and 4 cases (8.3%) showed focal CD34 staining, 3 cases (6.25 %) mimicked the staining pattern of HCC cells. In group B or benign mimickers, in contrast to HCC, no samples from the adjacent tissue areas of chronic hepatitis showed any positive staining of sinusoids for CD34. Therefore, CD34 staining can serve as a valuable marker to distinguish HCC from benign liver lesions like hepatitis or cirrhosis, and it is a sensitive, though not highly specific, marker for differentiating HCC from non-malignant neoplastic nodules.

Gottschalk *et al.*, 1992 evaluated the immunohistochemical staining patterns characteristic of hepatocellular carcinoma (HCC) using CD34 and factor

VIII antibodies were compared with those of other hepatic lesions to determine if these stainings can be used as a diagnostic criterion. These included HCC (14 cases), metastatic tumor (14 cases) and nonneoplastic liver lesions (16 cases). He suggested immunoperoxidase staining with CD34 and factor VIII be performed on the cell block sections from FNAs in any problematic hepatic case [9].

Kimura *et al.*, 1998 - quantitatively evaluated angiogenesis by CD34 immunohistochemistry in liver cirrhosis (LC), adenomatous hyperplasia (AH), and HCC, and proliferative activity estimated by Ki-67 immunohistochemistry. Angiogenesis was evaluated by CD34 immunohistochemistry using monoclonal antibody HPCA-2, and tumor proliferative activity was evaluated using monoclonal antibody MIB-1. Although they used an image analysis system to assess the microvessel density as the area percentage of the endothelial area. Angiogenesis was generally observed in HCC and there was no significant difference among all clinical stages and histological grades of HCC. On the other hand, the staining of CD34 was partly observed in sinusoids of AH, although no positive staining was seen in any sinusoids of LC [3].

The proliferative activity was significantly correlated with the clinical stage and histological grade of HCC. Their results indicate that the quantitation of angiogenesis does not provide significant prognostic information in HCC, but that it may have diagnostic value in distinguishing HCC from non-HCC.

Shuzhe *et al.*, in 2013 aimed to evaluate a panel of immunostaining markers (including GP73, GPC3, DCP, CD34, and CD31) as well as reticulin staining to distinguish HCC from the mimickers. Their results revealed that CD34 immunostaining and reticulin staining were highly sensitive for the diagnosis of HCC [10].

Andrea *et al.*, 2020 investigated the utility of digital protocols for Ki-67 immunohistochemistry quantitative analysis ("hot spot" method) in the setting of well-differentiated hepatocellular neoplasms. Resection cases of typical hepatic adenomas (HAs) (n = 40), atypical HAs (n = 9), and well-differentiated hepatocellular carcinomas (WD HCCs) (n = 56) were selected. The proliferative rate of HAs (typical, median 1.2% (range 0-7.4%) and atypical, median 1.0% (range 0.3-3%)) was significantly lower than that of WD HCCs (median 4.5%, range 0-49.8%) ($P < 0.0001$). Only a few (7.5%) of the adenomas (all inflammatory/telangiectatic type) had proliferative rates higher than 4%, compared to most (51%) of HCCs. He concluded that Ki-67 is a potentially useful adjunct marker in the evaluation of WD hepatocellular neoplasms, as "hot spot" proliferative rates are consistently very low in HAs but vary significantly in WD HCCs [11].

Grigioni *et al.*, have also found higher proliferative rates in HCCs (15–50% of tumor cells, which correlated well with Edmondson-Steiner grading) compared to five neoplastic lesions which did not appear malignant by the criteria utilized at the time. The benign hepatocellular lesions showed low proliferative rates, similar to that of normal or cirrhotic livers. These studies, however, did not restrict their cases to well differentiated HCCs which could potentially be mistaken for HAs on routine stains, had small numbers of HAs.

CONCLUSION

Our data demonstrates that IHC expression of CD 34, SMA & Ki-67 is essentially sensitive with a specific pattern of immune-expression, in early well differentiated hepatocellular carcinomas as compared to benign mimickers. Most HCC cells showed a strong diffuse CD 34 staining pattern in contrast to the negative expression pattern in surrounding non-tumor liver cells (hepatitis associated hepatocytes, cirrhotic hepatocytes, or normal hepatocytes). Hence, CD 34, SMA & Ki-67 can be significant helpful tool for reaching upto definite diagnosis of early well differentiated hepatocellular carcinomas when disease is yet at early stage & in a setup where specific markers of malignant hepatic tumors are not available available.

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