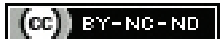# Use of Immunohistochemical Markers in Differentiation of Hepatocellular Carcinoma from Intrahepatic Cholangiocarcinoma and Metastatic Adenocarcinomatous Deposits in the Liver from Colon: A Cross-sectional Study

C ARUN PRABHAKARAN[1], V RAMYA[2], K SHANMUGAM[3], S BRIHADISVARAR[4], K CHANDRAMOULEESWARI[5]

ABSTRACT

Introduction: Hepatocellular Carcinoma (HCC) is a major worldwide health problem due to its high incidence and mortality rates. The liver often becomes a site for metastasis from various primary locations, benefiting from its abundant blood supply. Distinguishing liver metastatic tumours from HCC can pose a diagnostic challenge, significantly impacting prognosis and treatment decisions.

Aim: To differentiate between HCC, Intrahepatic Cholangiocarcinoma (ICC), and metastatic colonic adenocarcinoma in the liver using Hep par-1, Cytokeratin (CK) 7, CK19, and CK20 as immunohistochemical markers. The manual Tissue Microarray (TMA) technique was employed for present study.

Materials and Methods: The present cross-sectional study was conducted in Department of Pathology at Government Stanley Medical College, Chennai, Tamil Nadu, India, spanning a three-year duration from July 2012 to June 2015. A total of 60 cases diagnosed histologically with HCC, ICC, and metastatic colonic adenocarcinoma in the liver were included. The manual TMA technique was used to create recipient blocks, and immunohistochemistry was performed to assess the expression of Hep par-1, CK7, CK19 and CK20. The sensitivity, specificity, Positive Predictive Value (PPV), and Negative Predictive Values (NPV) of these markers in HCC, ICC, and metastatic colonic adenocarcinoma in the liver were analysed and tabulated using statistical software Statistical Package for Social Sciences (SPSS) version 16.0.

Results: The study included a total of 60 cases, with 40 (66.7%) males and 20 (33.3%) females, ranging in age from 27 to 73 years with a mean age of 51.3 years. Among the cases, there were 30 (50%) cases of HCC, 14 (23%) cases of ICC, and 16 (27%) cases of metastatic colonic adenocarcinoma in the liver. The sensitivity, specificity, and PPV of Hep par-1 in distinguishing HCC from ICC and metastatic deposits were 80%, 100%, and 100%, respectively. The NPV of Hep par-1 in distinguishing HCC from ICC and metastatic deposits was 70% and 72%, respectively. The sensitivity, specificity, PPV, and NPV of CK7 in distinguishing ICC from HCC were 3.3%, 50%, 6.3%, and 34.1%, respectively. The sensitivity, specificity, PPV, and NPV of CK19 in distinguishing HCC from ICC and metastatic deposits were 0%, 50%, 0%, and 33.3%. The sensitivity, specificity, PPV, and NPV of CK20 in distinguishing HCC from ICC and metastatic deposits were 0%, 53.3%, 0%, and 34.8%, respectively.

Conclusion: In conclusion, it was found that a panel of markers including Hep par-1, CK7, CK19 and CK20 can differentiate between HCC, ICC, and metastatic colonic adenocarcinoma in the liver. This differentiation is crucial for determining the appropriate treatment for patients by understanding the exact behaviour of the tumour.

INTRODUCTION

The global incidence of HCC is on the rise, and projections indicate that by 2025, over one million individuals will be affected [1]. This cancer is also renowned for its histomorphologic diversity, making the differentiation of HCC from ICC and metastatic adenocarcinomatous lesions originating from various organs a challenging task in histopathology. Several factors contribute to this challenge: a) the wide array of neoplasms that can originate from hepatocytes; b) the liver's susceptibility to metastases, which can closely resemble different variants of primary HCC; and c) the limitations of serum Alpha Fetoprotein (AFP) in effectively distinguishing poorly differentiated HCC from ICC and metastatic carcinomatous deposits from other primary sites.

Several Immunohistochemical (IHC) markers have been employed in prior studies. Among these, Hep par-1 has consistently emerged as the most sensitive and specific IHC marker for HCC [Table/Fig-1,2]. Additionally, CK7 and CK19 have been utilised to identify ICC [Table/Fig-3], while CK20 is commonly employed to detect
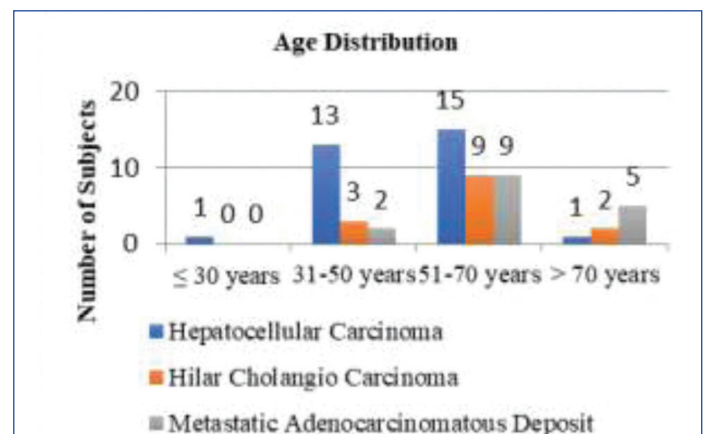

[Table/Fig-1]: Age-wise distribution of cases.

metastatic adenocarcinomatous deposits in the liver originating from the colorectal region [2-5].

Gender distribution	Hepatocellular Carcinoma (HCC)	Percentage (%)	Hilar cholangiocarcinoma	Percentage (%)	Metastatic adenocarcinomatous deposit	Percentage (%)
Male	18	60.00	12	85.71	10	62.50
Female	12	40.00	2	14.29	6	37.50
Total	30	100	14	100	16	100

[Table/Fig-2]: Gender-wise distribution of cases.
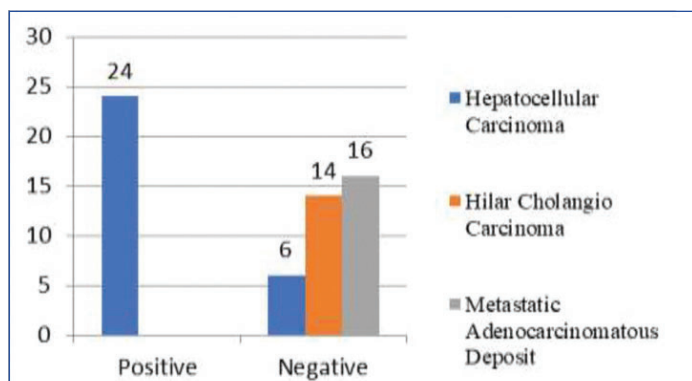
[Table/Fig-3]: Distribution of Hep par1 reactivity in all three malignancies.

The concept of TMAs originated in 1857, credited to Dr. Hector Battifora's innovative 'sausage' blocks. These blocks allowed multiple tissues from various organs to be combined in the same block, facilitating the study of antigen/protein reactivity [6]. Today, there are commercially available instruments, such as those from Beecher, capable of creating microarray blocks that can hold up to 1,000 cores. The next significant advancement in TMA development was described by Wan et al., who utilised a 16-gauge needle to manually extract cores from tissue blocks and arrange them in a recognisable pattern within a multi-tissue straw [7]. This technique enables researchers to perform all the same histological analyses that are conventionally done using formalin-fixed paraffin-embedded tissue sections.

The present study aimed to differentiate HCC from ICC and metastatic colonic adenocarcinoma in the liver using IHC markers with a manual TMA technique, thereby reducing the amount of antibodies used and preserving the donor tissue block.

MATERIALS AND METHODS

The present cross-sectional study was conducted at Government Stanley Medical College for a period of three years, from July 2012 to June 2015. A total of 60 specimens were taken for present study.

All procedures performed in the current study were approved by the Institutional Ethical Committee ECR/131/Inst/TN/2013/RR-22. Informed consent was obtained from all individual participants included in the study.

Inclusion and Exclusion criteria: The criteria used for the selection of cases were previously histologically diagnosed cases of HCC, ICC, and metastatic secondaries from the colorectal region in the liver (Trucut biopsies and resection specimens). Exclusion criteria were benign tumours, mesenchymal tumours, and paediatric tumours of the liver.

Study Procedure

For all the 60 cases, age and sex were recorded. The slides were screened, and the areas of interest were marked with a marker pen, which were again marked in the donor block. Using a 14-gauge jamshidi bone marrow aspiration needle, the recipient blocks were cored out, and for apparent identification, the design of the cores should be asymmetrical. Using a 16-gauge needle, the test cores were taken out from the area of interest in the donor block and were placed in the recipient block as per the microarray design [Table/Fig-4]. Each recipient block contains both controls for each IHC marker and test tissue cores. Sections were taken and subjected to IHC by Horse Radish Peroxidase (HRP) polymer technique.

Antigen retrieval was performed using the Tris Ethylenediamine Tetraacetic acid (Tris EDTA) buffer by the pressure cooker method. The endogeneous peroxide was blocked using peroxidase block for five minutes. Slides were then washed in two changes of Tris EDTA buffer for five minutes each. The primary antibody was then used to incubate the slides for 60 minutes. Then the slides were washed in two changes of Tris EDTA buffer for five minutes each. Incubation was done with the target binder for 15 minutes. Then the slides were washed in two changes of Tris EDTA buffer for five minutes each. Incubation with HRP-labeled polymer for 15 minutes was performed. Then the slides were washed in two changes of Tris EDTA buffer for five minutes each. They were incubated with 3-3'-Diaminobenzidine (DAB) substrate chromogen, which results in brown-colored staining. The slides were then rinsed in water, counterstained with Hematoxylin, washed in water, dehydrated, cleared, and mounted.

Evaluation of immunostaining: Hep Par 1 (clone OCH1E5.2.10): In present study, a mouse monoclonal antibody that shows granular cytoplasmic positivity in immunostaining was used. The staining was observed in normal and neoplastic hepatocytes. The intensity of staining was scored [8] as follows: 0=no reactivity; 1=less than 5% of cancer cells positive; 2=5-25% positive; 3=25-50% positive; 4=50-75% positive; 5=75-90% positive; and 6=more than 90% of tumour cells positive.

CK7, 19, 20: In present study, rabbit monoclonal antibodies that show brown cytoplasmic and membranous staining were used. Positive immunoreactivity of CK7 and 20 was defined as more than 20% of cells with cytoplasmic and membranous staining [9]. CK19 positivity was taken as 20%.

STATISTICAL ANALYSIS

The expression of Hep Par 1, CK7, 19, and 20 was analysed, and the results were tabulated. The sensitivity, specificity, PPV, and NPV of the markers in HCC, ICC, and metastatic colonic adenocarcinoma in the liver were calculated using Statistical software Statistical Package for Social Sciences version 16.0.

RESULTS

In present study, 30 (50%) of the samples were cases of HCC, 14 (23%) were intrahepatic cholangiocarcinoma, and 16 (27%) were metastatic adenocarcinomatous deposits in the liver from the colon. The study included an age range of 27 to 80 years. Out of the 30 cases of HCC, 15 (50%) were in the age group of 51 to 70 years, 13 (43.33%) were in the age group of 31 to 50 years, and one (3.33%) case each were below 30 years and above 70 years [Table/Fig-1]. Among the 14 cases of ICC, 9 (64.29%) were in the age group of 51 to 70 years, 3 (21.43%) were in the age group of 31 to 50 years, and 2 (14.29%) cases were above 70 years. Regarding the 16 cases of metastatic deposits from the colon, 9 (56.25%) were in the age group of 51 to 70 years, 2 (12.5%) were in the age group of 31 to 50 years, and 5 (31.25%) were above 70 years. Among the total 60 cases of HCC, cholangiocarcinoma, and metastatic adenocarcinomatous deposits, 33 (55%) cases were between 51 to 70 years. The incidence of metastatic adenocarcinomatous deposits in the liver (five cases) was higher compared to HCC (one case) and ICC (two cases) in individuals above 70 years. Only one case (3.33%) of HCC was observed below 30 years. The incidence of HCC, cholangiocarcinoma, and metastatic adenocarcinomatous deposits in the liver was notably higher among males (40 cases)

compared to females (20 cases). Among the 40 males, 18 (45%) were diagnosed with HCC, 12 (30%) with ICC, and 10 (25%) with metastatic deposits. Conversely, among the 20 females, 12 (60%) were affected by HCC, 2 (10%) by ICC, and 6 (30%) by metastatic deposits [Table/Fig-2].

Out of the 30 cases of HCC, 11 (36.7%) were categorised as well-differentiated, 3 (10%) as moderately differentiated, and 16 (53.3%) as poorly differentiated HCCs. Remarkably, within these 30 cases, six cases were negative for Hep par-1, and all six belonged to the group of poorly differentiated HCC. Within the well-differentiated group, out of 11 cases, six demonstrated score 6 positivity [Table/Fig-3-5]. Four exhibited score 5 positivity, and one displayed score 4 positivity. In the moderately differentiated group comprising three cases, one showed score 6 positivity, one had score 5 positivity, and another displayed score 3 positivity. In the poorly differentiated group, six cases were negative, seven exhibited score 2 positivity, and three cases displayed score 1 positivity. Notably, Hep par-1 was consistently negative in all 100% cases of intrahepatic cholangiocarcinoma (14 cases) and metastatic adenocarcinomatous deposits in the liver (16 cases) originating from the colon. CK7 exhibited positive expression in 14 (100%) of cholangiocarcinoma [Table/Fig-6], 1 (3.33%) of HCC, and 1 (6.25%) in metastatic adenocarcinomatous deposits in the liver from the colon. CK7 was negative in 29 (96.67%) of HCC and 15 (93.75%) of metastatic adenocarcinomatous deposits in the liver from the colon.


[Table/Fig-4]: Manual TMA block.
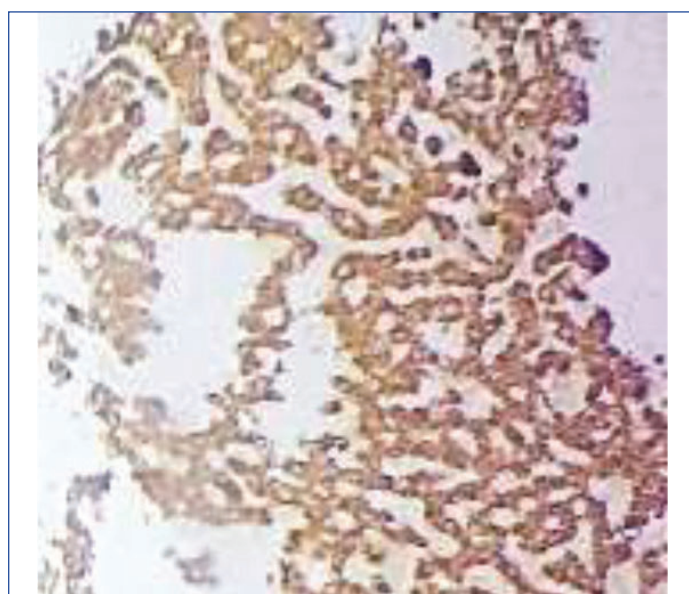
[Table/Fig-5]: Hep par1 positivity in well-differentiated HCC (6+positivity) (IHC, 10X).
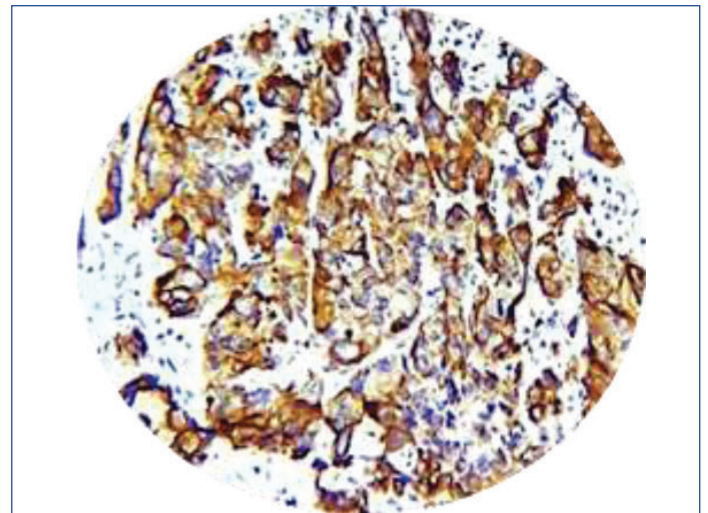
[Table/Fig-6]: CK7 positivity in cholangicarcinoma (IHC 40X).

CK19 was positive in 5 (35.71%) of cholangiocarcinoma [Table/Fig-7] and 10 (62.50%) in metastatic adenocarcinomatous deposits in the liver from the colon. CK19 was negative in 30 (100%) of HCC, 9 (64.29%) of cholangiocarcinoma, and 6 (37.50%) of metastatic adenocarcinomatous deposits in the liver from the colon.
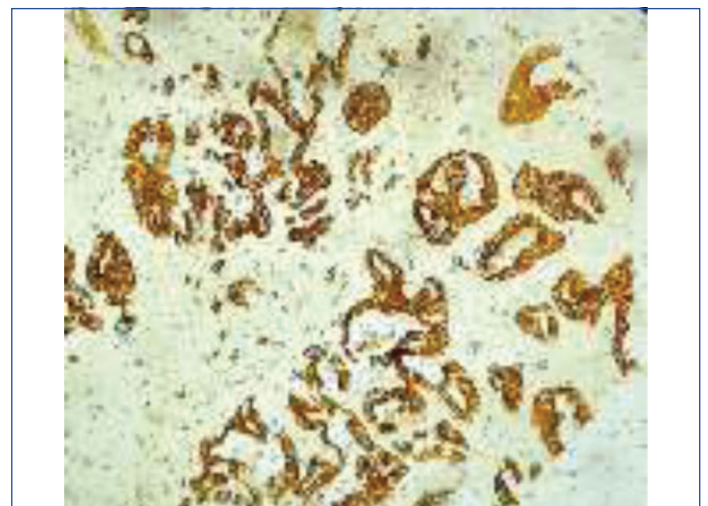
[Table/Fig-7]: CK19 positivity in cholangicarcinoma (IHC 10X).

CK20 was positive only in 14 (87.50%) of metastatic adenocarcinomatous deposits [Table/Fig-8] in the liver from the colon. CK20 was negative in 30 (100%) of HCC and 14 (100%) of cholangiocarcinoma, with 2 (12.50%) of metastatic adenocarcinomatous deposits in the liver from the colon [Table/Fig-9].
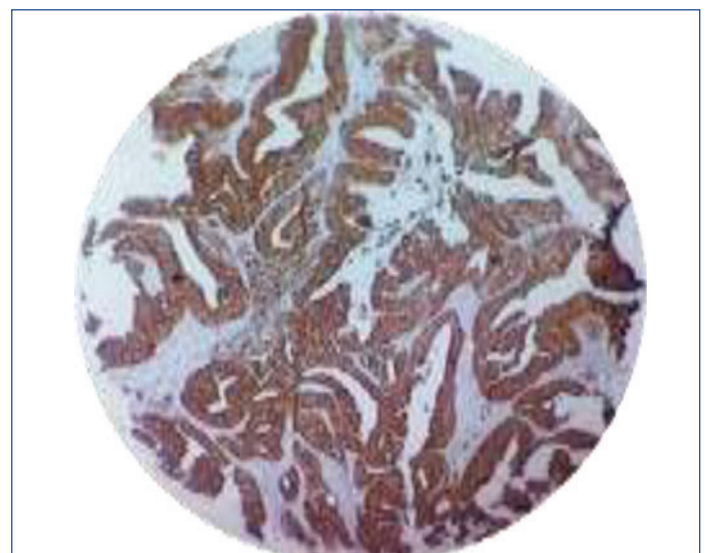
[Table/Fig-8]: CK20 positivity in metastatic adenocarcinomatous deposit (IHC,10X).

Parameters	HCC (30)		Intrahepatic cholangiocarcinoma (ICC) [14]		Metastatic adenocarcinomatous deposit [16]	
	n (cases)	%	n (cases)	%	n (cases)	%
Hep par-1 positive	24	80%	0	-	0	-
Hep par-1 negative	06	20%	14	100%	16	100%
CK7 positive	1	3.33%	14	100%	1	6.25%
CK7 negative	29	96.67%	0	-	15	93.75%
CK19 positive	0	-	5	35.71%	10	62.5%
CK19 negative	30	100%	9	64.29%	6	37.5%
CK20 positive	0	-	0	-	14	87.5%
CK20 negative	30	100%	14	100%	2	12.5%

[Table/Fig-9]: Expression of Hep par-1, CK7, CK19 and CK20 in HCC, intrahepatic cholangiocarcinoma and metastatic adenocarcinomatous deposit.

The diagnostic parameters for Hep Par1 in distinguishing HCC from ICC and metastatic deposits are as follows: sensitivity stands at 80%, specificity at 100%, and the PPV at 100%. The NPV for Hep Par1 in separating HCC from ICC and metastatic deposits is 70% and 72%, respectively. For CK7 in distinguishing ICC from HCC, the diagnostic parameters are as follows: sensitivity is 3.3%, specificity is 50%, the PPV is 6.3%, and the NPV is 34.1%. In the case of CK19 for distinguishing HCC from ICC and metastatic deposits, the diagnostic parameters are as follows: sensitivity is 0%, specificity is 50%, the PPV is 0%, and the NPV is 33.3%. Finally, for CK20 in distinguishing HCC from ICC and metastatic deposits, the diagnostic parameters are as follows: sensitivity is 0%, specificity is 53.3%, the PPV is 0%, and the NPV is 34.8% [Table/Fig-10]. Some peculiar histopathological features of HCC and ICC have been shown in [Table/Fig-11-13].

| HPE	Sensitivity	Specificity	PPV	NPV
Hep Par1 in HCC vs Meta	80	100	100	72
Hep Par1 in HCC vs ICC	80	100	100	70
CK7 in ICC Vs HCC	3.3	50	6.3	34.1
CK19 in HCC Vs ICC and Mets	0	50	0	33.3
CK20 in HCC Vs ICC and Mets	0	53.3	0	34.8

[Table/Fig-10]: Sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Values (NPV) of Hep par-1, CK7, CK19 and CK20.
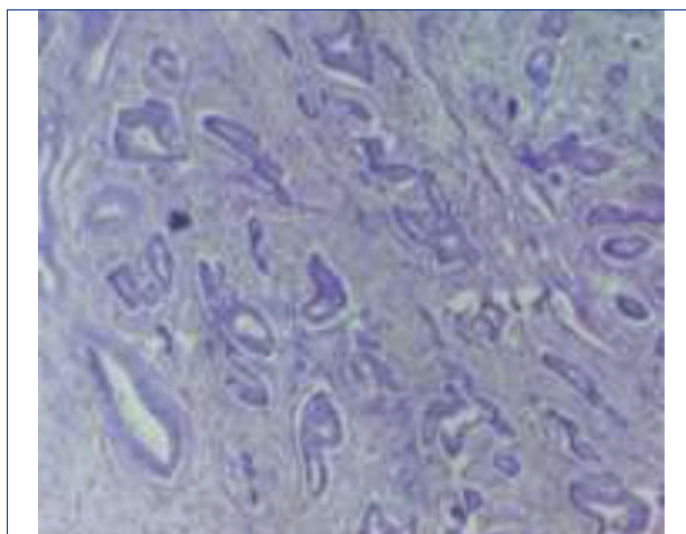vs: Versus; Meta: Metastatic adenocarcinoma
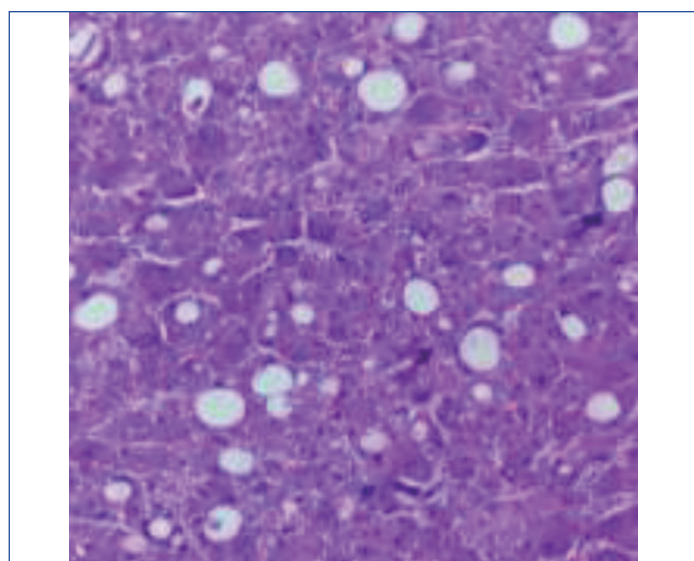
[Table/Fig-11]: Well-differentiated HCC, HCC {Hematoxylin and Eosin (H&E) 10X}.

DISCUSSION

Hepatocellular Carcinoma (HCC) is the predominant primary malignant liver neoplasm, accounting for approximately 80% of cases. Cirrhosis
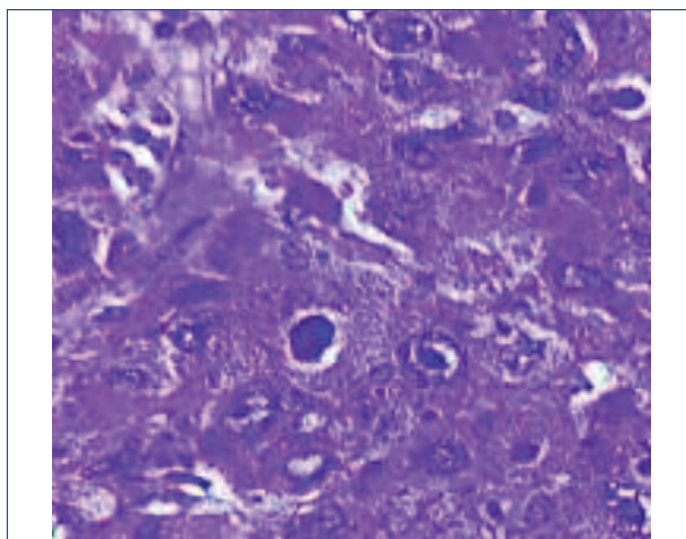
[Table/Fig-12]: Poorly differentiated HCC {Hematoxylin and Eosin (H&E) (IHC 40X)}.

[Table/Fig-13]: Intrahepatic cholangiocarcinoma, {Hematoxylin and Eosin (H&E), 10X}.

forms the backdrop for more than 80% of HCC cases, typically emerging between the third and sixth decades of life. The primary culprits behind HCC are chronic viral hepatitis, excessive alcohol consumption, and non alcoholic fatty liver disease. In present study, the age range of patients with HCC spanned from 27 to 73 years, with a mean age of 51.3 years [Table/Fig-1]. A 2014 study by Acharya SK et al., on HCC epidemiology in India found a presentation age range of 40 to 70 years [10]. For ICC, patients ranged in age from 35 to 80 years, with a mean age of 57.43 years. Yusoff AR et al., conducted a survival analysis of cholangiocarcinoma and unveiled a mean diagnosis age of 61 years [11]. In cases of metastatic adenocarcinomatous deposits in the liver originating from the colorectal region, patient ages spanned from 32 to 89 years, with a mean age of 63.19 years. Manfredi S et al., conducted a study on liver colorectal cancer metastases and indicated a peak incidence between ages 65 and 74 years [12].

Across all three liver malignancies, incidence rates were consistently higher in males compared to females [Table/Fig-2]. Acharya SK's 2014 study on HCC epidemiology in India reported a male-to-female ratio of 4:1 [10]. El-Serag's HB global study on HCC epidemiology suggested that men are at an increased risk, partly due to a higher incidence of viral hepatitis and alcoholic cirrhosis [13]. Wu EM et al., emphasised the male predominance in HCC, with an incidence two to four times higher in males than females [14]. Manfredi S et al., conducted a study on liver colorectal cancer metastases and reported a sex ratio of 2:1 [12].

The expression of Hep Par1 varied in the well- and moderately-differentiated HCC group, ranging from 1 to 6, whereas in the poorly differentiated group, all cases showed negativity [Table/Fig-3].

Studies by Mivervini MI et al., and Chu PG et al., have both noted that poorly differentiated HCCs are more likely to lack Hep Par1 expression compared to their better-differentiated counterparts. This suggests that poorly differentiated HCCs lose their reactivity to Hep Par1 [15,16]. In present study, Hep Par1 demonstrated a sensitivity of 80% and a specificity of 100%. The PPV and NPV were 100% and 72%, respectively, in the differentiation of HCC from ICC and colorectal region secondaries [Table/Fig-7]. Hanif R and Mansoor S evaluated Hep par-1 in distinguishing HCC from metastatic carcinoma and found a sensitivity of 83.3%, specificity of 96.6%, and positive and NPV and accuracy of 96.5%, 85.2%, and 90%, respectively [8].

In present study, authors compared the expression of CK7, CK19, and CK20 with the findings of Shimonoshi et al. They concluded that CK7, CK19, and CK20 are valuable markers for distinguishing ICC from metastatic adenocarcinomas in the liver originating from colorectal regions. In their study, CK7 was positive in 97% of cases, CK19 in 92% of ICC cases, and CK20 in 81% of metastatic adenocarcinomas in the liver from colorectal regions. Notably, the expression of CK19 in ICC decreased with tumour differentiation [17]. These results indicate that the reactivity of bile duct-type CK is reduced or lost in a small number of cholangiocarcinomas during neoplastic transformation or tumour development [18]. The present study employed manually made TMAs, offering several advantages, including cost-effectiveness, time efficiency, reduced consumption of IHC markers, and the ability to process a large number of cases rapidly. Shebl AM et al., also used a 1 mm core size in their study, created using a mechanical pencil tip, which offers ease in sampling from donor blocks and avoids splitting artifacts during sectioning [19]. In present study, authors utilised the same 1.0 mm core size, yielding consistent results.

Limitation(s)

Multiple cores must be taken in the case of heterogeneous tumours to avoid false negative results.

CONCLUSION(S)

The present study findings affirm that employing a panel of IHC markers, such as Hep par-1, CK7, CK19, and CK20, plays a pivotal role in effectively distinguishing among HCC, ICC, and metastatic adenocarcinomatous deposits within the liver originating from the colorectal region. This approach not only serves as a valuable tool for confirming histopathological diagnoses but also aids in the strategic planning of treatment protocols. The utilisation of a straightforward and economical manual TMA method demonstrates cost-effectiveness and resource optimisation, particularly within the confines of a tertiary care centre. Furthermore, it is worth noting that this methodology allows for the preservation of the original paraffin-embedded donor tissues, thereby facilitating potential future research endeavors.

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• Was informed consent obtained from the subjects involved in the study?   Yes
• For any images presented appropriate consent has been obtained from the subjects.   NA