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ROLE OF IMMUNOHISTOCHEMISTRY IN DIFFERENTIATING PRIMARY HEPATOCELLULAR CARCINOMA USING MANUAL TISSUE MICROARRAY TECHNIQUE AND ITS ADVANTAGE

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Abstract

Hepatocellular carcinoma (HCC) is a leading cause of liver cancer worldwide, posing diagnostic and therapeutic challenges, particularly in distinguishing it from metastatic liver tumors. This study evaluates the role of tissue microarray (TMA) technology and immunohistochemical markers, specifically Hep Par-1 and alpha-fetoprotein (AFP), in the differentiation and characterization of HCC.Thirty cases of HCC were analyzed, including 11 well-differentiated, 3 moderately differentiated, and 16 poorly differentiated tumors. TMA construction was performed, and immunohistochemistry was conducted to assess Hep Par-1 and AFP expression. Hep Par-1 demonstrated granular cytoplasmic positivity, with a sensitivity of 80% and specificity of 100%. Reactivity decreased with poorer differentiation, as all six negative cases were poorly differentiated. AFP positivity was observed in 45% of cases, with no significant correlation to differentiation level.Age distribution revealed a peak incidence between 31 and 70 years, with a male predominance (60%). Comparative analyses with previous studies confirmed consistent epidemiological trends and highlighted the diagnostic challenges in poorly differentiated HCC.The findings reaffirm Hep Par-1 as a reliable marker for HCC diagnosis, particularly in well-differentiated cases. AFP was less reliable due to variability in expression. The use of TMAs enhanced efficiency and reduced costs, supporting their utility in histopathological research. These results underscore the importance of combining morphological, immunohistochemical, and clinical data for accurate HCC diagnosis and management.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most prevalent type of primary liver cancer, posing significant global health challenges due to its frequent occurrence and high mortality rates. Liver metastases from various primary sites are common due to the liver's rich blood supply, making metastatic cancer the leading malignant tumor affecting the liver in adults. Distinguishing between liver metastases and HCC can be diagnostically challenging, impacting prognosis and treatment decisions.

Morphological analysis is initially employed to establish a differential diagnosis, with further refinement through histochemical and immunohistochemical studies when needed. Immunohistochemistry plays a crucial role when morphological features and identification of secretory substances are inconclusive. Among the immunohistochemical markers, Hep Par-1 has been identified as highly sensitive and specific for HCC, while cytokeratins 7, 19, and 20 are typically absent in hepatocellular carcinomas but present in various adenocarcinomas, including cholangiocarcinomas.

Recent advances in pathology include the introduction of tissue microarrays, which offer a high-throughput method for analyzing multiple specimens simultaneously. These microarrays consist of numerous small representative tissue samples from different cases on a single slide, allowing for efficient analysis using various techniques such as histochemical stains, immunostaining, in situ hybridization, and tissue micro-dissection. This technology has proven to be highly efficient, reducing duration and costs, particularly with expensive reagents.

METHODOLOGY

For all the 23 cases, details of age, sex and other relevant clinical data were recorded.

Microscopically diagnosed cases of primary hepatocellular carcinoma (well differentiated, moderately differentiated and poorly differentiated) in liver from colorectal region in liver biopsies specimens were selected randomly irrespective of age and sex.

Method of data collection:

All Liver Biopsies and Resection specimens received in the Department of Histopathology, LN medical College were included in the study. 10% Neutral buffered formalin was used as fixative. Appropriate tissues were sampled and the tissues were processed in various grades of alcohol and xylol using automated histokinette (Automated Tissue processor). Paraffin blocks were prepared and sections of 5 micron thickness were cut and stained using H& E technique and examined under the microscope for histopathological diagnosis and were taken up for the study(Using inclusion and Exclusion criteria). All the selected cases were included in the construction of tissue microarray.

Construction of Tissue Microarray

H & E slides were screened and the areas of interest were marked with marker pen which were again marked in the donor block. The recipient blocks were made by coring the paraffin block using 14 gauge bone marrow aspiration needles and the arrangement of the cores should be asymmetrical.

The cores from the donor block were taken from the areas of interest using 16 gauge needle. The diameter of the core was 1mm. These cores were placed in the recipient blocks as per our TMA design. This was placed in incubator at 37^{o}C for 24 hrs and kept in freezer compartment of refrigerator before sectioning. Each recipient block contains both controls and test tissue cores. The controls for Hep par1, AFP are normal liver tissue.

Sections were taken for IHC at 4 micron thickness in chrome alum coated slides using semi-automated microtome with disposable blades. The slides were kept in incubator at 70oC for an hour.

Only 23 cases were studied in this study in view of less availability of carcinomas of liver in 2012- 2014.

Sections were subjected to antigen retrieval technique by pressure cooker method using TRIS EDTA (Ph 9) buffer solution and then treated by HRP (horse radish peroxidase) polymer technique.

HRP polymer Technique:

1. The sections were deparaffinised in xylene or xylene substitutes
2. Rehydrated through graded alcohols
3. The slides were then washed in running tap water
4. The antigen retrieval was performed using the appropriate buffer(TRIS EDTA) by pressure cooker method.
5. The endogeneous peroxide was blocked using peroxidase block for 5 mins
6. Slides were then washed in 2 changes of TBS buffer for 5 mins each.
7. Primary antibody was then used to incubate the slides for 60 mins.
8. Then the slides were washed in 2 changes of TBS buffer for 5 mins each.
9. Then incubation was done with target binder for 15 mins
10. Then the slides were washed in 2 changes of TBS buffer for 5 mins each.
11. Then incubation with HRP labeled polymer for 15 mins
12. Then the slides were washed in 2 changes of TBS buffer for 5 mins each.
13. Then incubated with 3-3’diamino benzidine(DAB) substrate chromogen working solution which results in brown colored staining.
14. The slides were then rinsed in water, counterstained in hematoxylin, washed in water, dehydrated , cleared and mounted to be examined.

EVALUATION OF IMMUNOSTAINING

Hep Par 1 – In this study we have used mouse monoclonal antibody which shows granular cytoplasmic positivity in immunostaining. The staining was observed in normal and neoplastic hepatocytes. The intensity of staining was scored [2] as-

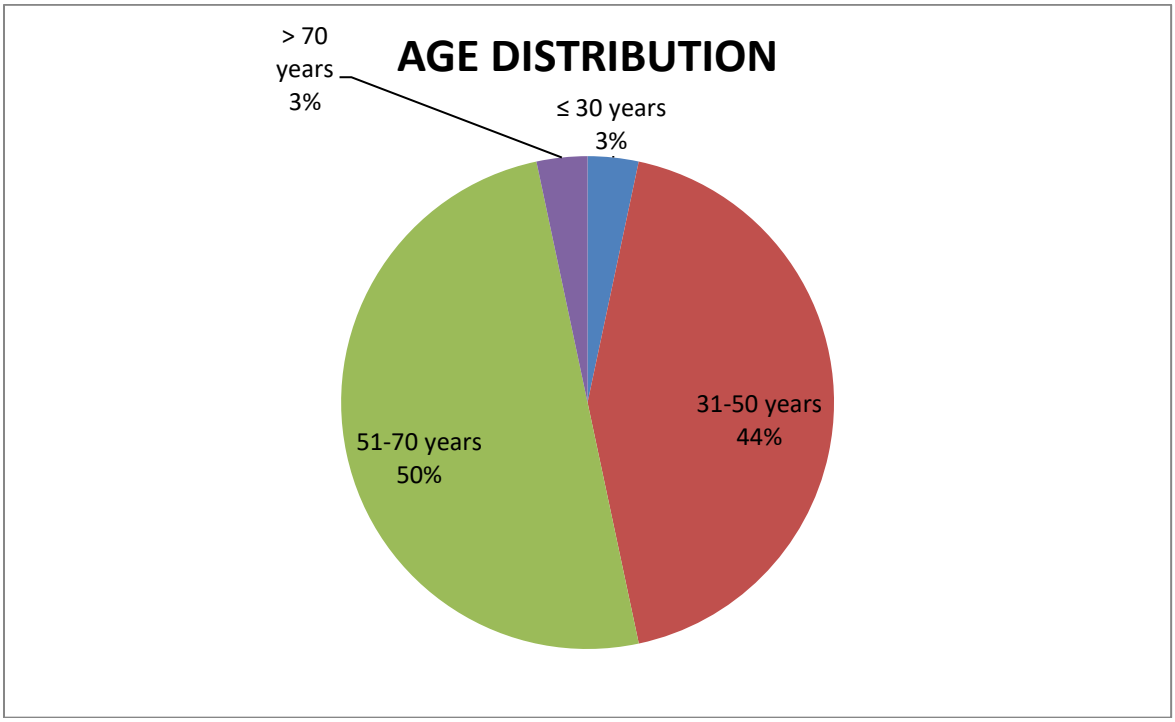
0 = no reactivity;
1 = less than 5% of cancer cells positive;
2 = 5 - 25% positive;
3 = 25 - 50% positive,
4 = 50 - 75% positive;
5 = 75 - 90% positive; and
6 > 90% of tumour cells positive.

RESULTS

In this present study we have taken 30 hepatocellular cases of liver biopsies and resection specimens. Out of the 30 samples studied majority of case Hepatocellular carcinoma in liver were between 51 to 70 years and a few less than 30 years. The study had a range of age group from 27 to 80years. Between 31 to 50 years, the incidence of hepatocellular carcinoma was high compared the other neoplasms in liver. More than 70 years, number of hepatocellular carcinoma cases also decreased. While there is only one case of Hepatocellular carcinoma below 30 years.

Age Distribution	HepatocellularCarcinoma	%
≤ 30 years	1	3.33
31-50 years	13	43.33
51-70 years	15	50.00
> 70 years	1	3.33
Total	30	100

TABLE 01: AGE WISE DISTRIBUTION OF CASES.

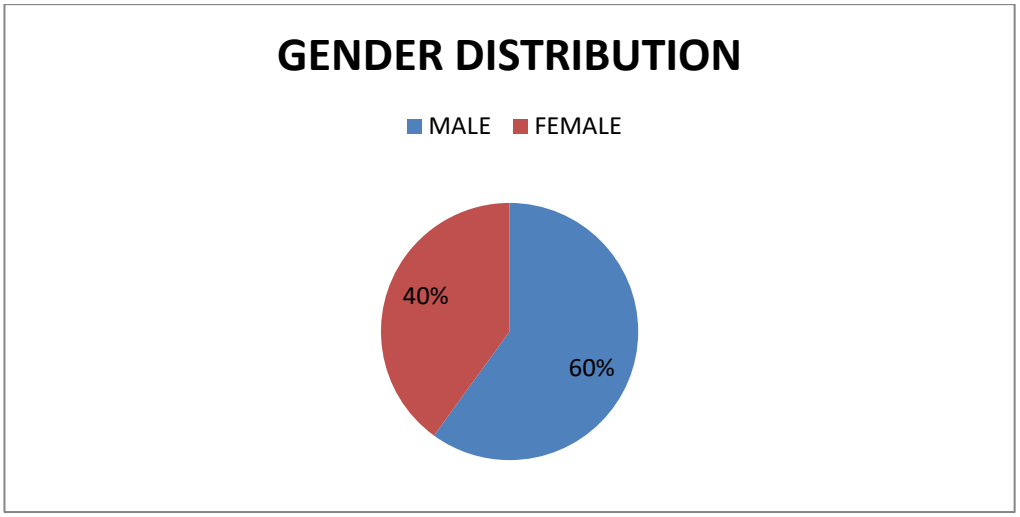


GRAPH NO. 01: AGE WISE DISTRIBUTION SHOW ON GRAPH

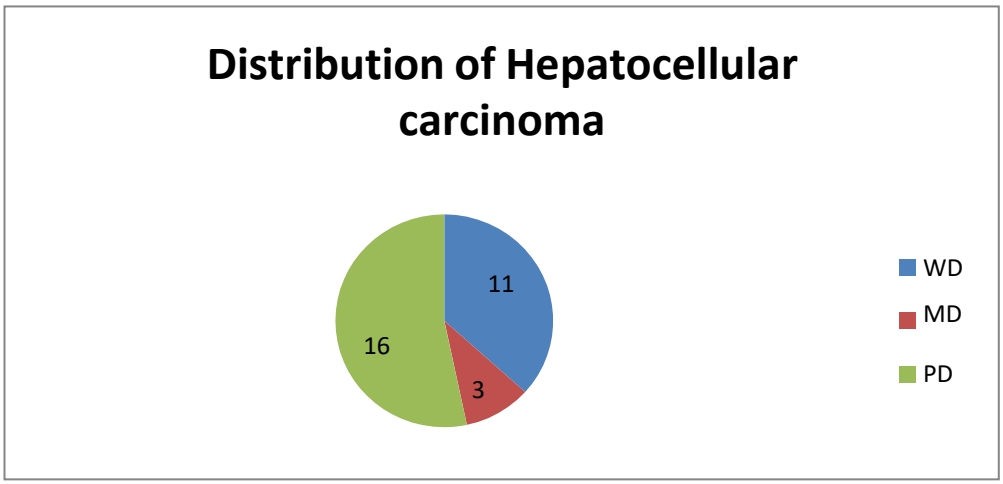
The incidence of Hepatocellular carcinoma in liver was common in males compared to that of females. The incidence of Hepatocellular carcinoma was high in both gender compared to the other malignancies.

Gender Distribution	HepatocellularCarcinoma	%
Male	18	60.00
Female	12	40.00
Total	30	100

TABLE NO.02: GENDER WISE DISTRIBUTION OF CASES



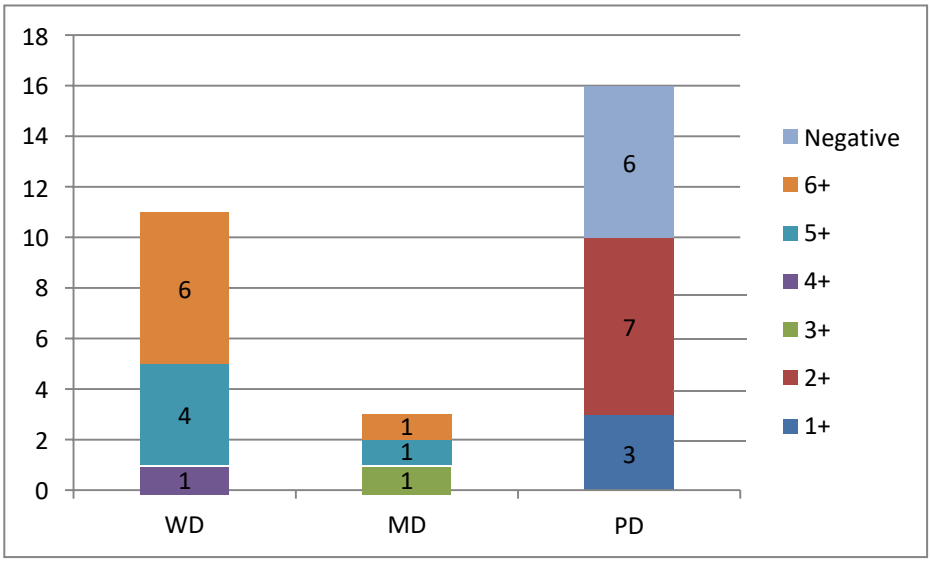
GRAPH NO.02: GENDER WISE DISTRIBUTION SHOW ON GRAPH



GRAPH NO.03: CASES DIFFERENTIATE BY GRADE WISE

DISTRIBUTION OF HEPATOCELLULAR CARCINOMA ACCORDING TO DIFFERENTIATION

In this study, out of 30 cases of hepatocellular carcinoma, 11 were well differentiated, 3 were moderately differentiated and 16 were poorly differentiated hepatocellular carcinomas. (Graph No. 03)

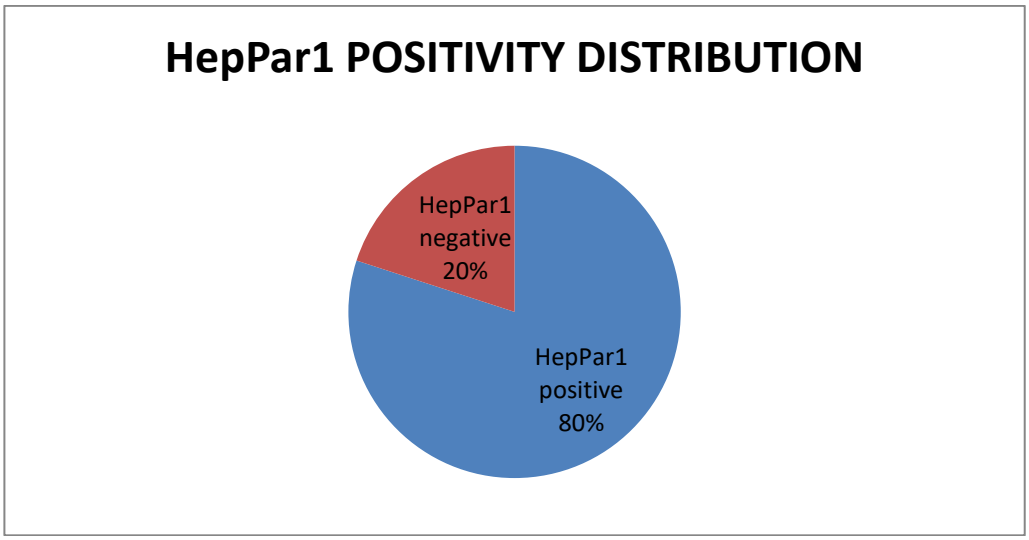


GRAPH NO.04: DISTRIBUTION OF HEPATOCELLULAR CARCINOMA ACCORDING TO Hep Par 1

REACTIVITY WITH RESPECT TO DIFFERENTIATION OF TUMOURS

The above graph(Graph 04) depicts the scoring of Hep par1 staining in different grades of Hepatocellular carcinoma. Out of 30 cases, 6 cases were negative and all 6 belongs to poorly differentiated hepatocellular carcinoma. In well differentiated group out of 11 cases, 6 were showing 6+ positivity,4 were showing 5+ positivity and 1 case showed 4+

positivity. In moderately differentiated group out of 3 cases, 1 case was 6+ positivity, 1 was 5 + positivity and one more showed 3+ positivity. In poorly differentiated group, 6 cases were negative, 7 cases showed 2+ positivity and 3 cases showed 1+ positivity.



GRAPH NO.05: DATA SHOWS HepPar 1 POSITIVITY ON GRAPH

In this study, out of 30 cases of Hepatocellular carcinoma, 24 cases were positive for Hep par1 and 6 cases were negative for Hep par1.

HepPar1Positivity	HepatocellularCarcinoma	%
HepPar1 Positive	24	80
HepPar1 Negative	6	20
Total	30	100

TABLE NO.03: HepPar1 POSITIVTY ON CASES

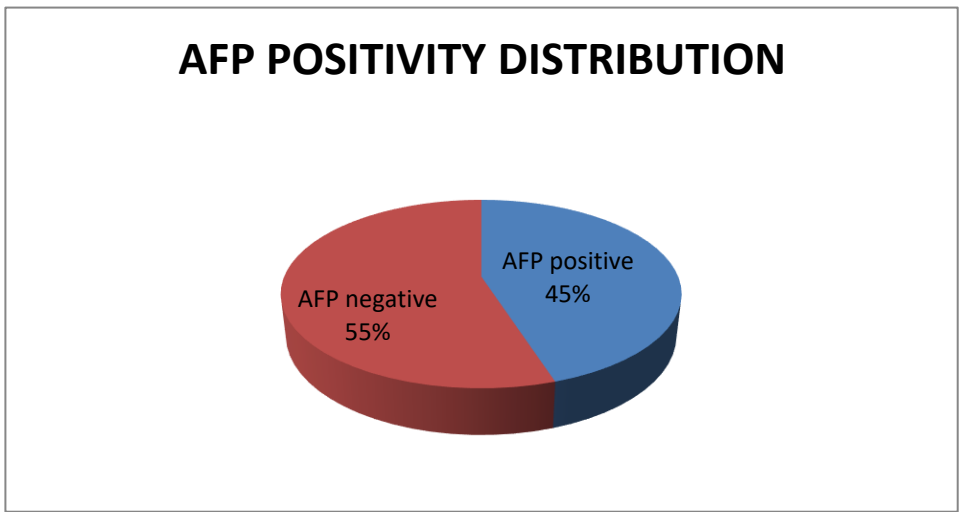
AFP REACTIVITY:

In this study, out of 30 cases of Hepatocellular carcinoma, 14 cases were positive for AFP and 16 cases were negative for AFP.

AFPPositivity	HepatocellularCarcinoma	%
AFP Positive	14	45
AFP Negative	16	55
Total	30	100

TABLE NO.04: AFP POSITIVITY ON CASES

Out of the 30 samples studied majority of case Hepatocellular carcinoma in liver were between 30 to 50 years and a few less than 70 years. The study had a range of age group from 27 to 80years. Between 31 to 50 years, the incidence of hepatocellular carcinoma was high compared the other neoplasms in liver. More than 70 years, number of hepatocellular carcinoma cases also decrease



GRAPH NO.06: AFP POSITIVITY SHOW ON CASES

DISCUSSION:

This cross sectional study was carried out in the Department of Pathology, Govt. Stanley Medical College. Total number of cases studied was 60, which included 30 cases of Hepatocellular carcinoma (11-welldifferentiated,3-moderatelydifferentiated,16-poorly differentiated)

1. AGE OF THE PATIENT

I our study the age of the patient with hepatocellular carcinoma range from 27 to 73 years with mean age of 51.3 years

In 2012, Hashem B. El-Serag, studied the epidemiology of HCC worldwide and concluded that in low risk population the incidence is >70 years and in high risk groups it is between 50 to 60 years

In 2014 Subrat K. Acharya studied the epidemiology of Hepatocellular carcinoma in India and concluded that the age ranges between 40 to 70 years at the time of presentation.

Sean F. Altekruse studied the geographic variation of Hepatocellular Carcinoma in the United States and stated that incidence was high in age group of more than 70 years.

2. GENDER

In our study in Hepatocellular Carcinoma of the liver, the incidence is high in males compared to that of females. 60% in Hepatocellular carcinoma male and 40% in female.

In 2014 Subrat K. Acharya studied the epidemiology of Hepatocellular carcinoma in India and concluded that the male to female ratio is 4:1 in India.

In 2012, Hashem B. El-Serag, studied the epidemiology of HCC world wide and concluded that men are at increased risk for HCC partly because they have a greater incidence of viral hepatitis and alcoholic cirrhosis. Sean F. Altekruse studied the geographic variation of Hepatocellular Carcinoma in the United States and stated that the male to female ratio is less than two-fold for ICC (1.4 to 1)

3. Hep Par1

Out of 30 cases, 6 cases were negative and all 6 belongs to poorly differentiated hepatocellular carcinoma. In well differentiated group out of 11 cases, 6 were showing 6+ positivity(Figure 21), 4 were showing 5+ positivity(Figure 26) and 1 case showed 4+ positivity. In moderately differentiated group out of 3 cases, 1 case was 6+ positivity, 1 was 5+ positivity and one more showed 3+ positivity. In poorly differentiated group, 6 cases were negative, 7 cases showed 2+ positivity and 3 cases showed 1+ positivity.

Razia Hanif evaluated the diagnostic utility of Hep par-1 in differentiating hepatocellular carcinoma from metastatic carcinoma and concluded that The sensitivity of Hep par-1 was 83.3%, specificity was 96.6%, positive and negative predictive values and accuracy were 96.5%, 85.2% and 90% respectively.

In our study the sensitivity of Hep par1 was 80%, specificity was 100%, positive and negative predictive values are 100% and 72% respectively[2].

Zhen Fan et al in his study in 2002 named Hep Par 1 Antibody Stain for the Differential Diagnosis of Hepatocellular Carcinoma: 676 tumors tested using tissue microarrays and conventional tissue sections revealed that out of 19 cases of HCC, 18 were positive. The one negative case was a poorly differentiated HCC. Hep Par 1 staining in HCC is frequently uneven and patchy compared to the more uniform staining of adjacent nonneoplastic liver[71].

Minervini et al. and Chu et al. observed that poorly differentiated HCCs are more likely to be negative for Hep Par 1 than better differentiated cases. This findings reveals that poorly differentiated HCCs loses its reactivity for Hep par1.Sugiki et al in 2004 revealed in his study that the negativity of Hep Par1 in few cases of HCC's can be explained by the uneven distribution of Hep Par1 in HCC.

4. AFP:

Out of 30 cases, 16 cases were negative and 14 cases shows positive.Chan *et al.* published a paper focusing on prognostic value of pre-operative alpha-fetoprotein (AFP) level in the patients receiving hepatectomy for hepatocellular carcinoma (HCC) (1). They collected 1,182 patients who had curative hepatectomy for HCC. The patients were divided into 3 groups: AFP <20, 20–400 and >400 ng/mL. The patients with AFP >400 ng/mL were younger than the patients with AFP <20 or 20–400 ng/mL. Because they were young, the patients with AFP >400 ng/mL have less incidence of comorbid diseases. The patients with AFP >400 ng/mL had larger tumors and up to 65.9% of the patients had vascular invasion. The 5-year overall and disease-free survival were significantly lower than the patients with AFP <20 ng/mL and the patients with AFP between 20 to 400 ng/mL. Dr. Chan also used receiver operating characteristic curve to search the optimal cut off value of AFP for disease-free and overall survival. They found that the survival rate was compatible to one stage up for disease-free survival if AFP was >9,000 ng/mL and one stage up for overall survival if AFP was >14,000 ng/mL. Finally, they claimed that pre-operative AFP level was a significantly prognostic factor to predict survival. High level of AFP >9,000 and >14,000 ng/mL warrant an up stage of the diseases for disease-free and overall survival, respectively.

Chao-Wei Lee,1,2,3 Hsin-I Tsai,2,3,4 Wei-Chen Lee,1,3 Shu-Wei Huang,3,5 Cheng-Yu Lin,3,5 Yi-Chung Hsieh,3,5 Tony Kuo,3,5 Chun-Wei Chen,3,5,*† and Ming-Chin Yu study that Introduction: serum alpha-fetoprotein (AFP) was routinely employed as a tumor marker for screening, diagnosis, and treatment follow-up of hepatocellular carcinoma (HCC). However, a substantial proportion of HCC patients had normal AFP level even at an advanced disease status. Few studies to date had tried to explore the nature and behavior of this normal AFP HCC (N-HCC). The purpose of this study was to investigate the clinicopathological characteristics and survival outcome of N-HCC after operation. In addition, potential tumor markers for N-HCC were also sought in an attempt to augment diagnostic ability. Methods: between 2005 and 2015, patients with hepatocellular carcinoma who were treated with hepatectomy in Chang Gung

Memorial Hospital Linkou branch were divided into two groups according to their preoperative serum AFP level (<15 ng/mL: NHCC; ≥15 ng/mL: abnormal AFP HCC (A-HCC)). Patient demographic data and clinicopathological variables were collected. Kaplan–Meier and Cox regression multivariate analyses were performed to identify significant risk factors for disease-free survival (DFS) and overall survival (OS) for N-HCC. ELISA and immunohistochemical (IHC) studies were employed to determine the diagnostic accuracy of various tumor markers. Results: a total of 1616 patients (78% male) who underwent liver resection for HCC were included in this study. Of them, 761 patients (47.1%) were N-HCC. N-HCC patients were significantly older with more comorbidities and less hepatitis virus infections. Furthermore, N-HCC had fewer early recurrences (49.6% vs. 60.8%, $p < 0.001$) and better DFS (44.6 months vs. 23.6 months, $p < 0.001$) and OS (94.5 months vs. 81.7 months, $p < 0.001$). Both ELISA and IHC studies demonstrated that glypican-3 (GPC3) would be a promising diagnostic tumor marker for N-HCC. Conclusion: N-HCC patients were significantly older and had less hepatitis virus infections or cirrhosis. Their tumors tended to be smaller, less vascular invaded, and well-differentiated. The carcinogenesis of N-HCC may thus not be identical to that of typical HCC. GPC3 would be a promising tumor marker for diagnosing N-HCC. Further study is warranted to validate our findings.

A R Hakeem 1 , R S Young, G Marangoni, J P A Lodge, K R Prasad Liver transplantation (LT) offers a possible cure for carefully selected patients with hepatocellular carcinoma (HCC). Studies report that preoperative alpha-fetoprotein (AFP) is a prognostic indicator that can predict survival and recurrence in this patients. To undertake a systematic review of available literature on preoperative AFP as a predictor of survival and recurrence following LT for HCC. A literature search was performed using Medline, Embase, Cochrane Library, CINAHL and Google scholar databases to identify studies reporting AFP as a prognostic marker in LT for HCC. Primary outcomes of interest were overall survival and recurrence. Secondary outcomes were correlation of pre-LT AFP with vascular invasion and grade of tumour differentiation. A total of 13 studies met the inclusion criteria (12,159 patients). The majority were male (9603, 78.9%). All were observational studies and only one prospective. Methodological quality was rated as poor for all studies, with selection and observer bias apparent for most cohorts. Reported survival rates and recurrence rates varied widely between the studies although overall demonstrated better outcomes for those with lower (<1000 ng/mL) pre-LT AFP levels. Similarly, rates of vascular invasion and poor tumour differentiation were higher in those with high pre-LT AFP levels. A quantity of AFP >1000 ng/mL is associated with poorer outcomes from liver transplantation for hepatocellular carcinoma. The quality of studies was generally poor and precluded valid statistical meta-analysis. There is a need to improve the performance and reporting of primary prognostic studies to facilitate high quality systematic review and meta-analysis.

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