DISSERTATION ON

ROLE OF IMMUNOHISTOCHEMISTRY(IHC) IN DIFFERENTIATING PRIMARY HEPATOCELLULAR CARCINOMA, CHOLANGIOCARCINOMA & SECONDARIES FROM COLORECTAL REGION USING MANUAL TISSUE MICROARRAY TECHNIQUE & ITS ADVANTAGES

Dissertation submitted to
TAMIL NADU DR. M.G.R. MEDICAL UNIVERSITY
CHENNAI

for
M.D. (PATHOLOGY)
APRIL 2016

Under the guidance of
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THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY
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CERTIFICATE

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ABBREVIATIONS

CC	-	Cholangiocarcinoma
CDC	-	Centre for Disease Control and prevention
CDX2	-	Caudal-related homeobox gene 2
CEA	-	Carcinoma Embryonic Antigen
CK	-	Cytokeratin
DAB	-	Diaminobenzidine
DPX	-	Dibutyl Phathalate Xylene
ER	-	Estrogen Receptor
Fig	-	Figure
GCDFP-15	-	Gross cystic disease fluid protein fraction-15
GIST	-	Gastrointestinal Stromal Tumour
HbsAg	-	Hepatitis B Surface antigen
HBV	-	Hepatitis B Virus
HBV DNA	-	Hepatitis B Virus Deoxy ribonucleic acid
HCC	-	Hepatocellular carcinoma
HCV	-	Hepatitis C Virus
Hep Par1	-	Hepatocyte Paraffin 1
ICC	-	Intrahepatic Cholangiocarcinoma
IHC	-	Immunohistochemistry

PAX 2	-	Paired-Box 2
PR	-	Progesterone Receptor
RCC	-	Renal Cell Carcinoma
TMA	-	Tissue Micro Array
TTF	-	Thyroid transcription factor

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INSTITUTIONAL ETHICAL COMMITTEE,

STANLEY MEDICAL COLLEGE, CHENNAI.

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Principal investigator: Dr. V.Ramya

Designation: PG in MD Pathology

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common histological type of primary liver carcinoma. HCC is a major health problem worldwide due to its high incidence and high rates of mortality.

- Liver is a common site of metastasis from many primary sites due to rich portal and systemic venous supply. Thus, metastatic cancer is the most common malignant tumour in adult liver.

- The distinction of liver metastatic tumour from HCC may present a diagnostic challenge that carries an impact on subsequent prognostication and therapeutic management.

- The morphology is used to establish a differential diagnosis and then histochemical and immunohistochemical studies are used to refine the diagnosis. Immunohistochemistry is helpful when morphology and identification of secretory substances fail.

- Among immunohistochemical markers, Hep par-1 has been reported as most sensitive and specific immunohistochemical marker for HCC.
- Cytokeratins 7, 19 and 20 were absent from most hepatocellular carcinomas but positive in many adenocarcinomas, including cholangiocarcinomas.

- Tissue microarray is a recent innovation in the field of pathology. A microarray contains many small representative tissue samples from hundreds of different cases assembled on a single histologic slide, and therefore allows high throughput analysis of multiple specimens at the same time.
- With this technology, tissue samples in the microarrays are amenable to a wide range of techniques, including histochemical stains, immunologic stains with either chromogenic or fluorescent visualization, in situ hybridization and even tissue micro-dissection techniques
- This method has proven to be extremely efficient, of shorter duration, and cost effective, especially with expensive reagents.[1,2,3,4]

AIMS AND OBJECTIVES

- To differentiate and to study the pattern of IHC markers like Hep-Par 1, CK7, CK19 and CK20 in primary hepatocellular carcinoma, cholangiocarcinoma and metastatic secondaries from colorectal region in liver.

- To demonstrate use of manual tissue microarray technique and its advantages of the same in IHC.

REVIEW OF LITERATURE

Hepatocellular carcinoma (HCC) is known for its histomorphologic heterogeneity. To differentiate HCC from their mimics is often a challenging issue in histopathology. The Reasons for this are: a) Variety of neoplasms that can arise from the hepatocytes, b) The liver is a target organ for metastases that can mimic variants of primary hepatocellular carcinoma, and c) The limitations of serum Alpha-fetoprotein (AFP) in differentiating the poorly differentiated HCC from intrahepatic cholangiocarcinoma (CC) and metastatic carcinomatous deposits from elsewhere.

Various immunohistochemical markers have been used for the identification of these tumors that include α-1-antitrypsin, carcinoembryonic antigen (CEA), factor XIIIa, ferritin, and albumin. However, their use to differentiate HCC from other neoplasms has been limited.[6,7,8,9,10]

Among immunohistochemical markers, Hep Par-1 has been used in various studies and reported as the most sensitive and specific immunohistochemical marker for HCC. CK 7, CK 19 for intrahepatic cholangiocarcinoma and CK 20 for Metastatic Adenocarcinomatous deposit in liver from colorectal region.[1,11,12,13]

EMBRYOLOGY

Liver primordium appears in the middle of 3rd week, as outgrowth of the endodermal epithelium at the distal end of the foregut. From this outgrowth, the hepatic diverticulum, or liver bud is formed and penetrates the septum transversum.

While hepatic cells continue to penetrate the septum, the connection between the hepatic diverticulum and the foregut (duodenum) narrows, forming the bile duct. A small ventral outgrowth arises from the bile duct, and which gives rise to the gallbladder and the cystic duct.

During further development, epithelial liver cords intermingle with the vitelline and umbilical veins, and thus forms hepatic sinusoids. The mesoderm of the septum transversum forms the hematopoietic cells, Kupffer cells and connective tissue cells.

After the invasion of hepatocytes into the entire septum transversum, the organ bulges caudally into the abdominal cavity, and the mesoderm of the septum transversum lying between the liver and the foregut and the liver and ventral abdominal wall become membranous, and forms the lesser omentum and falciform ligament respectively.

Anatomy

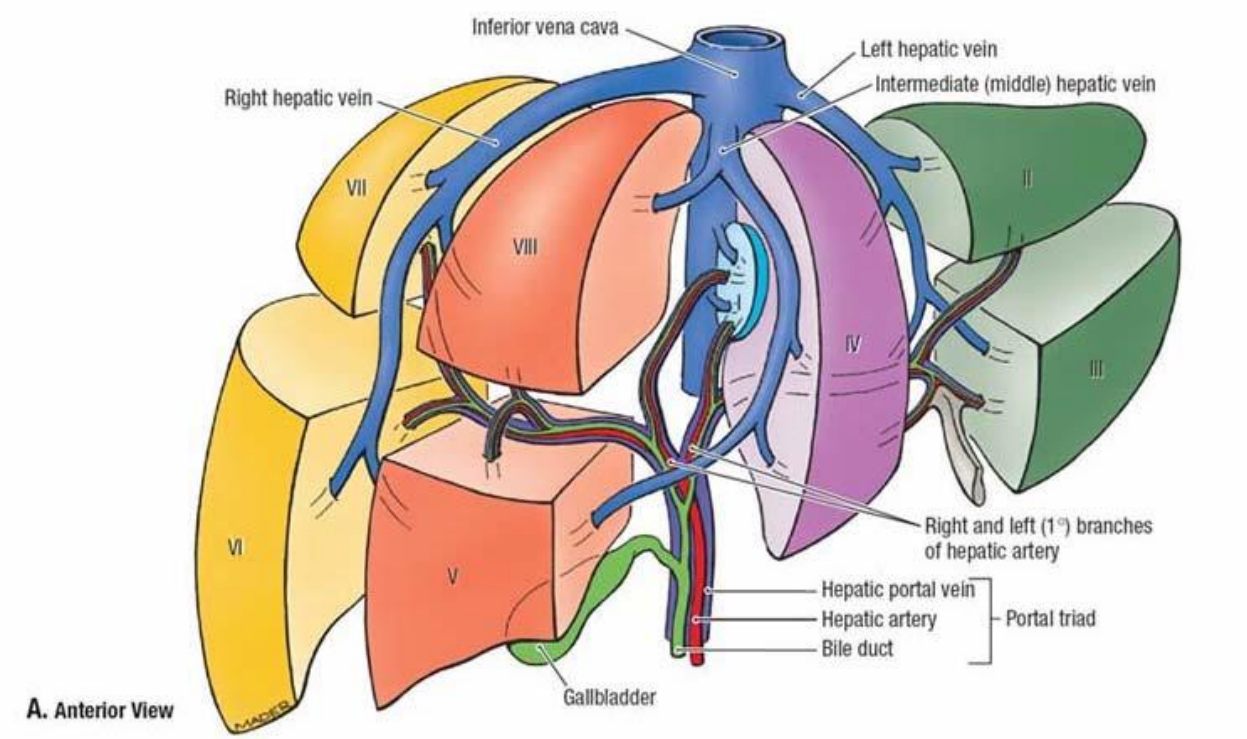


Fig 1 Gross anatomy of the liver

The Liver is the largest single wedge shaped organ in the human body. In an adult, it weighs about 1400 to 1600 kilograms and located in the right hypochondrium, behind the lower ribs. The liver is divided into four lobes: the right (the largest lobe), left, quadrate and caudate lobes.

The arterial supply of the liver is by the portal vein and hepatic artery. Venous drainage is by the hepatic vein. It is connected to the diaphragm and abdominal walls by five ligaments. The main functions of the gall

bladder are the storage, concentration, acidification and delivery of bile to small intestine.

Histology

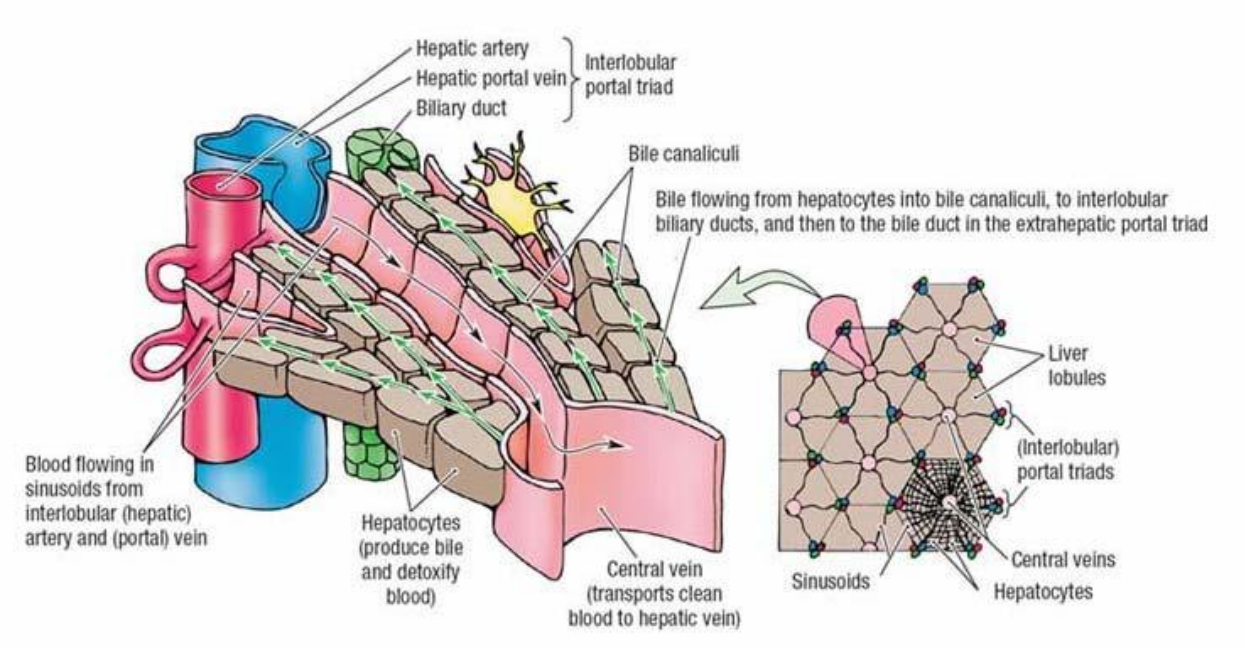


Fig 2 Histology of the liver

Liver is divided into many functional units called Lobules and these are hexagonal in shape. Lobules are formed of tightly packed, plates of epithelial cells called as hepatocytes radiating from a central vein. The outer surface of the liver is covered by a capsule composed of fibrous tissue called Glisson's capsule.

Hepatocytes are large polyhedral cells with round nuclei with peripherally dispersed chromatin and prominent nucleoli. The nuclei shows pleomorphism

and > 50% of the hepatocytes are diploid and some are even polyploid. Binucleate cells are also seen in normal liver.

The abundant cytoplasm is eosinophilic with few basophilic granules due to presence of numerous mitochondria and rough endoplasmic reticulum respectively. The sinusoids are lined by flattened endothelial lining cells.

The portal triad contains three main structures. They are the branch of the hepatic portal vein, smaller diameter thick-walled vessels are terminal branches of the hepatic artery and the bile ductules.

Between the hepatocytes, bile canaliculi are present which drains into collecting ducts (canals of Hering). These drains into the bile ductules which in turn drains into intrahepatic ducts and into the right and left hepatic ducts, the common hepatic duct and then to the duodenum via the common bile duct. The portal tracts are often called as portal triads. Lymphatics are also present in the portal tracts, but since their walls are delicate and often collapsed they are not easily made out.

The layer of hepatocytes immediately bordering the portal triad is known as the limiting plate. Between the anastomosing plates of hepatocytes, there are sinusoids which receives blood from both the portal and hepatic arterial systems.

Physiology

The functions of the liver can be classified broadly as metabolic, synthetic, storage, catabolic, and excretory.

METABOLIC FUNCTIONS**:**

Liver plays several roles in carbohydrate metabolism. It synthesizes and stores glycogen via glycogenesis and glycogenolysis. It is also responsible for gluconeogensis and cholesterol synthesis.

SYNTHETIC FUNCTIONS**:**

Liver synthesize plasma proteins that includes albumin, coagulation factors(I, II, V, VII, IX, X, XI), complements, acute phase reactants, and binding proteins for vitamin A, iron and copper.

STORAGE FUNCTIONS**:**

Liver stores various substances like glycogen, copper, iron, lipid-soluble vitamins(vitamin A, D), iron, copper and triglycerides.

CATABOLIC FUNCTIONS**:**

Liver catabolises endogenous substances, hormones, serum proteins, and plays an vital role in the detoxification of drugs.

EXCRETORY FUNCTIONS:

Bile is the primary excretory product of the liver which is a mixture of bile acids, conjugated bilirubin, cholesterol, phospholipids, and electrolytes.[14,15,16]

Pathology of Hepatocellular carcinoma

Many etiologic factors are implicated in etiopathogenesis of HCC, most important being HBV and HCV infection, and association with cirrhosis. Genesis of HCC is linked to prolonged infection with HBV. The evidence in support is both epidemiologic and direct. The incidence of HBsAg positivity is higher in HCC patients. There is more direct evidence of integration of HBV-DNA genome in the genome of tumour cells of HCC.

Conditions Associated with Hepatocellular Carcinoma *[17]*

1.Cirrhosis	
Alcohol	High
Hepatitis C	High
Hepatitis B	High
Autoimmune chronic active hepatitis	High
Cirrhosis due to non alcoholic fatty liver disease	Moderate
Crytogenic cirrhosis	Moderate
Primary Biliary Cirrhosis	**Low**
2.Metabolic diseases	
alpha1-Antitrypsin deficiency	Moderate
Ataxia telangiectasia	Moderate

Types 1 and 3 glycogen storage disease	Moderate
Galactosemia	Moderate
Citrullinemia	Moderate
Porphyria cutanea tarda	Moderate
Wilson's disease	Low
3.Environmental	
Thorotrast	Moderate
Androgenic steroids	Moderate
Cigarette smoking	Moderate
Aflatoxin	Moderate

Long-standing HCV infection has emereged as a major factor in the etiology of HCC, generally after more than 30 years of infection. The patients having anti-HCV and anti-HBc antibodies together have three times higher risk of developing HCC than in those with either antibody alone. HCV infection after a long interval produces cirrohosis more often prior to development of HCC, while in HCC following HBV infection half the cases have cirrohosis and remainder have chronic hepatitis.

It is also possible that HBV and HCV infection act synergistically to predispose to HCC.

Cirrhosis of all etiologic types is more commonly associated with HCC but the most frequent association is with macronodular post-necrotic cirrhosis. The mechanism of progression to HCC appears to be chronic regenerative activity in cirrhosis, or that the damaged liver in cirrhosis is rendered vulnerable to carcinogenic influences. Liver cell dysplasia identified by cellular enlargement, nuclear hyperchromatism and multinucleate cells, is found in 60% of cirrhotic livers with HCC and in only 10% of non-cirrhotic livers. It has been observed that alcoholics have about four-fold increased risk of developing HCC. It is possible that alcohol may act as co-carcinogen with HBV or HCV infection, but alcohol does not appear to be a hepatic carcinogen per se.

An important mycotoxin, aflatoxin B1, produced by a mould Aspergillus flavus, is carcinogenic; it may act as a co-carcinogen with hepatitis B or may suppress the cellular immune response. A number of chemical carcinogens can induce liver cancer in experimental animals. These include butter-yellow and nitrosamines used as common food additives.

Limited role of various other factors in HCC has been observed. These include the following: i) haemochromatosis; ii) α-1-antitrypsin deficiency; iii) prolonged immunosuppressive therapy in renal transplant patients; iv) other

types of viral hepatitis; v) tobacco smoking; and vi) parasitic infestations such as clonorchiasis and schistosomiasis.

Gross Pathology of HCC

The background liver shows cirrhosis in the majority of cases. Tumors can be classified as massive when a solitary large mass is seen, nodular when multiple discrete nodules are seen, and diffuse when multiple small indistinct nodules are seen. Tumors less than 2 cm in diameter are referred to as small HCC; these small tumors usually lack gross vascular invasion, necrosis, or hemorrhage. Tumors are generally soft and may be paler than the adjacent liver or bile stained. Irregular borders and satellite nodules can be present. HCC has a tendency for vascular invasion. Portal and hepatic veins can be involved.

The grading of HCC is based on differentiation of tumor and is based on the system developed by Edmondson and Steiner in 1954. Well-differentiated tumors show a pseudoacinar or thin trabecular pattern and mild nuclear atypia. Most of the tumors are less than 3 cm, and fatty change is often present(**Figure 11**). Moderately differentiated tumors have more cytologic and architectural variability with wider trabeculae and more pronounced cytologic atypia. Multinucleated and giant tumor cells can be seen focally(**Figure 12**). Poorly differentiated tumors often show a solid growth pattern accompanied by moderate to marked nuclear pleomorphism

(**Figure 13**). Undifferentiated tumors also show a solid growth pattern with no apparent hepatocellular differentiation and may include sarcomatoid components.[18]

Pathogenesis of Cholangiocarcinoma

The pathogenesis of cholangiocarcinoma is poorly understood, the incidence and mortality is in increasing trend.[19] It is often diagnosed late and survival is poor. About 15-20% of liver and bile duct cancers are cholangiocarcinoma. Like gallbladder cancers the incidence increases with age. The term cholangiocarcinoma includes intrahepatic, perihilar and distal extrahepatic tumours of biliary tracts. The perihilar tumours involving the bifurcation of hepatic ducts also termed as Klatskin tumour, from Klatskin's original description in 1965.

The perihilar bile duct tumours were classified by Bismuth et al. as tumours below the confluence of the left and right hepatic ducts (type I), tumours reaching the confluence(typeII), tumours occluding the common hepatic duct and either the right or left duct (types IIIa and IIIb, respectively), and tumours that are multicentric or that involve the confluence and both the right and left hepatic ducts(type IV).[20]

Most cholangiocarcinomas involve perihilar and distal extrahepatic bile ducts.

RISK FACTORS[21]

1. Hepatolithiasis
2. Liver flukes
3. Biliary cystic disease
4. Primary sclerosing cholangitis
5. Radionucleotides
6. Chemical carcinogens

The evolution of cellular changes most probably can be described as a multistep sequential progrssion of metaplasia, dysplasia and finally neoplasia. Conversion of normal to malignant bile duct tissue probably requires a number of successive mutations. Oncogenes are K-ras, c-myc, c-neu, c-erb-b2 and c-met. Tumour suppressor genes are p53 and bcl-2.[22,23]

Gross Pathology. The three macroscopic types of Intrahepatic cholangiocarcinoma: mass forming, periductal infiltrating, and intraductal. Mass forming is the most common appearance of intrahepatic cholangiocarcinoma and is characterized by a localized tumor with a distinct border that grows radially without periductal or intraductal spread. The periductal-infiltrating type infiltrates along the bile duct and is often associated with stricture and involvement of periductal connective tissue. Both these types are usually firm, white-tan lesions because of a dense fibrous stroma. Advanced cases can show mixed patterns of growth.

Microscopic Features. *M*ajority (>95%) of these tumors are adenocarcinomas. The well-differentiated tumors show tubular, papillary, and cord-like patterns, and cytologic atypia can be minimal. Intracytoplasmic lumina, focal cribriform architecture, nuclear stratification, and intraluminal cellular debris favor carcinoma over a benign process. Nucleoli are often less prominent compared with HCC. Mucin can be demonstrated in most cases. A prominent desmoplastic stroma is characteristic of cholangiocarcinoma. Occasionally, the tumor cells form small narrow tubular structures resembling ductules or canals of Hering

METASTATIC LIVER DISEASE

Metastatic tumours are the most common malignant liver lesion in adults.[24]

By haematogenous, lymphatic or transperitoneal spread, almost all malignant neoplasms and haematological malignancies, can secondarily involve the liver and cause metastatic disease.[25]

In case of liver metastasis, the primary tumour most frequently is located in colon, pancreas, stomach, breast, oesophagus, genitourinary organs[26]. Lung cancer can metastasize to liver.[27] Adenocarcinoma was the most frequent histological type of metastases in liver. Lymphoma constituted 0.4% of all tumours.[28,29] In children, neuroblastoma, nephroblastoma and rhabdomyosarcoma are the most frequent sources of metastases to the liver.[30] The liver metastases can occur in patients with Hodgkin's

lymphoma and non-Hodgkin's lymphoma at the time of diagnosis and even leukemias can also involve the liver.[27]

Diagnosis of malignant melanoma[26] is very difficult due to variable morphological features and using specific IHC markers gives the diagnosis.

To identify the metastasis from breast primary tumour, gross cystic disease fluid protein fraction-15 (GCDFP-15) and/or mammaglobin can be used. Breast cancers of luminal molecular type express oestrogen (ER) and progesterone receptors (PR).

Use of LCA (leukocyte common antigen) can differentiate HCC from haematological neoplasms. The CK5/6 and CK 34betaE12 in association with strong nuclear reactivity with p63 protein are useful to diagnose squamous cell carcinoma.[31,32]

Metastatic colorectal carcinomas can be recognised by diffuse intensive cytoplasmic expression of CK20 and nuclear expression of CDX2.[33,34]

Neuroendocrine tumours are characterised by strong cytoplasmic expression of chromogranin A and synaptophysin and negativity for Hep Par 1.

PAX-2 is used as marker of metastatic renal cell carcinoma.

WHO HISTOLOGICAL CLASSIFICATION OF TUMOURS OF LIVER AND INTRAHEPATIC BILE DUCTS - 2010

Epithelial tumours

Benign

- Hepatocellular adenoma
- Intrahepatic bile duct adenoma
- Intrahepatic bile duct cystadenoma
- Biliary papillomatosis
- Focal nodular hyperplasia

Malignant

- Hepatocellular carcinoma
- Bile duct cystadenocarcinoma
- Intrahepatic cholangiocarcinoma
- Hepatoblastoma
- Combined hepatocellular and cholangiocarcinoma
- Undifferentiated carcinoma

Non-epithelial tumours

Benign

- Angiomyolipoma
- Haemangioma
- Infantile haemangioendothelioma

- Lymphangioma and lymphangiomatos is

Malignant

- Angiosarcoma
- Epithelioid haemangioendothelioma
- Rhabdomyosarcoma
- Embryonal sarcoma
- Others

Miscellaneous Tumours

- Kaposi sarcoma
- Solitary fibrous tumour
- Teratoma
- Yolk sac tumour
- Carcinosarcoma
- Rhabdoid tumour

Haemopoietic and lymphoid tumours

Secondary tumours

Epithelial abnormalities

- Dysplastic nodules
- Bile duct abnormalities
- Liver cell dysplasia
- Intraepithelial carcinoma

TNM Staging Scheme for Carcinoma of Liver and Intra hepatic bile ducts (AJCC 2010)

PRIMARY TUMOR (T)

TX : Primary tumor cannot be assessed

T0 : No evidence of primary tumor

T1 : Solitary tumor without vascular invasion

T2 : Solitary tumor with vascular invasion or multiple tumors none >5 cm

T3 : Multiple tumors >5 cm or tumor involving a major

branch of the portal or hepatic vein(s)

T4 : Tumor(s) with direct invasion of adjacent organs other than the gallbladder or w ith perforation of visceral peritoneum

REGIONAL LYMPH NODES (N)

NX : Regional lymph nodes cannot be assessed

N0 : No regional lymph node metastasis

N1 : Regional lymph node metastasis

DISTANT METASTASIS (M)

MX : Distant metastasis cannot be assessed

M0 : No distant metastasis

M1 : Distant metastasis

STAGE GROUPING

Stage I	T1	N0	M0
Stage II	T2	N0	M0
Stage IIIA	T3	N0	M0
Stage IIIB	T4	N0	M0
Stage IIIC	Any T	N1	M0
Stage IV	Any T	AnyN	M1.

ROLE OF IMMUNOHISTOCHEMISTRY IN DIFFERENTIATING PRIMARY HEPATOCELLULAR, CHOLANGIOCARCINOMA AND METASTATIC ADENOCARCINOMA IN COLORECTAL REGION

IHC markers are used to study HCC and to differentiate hepatocellular carcinoma from other benign and malignant mimics, especially intrahepatic cholangiocarcinoma and metastatic adenocarcinoma.[35,36]

Hurlimann J et al in 1991, studied that C-reactive protein is a sensitive and specific marker for HCC. Factor XIII a, can also be used to stain HCC.[39]

In 2000, T Shimonishi et al demonstrated that the combined immunostaining of cytokeratins 7, 18, 19 and 20 is useful to differentiate intrahepatic cholangiocarcinomas from metastatic adenocarcinomas in liver and from colorectal and gastric regions.[42]

Zhen Fan, M.D. et al in 2003 studied that Hep Par 1 as a useful marker for HCC. It is not 100% specific because it stains few non hepatic malignancies. Hence Hep Par 1 in addition to other positive and negative markers of HCC is recommended for the differential diagnosis of HCC.[41]

In 2004, Varma and Cohen et al mentioned that among the many diagnostic markers studied, pCEA, HepPar 1, CD34, CK 7, CK 19, and CK 20 have been found to be valuable in distinguishing HCC from metastatic neoplasms of extrahepatic sites.[36]

Goodman ZD et al studied in 2007, that as the neoplastic cells of hepatocellular carcinoma mimics the normal liver cells both by functional and morphological characters. Hence it is difficult to differentiate using immunostains, even though if present they are not 100% specific.[37]

In 2009, Ali Sawan et al studied that CK7 and CA19-9 positive staining can exclude a diagnosis of hepatocellular carcinoma, but cannot discriminate between metastatic carcinoma (from stomach and pancreaticobiliary origin) and cholangiocarcinoma. The CK20+/CK7–ve phenotype indicates metastatic intestinal adenocarcinoma, most often from the colon or rectum.[40]

In 2012, Daniela Fanni et al revealed in his study that there are no stains that can absolutely distinguish well-differentiated hepatocellular carcinoma from hepatic benign lesions, Even then, selective IHC markers can be used in addition to other histopathological features and can establish the diagnosis of hepatocellular carcinoma.[38]

Dana T.Timek et al in 2012 demonstrated that Arg-1 has a similar sensitivity and higher specificity in differentiating a non-HCC from HCC when compared with HepPar-1 and glypican-3. These 3 markers are recommended as the most effective panel for small tissue biopsy or FNA specimens in the distinction of HCC from metastatic carcinoma.[43]

TISSUE MICRO ARRAY

The tissue microarray (TMA) technique has been in use for 15 years. The origin of TMAs can be attributed to Dr Hector Battifora's humble 'sausage' blocks (**Figure 3**), in which a number of tissues, from different organs, were put together in the same block and the reactivity of antigen/protein was studied. The disadvantage of this technique was when tumors or tissues from the same organ were put together it was difficult and impossible to identify them back.[44]

The technology was first described in 1987, but its was used 11 years later, when Kononen and colleagues further modified and developed a device that could rapidly and reproducibly produce TMAs (Kononen *et al.*, 1998).[46]

There are expensive commercially available instruments(Beecher) for making microarray blocks, which can used to array upto 1,000 cores in the same block.

The next step in the development of TMA was described by Wan et al. who used a 16-gauge needle to manually bore cores from tissue blocks and array them in a multi-tissue straw in a recognizable pattern. [45]

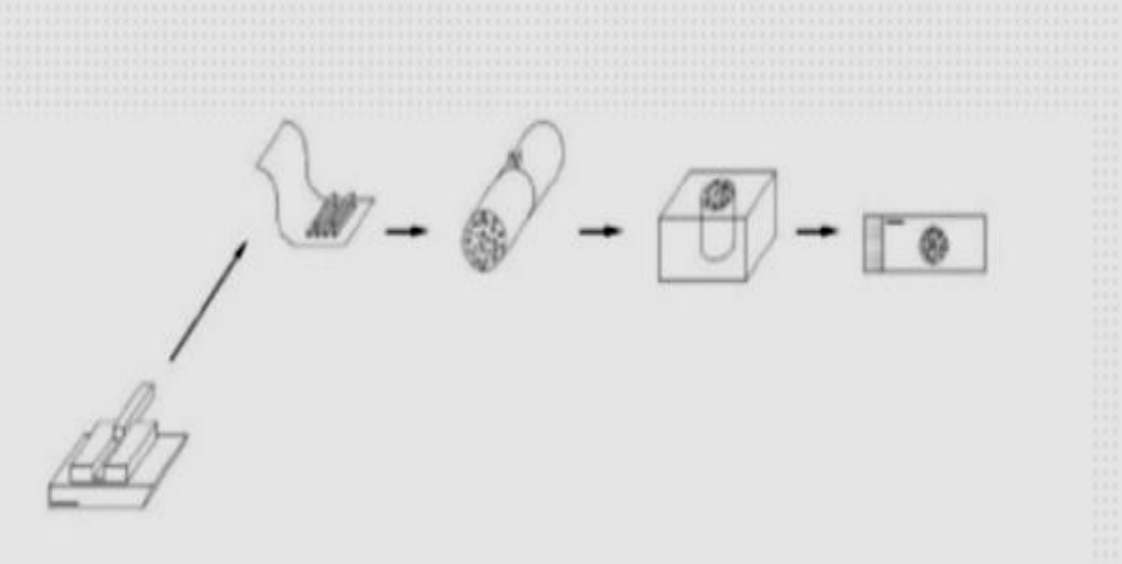


Fig 3 Demonstration of Battifora's Sausage blocks

Suk Jin Choi et al concluded that simple and inexpensive construction of high-density and high-quality TMAs can be made by using paraffinized agarose gels as recipient blocks.[47]

APPLICATIONS

TMA shifts the research from basic to clinical and enables researchers to look for expression of specific protein on tissue samples from a large cohort of patients by immunohistochemistry.

All researches currently done in conventional histological sections from formalin fixed paraffin embedded tissue are possible using TMA.

Types of TMAs

These are the commonly used types of TMAs used depending upon the need in the study design.

1. **Cell line arrays:** Used to detect the specificity of an antibody in finding the proteins.
2. **Random tissue/tumor arrays:** Used for surveillance of antibodies which were already in use.
3. **Consecutive case array:** Used to build TMA array from specific tissue of interest.
4. **Tumor characteristic-based array:** A special type of array used to evaluate a single parameter of a tumour such as patient age or tumor grade.
5. **Progression arrays:** These types of arrays are used to analyze the role of protein(s) in cancer progression.
6. **Outcome based arrays** [50,51]

Tissue microarray technique

Microarray is a technique of organizing minute amounts of tissues of interest on a solid support.[52] Tissue microarrays are composite paraffin blocks constructed by extracting cylindrical tissue core "biopsies" from different paraffin donor blocks and re-embedding these into a single recipient (microarray) block at defined array coordinates.

At first, the donor blocks (invariably stored paraffin blocks) are retrieved and sectioned to produce standard microscopic slides that are stained with hematoxylin and eosin. Routinely examines the slides to mark the area of interest, which is commonly an area of cancer depending upon the study design, after which the samples can be taken from the area of interest and arrayed.[53]

A tissue microarray instrument is used to acquire a tissue core from the donor block. This core is then placed in the empty cores, which are already made in an empty paraffin block—the recipient block. The core is placed at a specifically assigned coordinate (X-Y guide), which is accurately recorded, typically on a spreadsheet. Using a microtome, the sections are cut at 5 μm from the tissue microarray blocks to generate tissue microarray slides for molecular and immunohistochemical analyses.

Manual Construction of a TMA

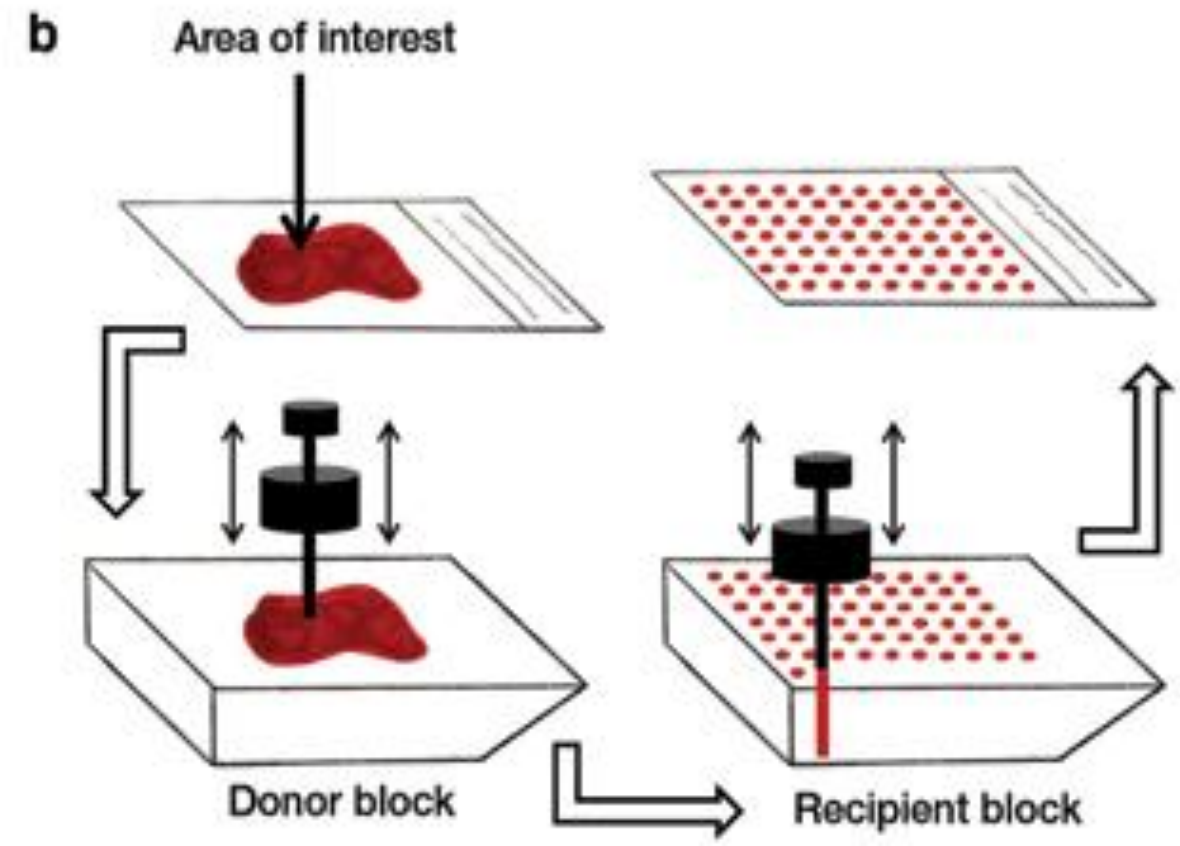


Fig 4 Diagrammatic representation of construction of Manual TMA

Donor block: The block from which a core of tissue considered for study, is called as the donor block. This is done by examining the H & E stained slides. The donor blocks should not contain poorly processed areas. Donor blocks have variable depths of residual tissue. These lengths depend upon the thickness of the original tissue when submitted for embedding, as well as the number of sections obtained from a block before TMA construction. The

tissue within the block should be at least 1 mm thick to allow for adequate core length. Preferably, 3 to 4 mm of tissue thickness is recommended.

Recipient block: The empty paraffin block in which the cores are placed is called as the recipient block. The cores should be placed towards the center of this block in order to prevent cracking of the block leaving around 3mm space from the margins. Multiple sections from the block for H & E, and Special studies should be cut at the same time to prevent wastage of tissue. ========While creating the recipient holes within the microarray block, residual paraffin remains within the hole. As the donor cores are inserted into the recipient block, the residual paraffin is pushed into the hole. If the depth of the hole is insufficient, the compacted paraffin prevents proper insertion of the core with excessive tissue extrusion. This results in unnecessary tissue loss during block facing. Sufficiently deep holes allow space for residual paraffin to be compacted within the recipient block and prevent needless loss of the donor cores. Although excessive extrusion of cores results in unnecessary loss of tissue, it is just as important to avoid insertion of the cores below the surface of the recipient block. Tissue length is inherently variable within the various cores. The length of tissue depends upon the thickness of tissue submitted for embedding, as well as the number of sections previously cut from the donor block. Therefore, many specimen cores will not span the entire depth of the TMA block. By inserting the cores below the surface of the block, multiple sections must be cut to face the

block and ensure all tissue discs are represented. Depending upon how deeply the cores were inserted, significant amount of tissue can be lost while attempting to ensure complete representation of all cores. Therefore, it is advisable to leave the cores slightly protruding from the recipient paraffin block.

The slight core protrusions can be corrected after completion of all core insertions. Space remains between the inserted cores and the recipient holes. Miniscule cracks form within the tissue cores and the recipient block during their various manipulations. These air pockets and cracks disrupt tissue discs during sectioning. By heating the TMA after core insertion, the cores are merged with the surrounding paraffi n, reducing the cracks and air pockets. Ideally, complete melting of the paraffin allows appropriate merging of the cores with the recipient block. Unfortunately, completely melted paraffin does not support cores within the recipient block, which quickly lose their orientation in the heated liquid paraffi n. Therefore, moderate heating to soften, but not melt, the TMA block is used to slowly merge the cores with the paraffin. Delicate pressure applied to the surface of the heated recipient block aids in the merging. This can be accomplished by applying gentle pressure with a glass slide to the surface of the block. This step evenly completes the insertion of the cores. Multiple cycles of heating and cooling are used to adequately merge the block with the tissue cores and complete the creation of a TMA block.----------

Size of the cores: TMA tissue discs contain a limited area of tissue. When selecting the area to core, focus should be directed toward identifying areas within the block that fulfill the study parameters and fit within the area of the recipient core position. Another consideration is the location of cells within tissue discs. Cells located on the periphery of the tissue disc are more susceptible to artifacts, such as nonspecific immunohistochemical staining of tissue edges, and can seriously confound the interpretation of the TMA study. Ideally, the cells of interest are located centrally within the tissue cores. Stylets for procurement of various core sizes are available, although some options are instrument dependent. For most instruments and protocols, diameter options range between 0.6 and 2 mm. Beecher Instruments offers a 3-mm- diameter core needle for its automated arrayer (ATA-27). In general, smaller core diameters result in greater specimen number in each array block, resulting in higher throughput.

The larger core diameters result in more tissue represented per specimen but fewer cases analyzed per slide. The decision of which diameter to use depends upon core-related artifact considerations, specimen number, and availability of donor tissue. During sectioning of the array, individual cores can fold, completely disrupting the tissue disc. The larger core sizes result in greater tissue disc area per section. Therefore, folding or disruption of larger cores more often results in partial obstruction of the individual sample. However, there is usually sufficient

intact tissue remaining to provide adequate analysis. With the larger core diameters, usually two cores are suffi cient to provide acceptable concordance rates. This reflects the greater tissue area available with the larger cores and their decreased likelihood of complete loss during sectioning artifact. On the other hand, the larger cores cause greater disruption of donor blocks. The larger needles are relatively more likely to crack and fragment the tissue block when punching cores. The fragmentation or disruption of the original block destroys its use for further studies. This is problematic if the tissue block is needed for further diagnostic or research purposes. In comparison, the smaller cores cause less disruption of the donor block and less artifact when the donor block is sectioned after core removal. Therefore, if the integrity of the original donor block is imperative, smaller core sizes should be considered.

Density: The maximum number of cores that can be placed on a single block is variable, depending on size of the core and block. Cores should start at least 3 mm away from the block edges, to prevent the paraffin from cracking.

Distance: Placement of many cores close to one another distorts the TMA. Cores located within the center of the block will be higher than the cores in the periphery, making it difficult to face the block without losing significant amount of tissue. Therefore, the cores within the recipient block

should be adequately spaced. Most protocols recommend a spacing of 0.7 to 0.8 mm between core.

ADVANTAGES

- Amplification of a scarce resource
- Simultaneous analysis of very large numbers of specimens.
- Uniformity
- Decreased assay volume, time and cost.
- Original block was not destroyed for diagnosis and thus conserves valuable tissue.
- Effective for assessing quality control assurance programs such as intra- and interlaboratory variation in immuno-histochemical and molecular studies and as a efficient tool.[54,55,56]

LIMITATIONS

- In case of heterogenous tumours, multiple cores must be taken in order to avoid false negative results.
- Requires experienced personnel and expensive equipment.
- High cost.[57]

Commercial TMA arrayer machines such as automated and semiautomatic tissue arrayers are expensive. A relatively simple and inexpensive alternative

is the use of lab-made recipient paraffin blocks and ordinary cannula-piercing needles, skin biopsy punches, and bone marrow biopsy needles.[58]

Chang Hwan Choi concluded that by using cannula piercing needles and recipient paraffin blocks, high density TMAs with varying core diameters (0.6 mm, 1 mm, 2 mm, 3 mm, and 5 mm) can be constructed at low cost. The arrays were fairly well established in terms of alignment of the tissue cores, so that we were able to appreciate the histologic and immunohistochemical features of the TMA sections without substantial difficulty.

By using TMA kits made from bone marrow biopsy needles, disposable skin biopsy punches and metallic ink cartridges of ballpoint pens, researchers are able to construct low cost TMAs.[58]

MECHANICAL PENCIL TIP NEEDLE METHOD(**Figure No. 5,6**)

Fig 5 & 6 Demonstration of Mechanical pencil tip technique

Abdelhadi M Shebl et al concluded that mechanical pencil tip technique is the most inexpensive easy technique among the literature. It also takes a reasonable amount of time and reduces antibody consumption. The major disadvantage of TMA technology is the cost, hence these inexpensive will improve the basic research(**Figure 5 & 6).** [59]

Mohamed a Elkablawy stated that conventional TV/radio telescopic antenna can be used to punch tissue cores manually from donor paraffin embedded tissue blocks which were pre-incubated at 40^{o}C. The technique was simple and caused minimal damage to the donor blocks. He concluded that this technique is easy to reproduce, quick, inexpensive and creates uniform blocks with abundant tissues without specialized equipment. It was found to improve the stability of the cores within the paraffin block and facilitated no losses during cutting and immunostaining.[60]

Fig 7 Usage of conventional TV Antenna in construction of TMA

Immunohistochemistry:

Immunohistochemistry involves two disciplines – immunology and histology. Immunohistochemistry is used to determine expression of particular antigen and its microanatomic location in the tissue. IHC uses antibodies to distinguish antigenic differences between the cells. These differences can specifically identify the lineage of cell populations and define biologically distinct population of cells within the same lineage.

Antigen retrieval technique was introduced by Shi and associates in 1991. It's a simple method that involves heating paraffin sections to a high temperature before IHC staining.The use of antiboby in IHC depends on sensitivity and specificity of antigen antibody reaction and the hybridoma technique provides limitless source of highly specific antibodies.

Detection systems :

Antibodies are labeled or flagged by some method to permit visualization – these include fluorescent substances , enzymes forming coloured reaction with suitable substrate (light microscopy) or heavy metals (electron microscopy).

Methods of IHC :

Direct conjugate labeled antibody method :

Antibody is attached with a label by chemical means and directly applied to tissue sections. It is a rapid and easy procedure and involves detection of multiple antigens which require separate incubation with specific antibodies.

Indirect sandwich method :

Enzymes are labeled with secondary antibody which is produced against primary antibody. The advantages are increased versatality , high working dilution of primary antibody and easy preparation of secondary antibodies against a primary antibody of different species.

Unlabelled antibody methods :

Enzyme bridge technique :

Here the labeled moiety is linked to the antigen solely by immunologic binding.

Peroxidase antiperoxidase method :

The principle of the PAP method is similar to that of the enzyme bridge method. The acronym PAP denotes the peroxidase antiperoxidase reagent that consists of antibody against horseradish peroxidase and horseradish peroxidase antigen in the form of a small, stable immune complex. Available evidence suggests that this

immune complex typically consists of two antibody molecules and three horseradish peroxidase molecules in the configuration. The PAP reagent and the primary antibody must be from the same species (or from closely related species with common antigenic determinants),whereas the bridge or linking antibody is derived from a second species and has specificity against the primary antibody.

Avidin biotin technique :

The high affinity between biotin and avidin is used in this technique ;. Biotin binds to the primary antibody and avidin binds to the enzyme thus attaching it to the biotinylated antibody. Disadvantage of this procedure is the presence of endogeneous biotin activity that produces non specific background staining.

Avidin biotin conjugate procedure :

Here the primary antibody is added followed by biotinylated secondary antibody and next preformed complexes of avidin and biotin horse radish peroxidase conjugate.

Biotin streptavidin system :

Streptavidin is used in place of avidin, Streptavidin complexes are more stable compared to avidin.

Immunogold silver technique :

This is used in ultrastructural immunolocalisation. Gold particles are enhanced by addition of several layers of silver.

Polymeric method :

This technique allows the binding of a large number of enzyme molecules to a secondary antibody via dextran backbone. The advantages of this technique are increased sensitivity , minimized non specific background staining and reduction in number of assay steps.

Alkaline phosphatase and anti alkaline phosphatase method :

The principle is the same as that of PAP method .

Tissue fixation , Processing and antigen retrieval techniques :

Tissues for IHC undergo fixation , dehydration and Paraffin embedding.

Fixation :

This is a critical step as preservation of morphology is essential for interpretation . 10 % neutral buffered formalin is used .It has the following advantages :

1. Good morphological preservation
2. Cheap , easily available , penetrates tissues well and sterilizes them.

3. Carbohydrate antigens are better preserved and does not interfere with the staining process.

The disadvantage of masking antigens during fixation can be overcome by antigen retrieval technique .

Antigen retrieval:

The following techniques are used for unmasking of the antigen :

1. Proteolytic enzyme digestion
2. Microwave antigen retrieval
3. Microwave and trypsin antigen retrieval technique
4. Pressure cooker antigen retrieval.

IHC MARKERS

Hep Par-1 (Hepatocyte Paraffin 1) is a monoclonal antibody that reacts with an epitope of liver mitochondria, with a typical granular pattern in most liver specimens. It also reacts with other normal or pathological structure, such as renal tubules and intestinal epithelium as well as with intestinal metaplasia in the stomach and esophagus[37]. It produces positive staining in the majority of cases of hepatocellular carcinoma and only a small percentage of other tumors, including some cholangiocarcinomas and metastatic adenocarcinomas from the stomach and other sites[64]. Few scientific literatures reported cases of hepatocellular carcinoma negative for

Hep Par-1, probably due to the uneven distribution of Hep Par-1 in hepatocellular carcinoma.[62,63] The expression of the marker decreases with decreasing differentiation of the tumour.

The commercially available Hep Par 1antibody (clone OCH1E5.2.10) stains normal and neoplastic hepatocytes. This antibody was developed in 1993 by Wennerberg et al.[65] using fixed liver as immunogen. The target antigen has not yet been fully determined.

Cytokeratins (CKs) represent the epithelial class of intermediate- sized filaments of the cytoskeleton. There are 20 subtypes of cytokeratin (CK) intermediate filaments. These have different molecular weights and demonstrate differential expression in various cell types and tumors. Among the most useful cytokeratins are CK7 and CK20. CK7 is found in many ductal and glandular epithelia, including lung, breast, ovary, and endometrium. CK20 is expressed in the gastrointestinal (GI) epithelium, urothelium, and Merkel cells. The combined expression patterns of CK7 and CK20 have been extensively studied in various primary and metastatic carcinomas. CK20 is expressed alone in the majority of intestinal adenocarcinoma and in Merkel cell carcinomas whereas CK7 is present without CK20 in most breast, lung and ovarian adenocarcinoma, and with CK20 in urothelial, pancreatic and gastric carcinomas. The CK7-/CK20+ expression pattern is known to be highly characteristic of colorectal

carcinomas, however, not all colorectal carcinomas show the CK7-/CK20+ expression pattern. Occasionally colorectal carcinomas may show significant CK7 expression and conversely, expression of CK20 may be seen in a variety of non-colorectal adenocarcinomas such as urothelial, gastric and pancreatobiliary tract carcinomas.[66]

MATERIALS AND METHODS

The study was carried out in the Department of Pathology, Govt. Stanley Medical College, from July 2012 to June 2015 after obtaining the approval from Institutional Human Ethical Committee (IHEC) of Govt. Stanley Medical College, Chennai.

Total of 60 specimens were taken for this study.

INCLUSION CRITERIA

Histologically diagnosed cases of primary hepatocellular carcinoma, cholangiocarcinoma and metastatic secondaries from colorectal region in liver. (Trucut biopsies and resection specimens).

EXCLUSION CRITERIA

Benign tumours of liver, tumours of liver in infancy, mesenchymal tumours of liver.

METHODOLOGY

For all the 60 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), intra hepatic cholangiocarcinoma and metastatic secondary deposits 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 Pathology, Govt. Stanley 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. 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, CK7,19,20 are normal liver tissue, moderately differentiated gastric carcinoma, cholangiocarcinoma and moderately differentiated colonic adenocarcinoma respectively.

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 70^{o}C for an hour.

Only 60 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;

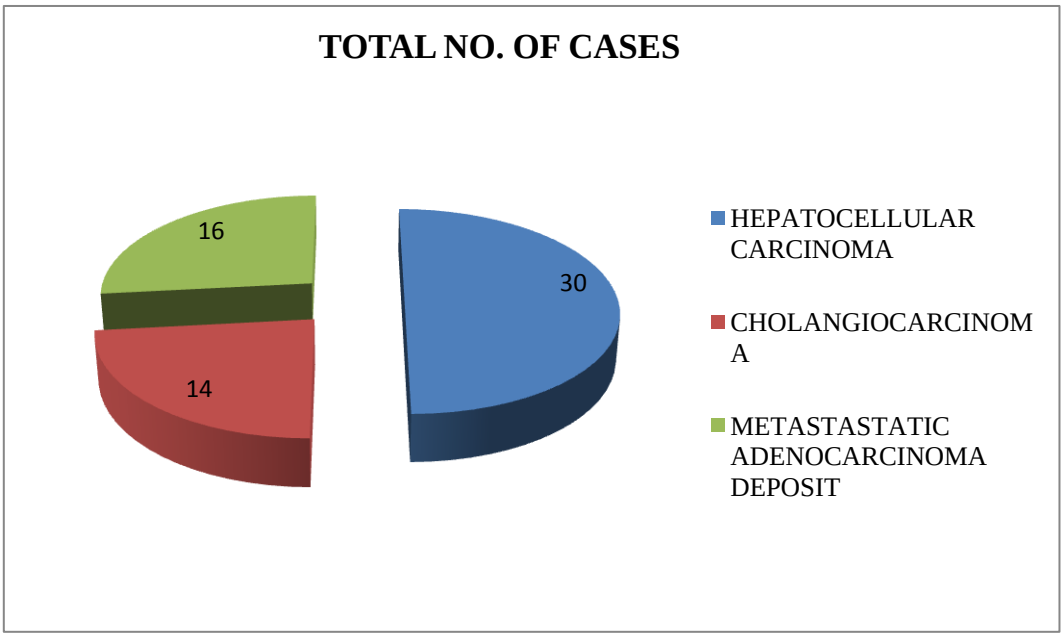
5 = 75 - 90% positive; and

6 > 90% of tumour cells positive.

CK 7, 19, 20 - In this study we have used rabbit monoclonal antibodies which shows brown cytoplasmic and membranous staining. Positive immunoreactivity was defined as more than 20% of cells staining with the proper pattern of reactivity[40].

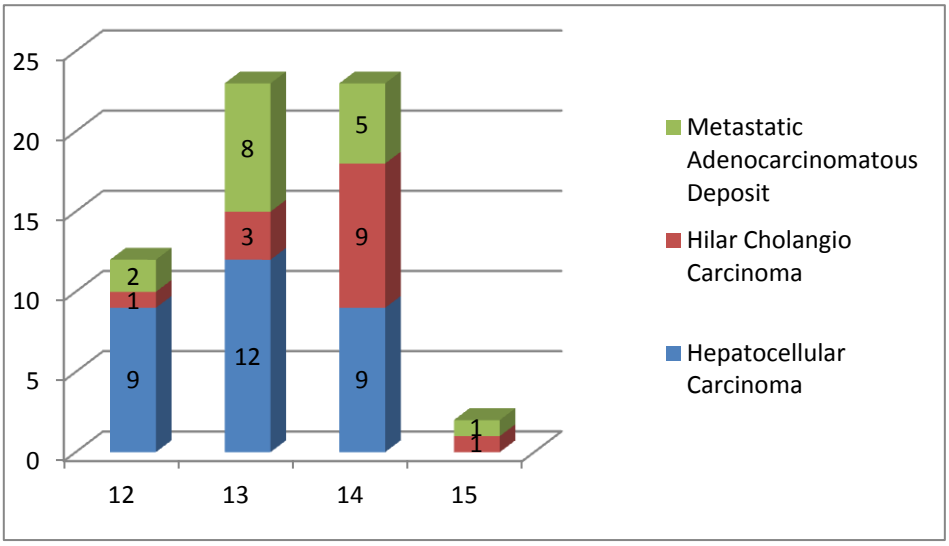
OBSERVATION AND RESULTS

In this present study we have included 60 cases of liver biopsies and resection specimens, out of which 30 were hepatocellular carcinoma, 14 were cholangiocarcinoma and 16 were metastatic adenocarcinomatous deposit in liver from colorectal region fulfilling the inclusion and exclusion criteria.



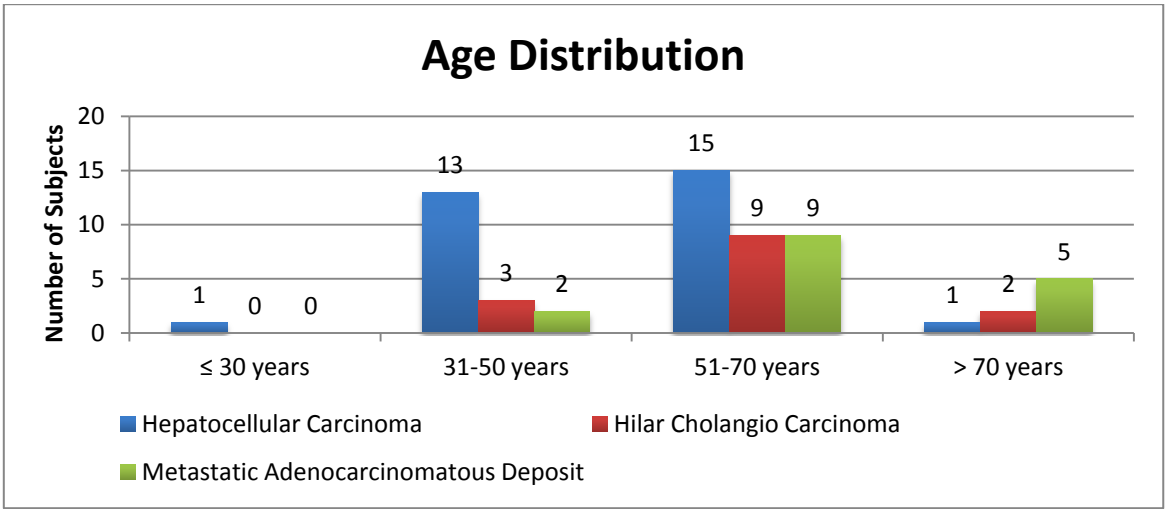
GRAPH NO.1- TOTAL NO. OF CASES

In this study(Graph no.1) 50% samples were cases of Hepatocellular carcinoma, 23% were Intrahepatic cholangiocarcinoma and 27% were metastatic adenocarcinomatous deposit in liver from colorectal region.



GRAPH NO.2- YEAR WISE DISTRIBUTION OF CASES

Out of the 60 samples studied, as depicted in the graph above(Graph no. 2), the samples of hepatocellular carcinoma was high compared to Intrahepatic cholangiocarcinoma and Metastatic adenocarcinomatous deposit in liver from colorectal region in 2012 and 2013. In 2014, the samples of Hepatocellular carcinoma and Intrahepatic cholangiocarcinoma were equal. In 2015, only 2 samples were studied which were Intrahepatic cholangiocarcinoma and Metastatic adenocarcinomatous deposit in liver from colorectal region.



GRAPH NO. 3- AGE WISE DISTRIBUTION OF CASES

Out of the 60 samples studied (Graph no. 3), majority of cases with Hepatocellular carcinoma, Cholangiocarcinoma and Metastatic Adenocarcinomatous deposit 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 two neoplasms in liver. More than 70 years, the incidence of Metastatic Adenocarcinomatous deposit in liver was high compared to other neoplasms in liver. While there is only one case of Hepatocellular carcinoma below 30 years.

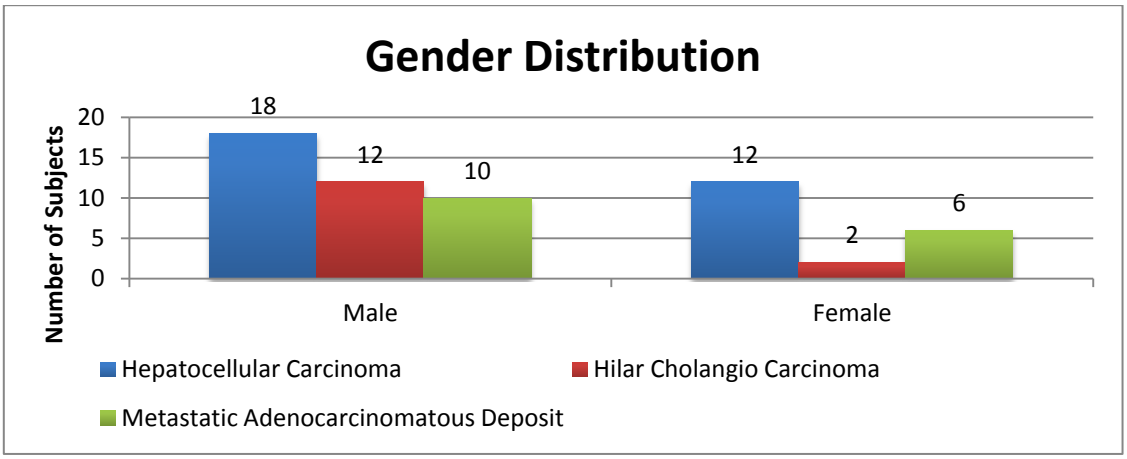
Age Distribution	Hepatocellular Carcinoma	%	Hilar Cholangio Carcinoma	%	Metastatic Adenocarcinomatous Deposit	%
≤ 30 years	1	3.33	0	0.00	0	0.00
31-50 years	13	43.33	3	21.43	2	12.50
51-70 years	15	50.00	9	64.29	9	56.25
> 70 years	1	3.33	2	14.29	5	31.25
Total	30	100	14	100	16	100

TABLE NO. 1- AGE WISE DISTRIBUTION OF CASES

Age Distribution	Hepatocellular Carcinoma	Hilar Cholangio Carcinoma	Metastatic Adenocarcinomatous Deposit
N	30	14	16
Mean	51.63	57.43	63.19
SD	10.85	12.16	14.26

TABLE NO. 2 COMPARISON OF MEAN AGE BETWEEN THE THREE STUDY GROUPS

The above table (Table no.2) depicts the mean age incidence of Hepatocellular carcinoma as 51.63 years, Cholangiocarcinoma as 57.43 years and Metastatic Adenocarcinomatous deposit in liver as 63.19 years.

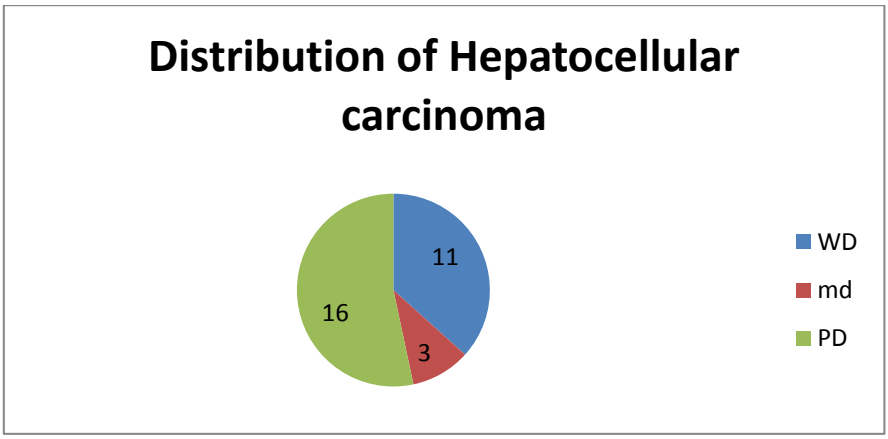


GRAPH NO. 4 GENDER DISTRIBUTION OF CASES

The incidence of Hepatocellular carcinoma, Cholangiocarcinoma and Metastatic Adenocarcinomatous deposit 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 two malignancies.(Graph no.4 and Table no.3).

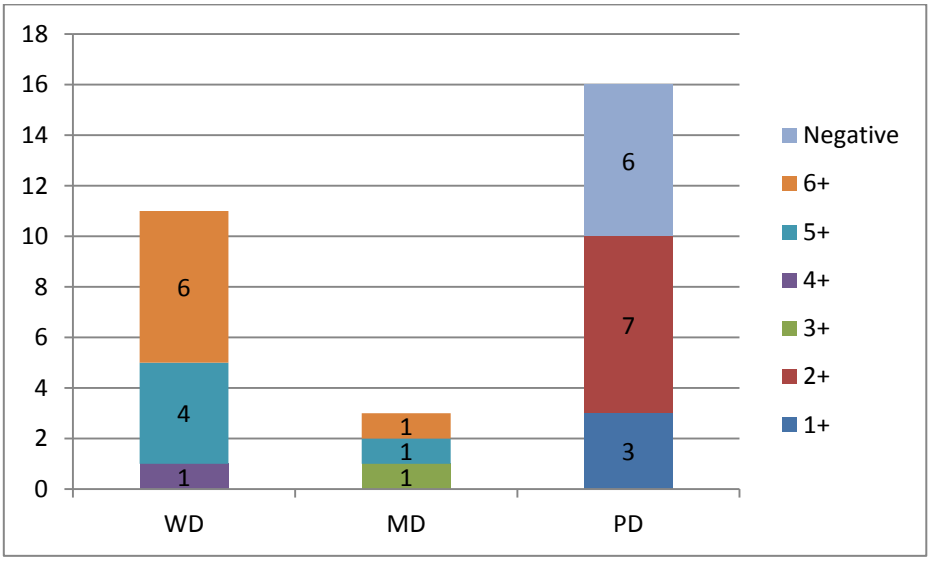
Gender Distribution	Hepatocellular Carcinoma	%	Hilar Cholangio Carcinoma	%	Metastatic Adenocarcinomatous Deposit	%
Male	18	60.00	12	85.71	10	62.50
Female	12	40.00	2	14.29	6	37.50
Total	30	100	14	100	16	100

TABLE NO.3- GENDER DISTRIBUTION OF CASES



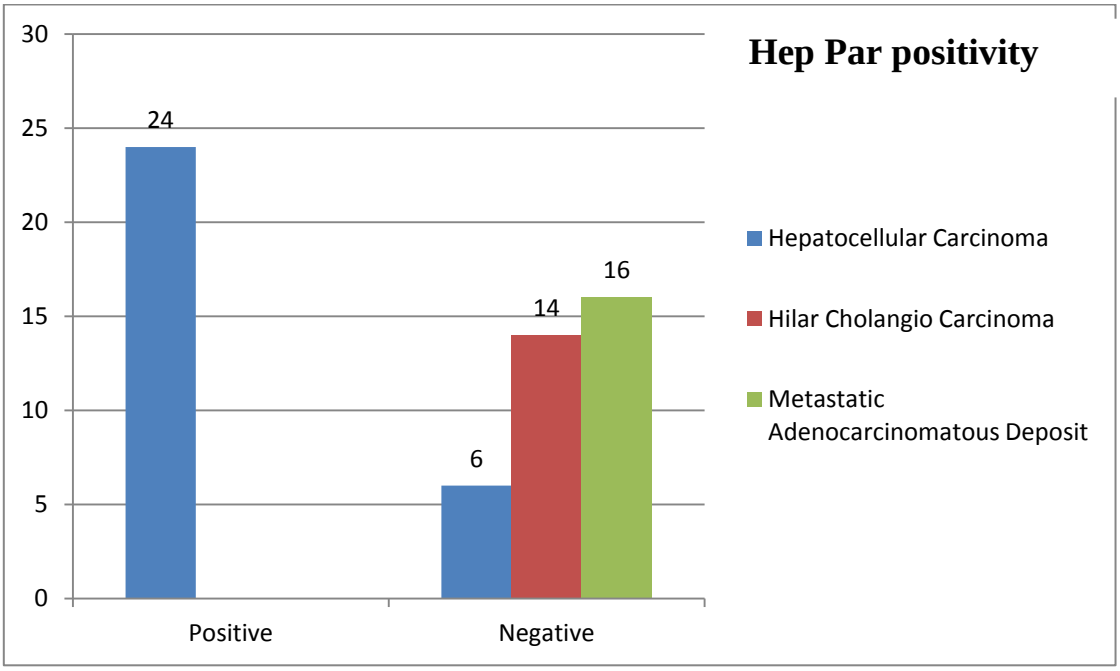
GRAPH NO. 5 – DISTRIBUTION OF HEPATOCELLULAR CARCINOMA ACCORDING TO DIFFERENTIATION

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. 5)



GRAPH NO.6 DISTRIBUTION OF HEPATOCELLULAR CARCINOMA ACCORDING TO Hep Par 1 REACTIVITY WITH RESPECT TO DIFFERENTIATION OF TUMOURS

The above graph(Graph no.6) 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.

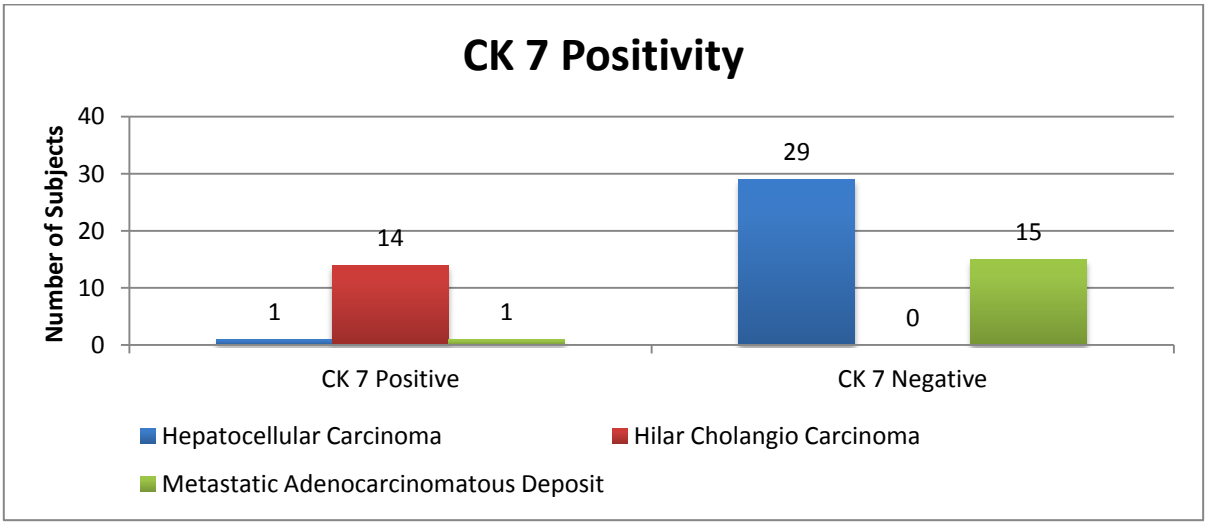


GRAPH NO. 7 DISTRIBUTION OF Hep par1 REACTIVITY IN ALL THREE MALIGNANCIES

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. Hep par1 was negative for 100% cases of Intrahepatic Cholangiocarcinoma and Metastatic Adenocarcinomatous Deposit in liver from colorectal region.(Graph No. 7 & Table no. 4)

Hep Par1 Positivity	Hepatocellular Carcinoma	%	Hilar Cholangio Carcinoma	%	Metastatic Adenocarcinomatous Deposit	%
Hep Par1 Positive	24	80	0	0.00	0	0.00
Hep Par1 Negative	6	20	14	100.00	16	100.00
Total	30	100	14	100	16	100

TABLE NO. 4 - DISTRIBUTION OF Hep par1 REACTIVITY IN ALL THREE MALIGNANCIES

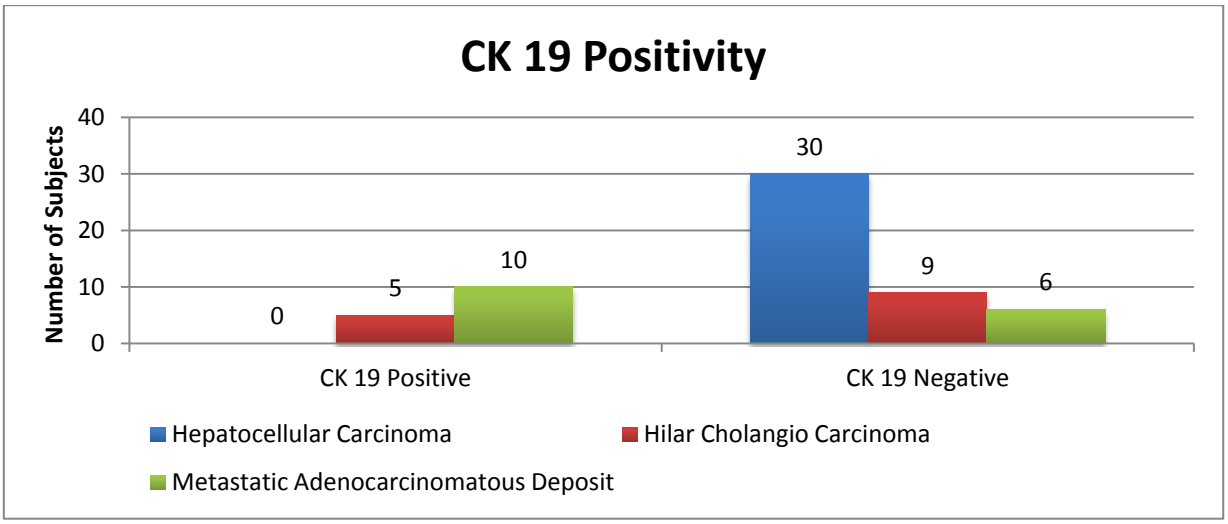


GRAPH NO. 8 - DISTRIBUTION OF CK 7 REACTIVITY IN ALL THREE MALIGNANCIES

CK 7 Positivity	Hepatocellular Carcinoma	%	Hilar Cholangio Carcinoma	%	Metastatic Adenocarcinomatous Deposit	%
CK 7 Positive	1	3.33	14	100.00	1	6.25
CK 7 Negative	29	96.67	0	0.00	15	93.75
Total	30	100	14	100	16	100

TABLE NO. 5- DISTRIBUTION OF CK 7 REACTIVITY IN ALL THREE MALIGNANCIES.

In this study,(Graph no. 8 & Table No. 5) CK 7 was positive in 100% cases of Cholangiocarcinoma, 3.33% 0f Hepatocellular carcinoma and 6.25% in Metastatic Adenocarcinomatous deposit in liver from colorectal region. CK 7 was negative in 96.67% cases of Hepatocellular carcinoma and 93.75% cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region.

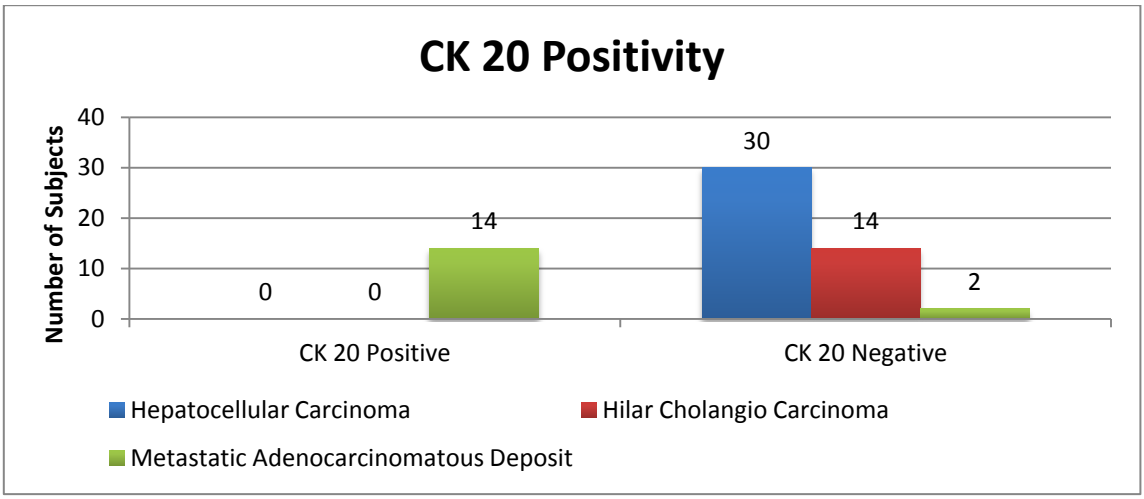


GRAPH NO. 9 - DISTRIBUTION OF CK 19 REACTIVITY IN ALL THREE MALIGNANCIES

CK 19 Positivity	Hepatocellular Carcinoma	%	Hilar Cholangio Carcinoma	%	Metastatic Adenocarcinomatous Deposit	%
CK 19 Positive	0	0.00	5	35.71	10	62.50
CK 19 Negative	30	100.00	9	64.29	6	37.50
Total	30	100	14	100	16	100

TABLE NO. 6 - DISTRIBUTION OF CK 19 REACTIVITY IN ALL THREE MALIGNANCIES

In this study, CK 19 was positive in 35.71% cases of Cholangiocarcinoma and 62.50% in Metastatic Adenocarcinomatous deposit in liver from colorectal region. CK 19 was negative in 100% cases in Hepatocellular carcinoma, 64.29% cases in Cholangiocarcinoma and 37.50% in Metastatic Adenocarcinomatous deposit in liver from colorectal region.(Graph no. 9 & Table No. 6)



GRAPH NO. 10- DISTRIBUTION OF CK 20 REACTIVITY IN ALL THREE MALIGNANCIES

CK 20 Positivity	Hepatocellular Carcinoma	%	Hilar Cholangio Carcinoma	%	Metastatic Adenocarcinomatous Deposit	%
CK 20 Positive	0	0.00	0	0.00	14	87.50
CK 20 Negative	30	100.00	14	100.00	2	12.50
Total	30	100	14	100	16	100

TABLE NO. 7 - DISTRIBUTION OF CK 20 REACTIVITY IN ALL THREE MALIGNANCIES

In this study, CK 20 was positive only in 87.50% cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region. CK 20 was negative in 100% cases of Hepatocellular carcinoma and Cholangiocarcinoma with 12.50% cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region.(Graph no. 10 & Table No. 7)

DISCUSSION

This cross sectional study was carried out in the Department of Pathology, Govt. Stanley Medical College. Total number of cases studied were 60, which included 30 cases of Hepatocellular carcinoma(11-well differentiated,3-moderately differentiated,16-poorly differentited), 14 cases of Intrahepatic cholangiocarcinoma and 16 cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region.

1.**AGE OF THE PATIENT**

In our study the age of the patient with Hepatocellular carcinoma ranges from 27 to 73 years with mean age of 51.3 years(Graph No.3,Table No.1 & 2).

In 2012, Hashem B. El-Serag, studied the epidemiology of HCC world wide and concluded that in low risk population the incidence is >70 years and in high risk groups it is between 50 to 60 years[68].

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.[67]

In our study the age of the patient with Intrahepatic cholangiocarcinoma ranges from 35 to 80 years, with mean age of 57.43 years.

Ahmad Ramzi Yusoff et al. studied the survival analysis of cholangiocarcinoma and revealed that the mean age of diagnosis is 61 years[69].

Sean F. Altekruse studied the geographic variation of Intrahepatic Cholangiocarcinoma, Extrahepatic Cholangiocarcinoma, and Hepatocellular Carcinoma in the United States and stated that incidence was high in all three cancers in age group of more than 70 years[70].

In our study the age of the patient with Metastatic Adenocarcinomatous deposit in liver from colorectal region ranges from 32 to 89 years with mean age being 63.19 years.

Sylvain Manfredi et al studied the epidemiology of liver colorectal cancer metastases and concluded that the peak incidence of metastasis is from 65 and 74 years[71].

2. **GENDER**

In our study in all the three malignancies of the liver, the incidence is high in males compared to that of females. 60% in Hepatocellular carcinoma, 85.71% in Intrahepatic cholangiocarcinoma and 62.5% in Metastatic Adenocarcinomatous deposit in liver from colorectal region.

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[67].

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.[68]

Sean F. Altekruse studied the geographic variation of Intrahepatic Cholangiocarcinoma, Extrahepatic Cholangiocarcinoma, and 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)[70].

Sylvain Manfredi et al studied the Epidemiology of Liver Colorectal Cancer Metastases and concluded that the sex ratio is 2:1[71].

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.[7,9]

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.[62]

4.CK 7,19 20 (Cytokeratin 7,19,20)

In this study, CK 7 was positive in 100% cases of Cholangiocarcinoma(**Figure 24**), 3.33% 0f Hepatocellular carcinoma and 6.25% in Metastatic Adenocarcinomatous deposit in liver from colorectal region. CK 7 was negative in 96.67% cases of Hepatocellular carcinoma and 93.75% cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region.

In this study, CK 19 was positive in 35.71% cases of Cholangiocarcinoma (**Figure 25**)and 62.50% in Metastatic Adenocarcinomatous deposit in liver from colorectal region(**Figure 22**). CK 19 was negative in 100% cases in Hepatocellular carcinoma, 64.29% cases in Cholangiocarcinoma and 37.50% in Metastatic Adenocarcinomatous deposit in liver from colorectal region.

In this study, CK 20 was positive only in 87.50% cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region(**Figure 23**). CK 20 was negative in 100% cases of Hepatocellular carcinoma and Cholangiocarcinoma with 12.50% cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region.

Shimonoshi et al concluded in his study in 2000 that CK 7,19 and 20 are useful to differentiate intrahepatic cholangiocarcinoma from metastatic adenocarcinomas in liver from colorectal regions; it also indicates the

primary focus of metastatic adenocarcinomas in livers. In his study CK 7 was positive in 97% and CK 19 was in 92% cases of Intrahepatic Cholangiocarcinoma. CK 20 was positive in 81% of metastatic adenocarcinomas in liver from colorectal region. The expression of CK 19 in intrahepatic cholangiocarcinoma was decreases with the differentiation of the tumour. The positivity of CK 19 was less in moderately and poorly differentiated cholangiocarcinomas compared to well differentiated cholangiocarcinoma. [42]

These results suggest that reactivity of bile duct–type cytokeratin was reduced or lost in a small number of cholangiocarcinomas during neoplastic transformation or tumor development.[48]

Rullier A et al in his study revealed that CK 7 was positive in 100% and Ck 20 was 47.36% in Intrahepatic Cholangiocarcinoma. In metastatic adenocarcinoma CK 20 was 100% and CK 7 was 24%.[72]

Bayrak et al in his study stated the CK 20 negative cases of colorectal adenocarcinoma can be confirmed by CDX2.[73]

TISSUE MICRO ARRAY

The advantages of manually made tissue micro array in our study is of:

- Low cost

- Less time consuming
- Less amount of IHC markers
- Large number of cases can be done in short time

Shebl et al made 1mm cores in their study using mechanical pencil tip and advantages of this size are easy to sample from donor blocks and there is no splitting artifacts in the hot water bath during sectioning.[58]

In our study we had also used 1.0mm core size and the results were the same.

Chang Hwan Choi et al in his study stated that the core loss in manual TMA array is 3% and in our study was 6%. [57]

Mohamed A Elkablawy et al used radio antenna to make cores of size 2,3,4mm size and they states that the larger the core size, the blocks will be uniform, there will be more stability of cores in the block and no loss of cores during cutting and sectioning[59].

SUMMARY AND CONCLUSION

The study was carried out in the Department of Pathology, Govt.Stanley Medical College, over a period of three years from July 2012 to June 2015 after obtaining the approval from Institutional Human Ethical Committee (IHEC) of Govt. Stanley Medical College, Chennai.

1) Total number of cases studied were 60, which included 30 cases of Hepatocellular carcinoma(11-well differentiated,3-moderately differentiated,16-poorly differentited), 14 cases of Intrahepatic cholangiocarcinoma and 16 cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region.

In this study we prepared manual tissue microarray blocks from the selected liver specimens fulfilling the inclusion and exclusion criteria.

TMA blocks are sectioned and Immunohistochemistry is done using Hep par1, CK 7, 19 and 20 to differentiate Hepatocellular carcinoma, Intrahepatic Cholangiocarcinoma and Metastatic Adenocarcinomatous deposit in liver from colorectal region.

2) Incidence of HCC, ICC and Metastatic adenocarcinomatous deposit in liver fron colorectal region was high between 51 to 70 years.

3) Incidence of HCC was high compared other tumours.

4) There was only a single case of HCC below 30 years.

5) The mean age incidence of Hepatocellular carcinoma was 51.63 years, Intrahepatic cholangiocarcinoma was 57.43 years and Metastatic Adenocarcinomatous deposit in liver was 63.19 years.

6) Incidence of HCC was high in both genders.

7) Out of 30 cases of hepatocellular carcinoma, 11 were well differentiated, 3 were moderately differentiated and 16 were poorly differentiated hepatocellular carcinomas.

8) Out of 30 cases of Hepatocellular carcinoma, 24 cases were positive for Hep par1 and 6 cases were negative for Hep par1. Hep par1 was negative for 100% cases of Intrahepatic Cholangiocarcinoma and Metastatic Adenocarcinomatous Deposit in liver from colorectal region.

9) CK 7 was positive in 100% cases of Cholangiocarcinoma, 3.33% 0f Hepatocellular carcinoma and 6.25% in Metastatic Adenocarcinomatous deposit in liver from colorectal region. CK 7 was negative in 96.67% cases of Hepatocellular carcinoma and 93.75% cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region.

10) CK 19 was positive in 35.71% cases of Cholangiocarcinoma and 62.50% in Metastatic Adenocarcinomatous deposit in liver from colorectal region. CK 19 was negative in 100% cases in Hepatocellular carcinoma, 64.29% cases in Cholangiocarcinoma and 37.50% in Metastatic Adenocarcinomatous deposit in liver from colorectal region.

11) CK 20 was positive only in 87.50% cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region. CK 20 was negative in 100% cases of Hepatocellular carcinoma and Cholangiocarcinoma with 12.50% cases of Metastatic Adenocarcinomatous deposit in liver from colorectal region.

By the end of this study we conclude that manual TMA technique is superior to conventional technique of Immunohistochemistry and automated Micro arrayer ---instruments in terms of cost, time consumption, amount of reagents used and preservation of tissue of interest.

Using this panel of markers-Hep par1, CK 7,CK 19 and CK 20 we can differentiate Hepatocellular carcinoma, Intrahepatic Cholangiocarcinoma and Metastatic Adenocarcinomatous deposit in liver from colorectal region. The diagnosis of these malignancies is very important because the treatment protocols differ for each of these malignancies.

FIGURES

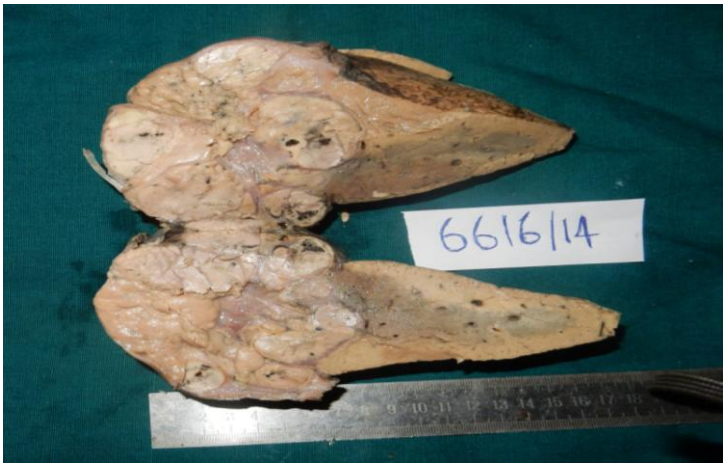


Fig 8 Gross picture of Hepatocellular carcinoma involving segment of liver

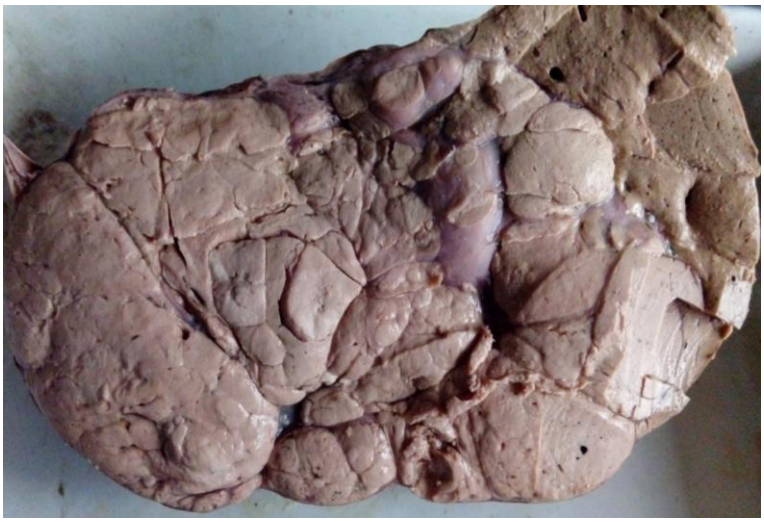


Fig 9 Gross picture of Hepatocellular carcinoma involving whole lobe

Fig 10 Gross picture of Cholangiocarcinoma

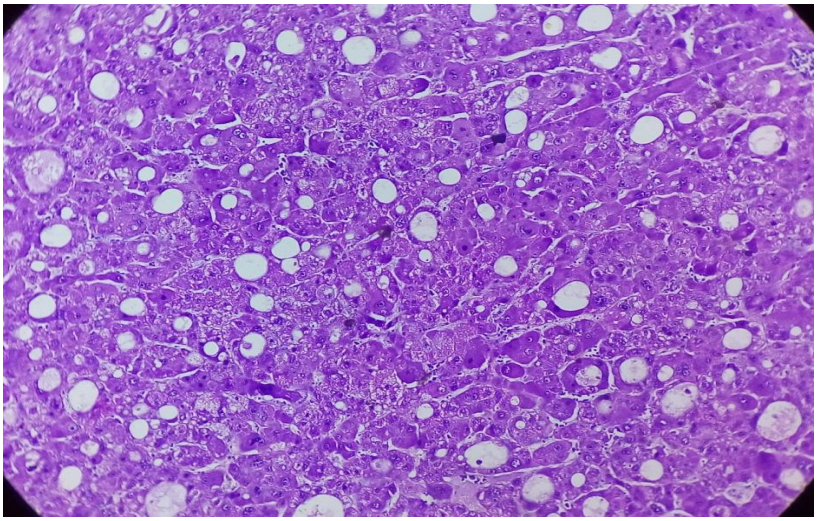


Fig 11 H & E picture of Well Differentiated Hepatocellular Carcinoma(10 X View)

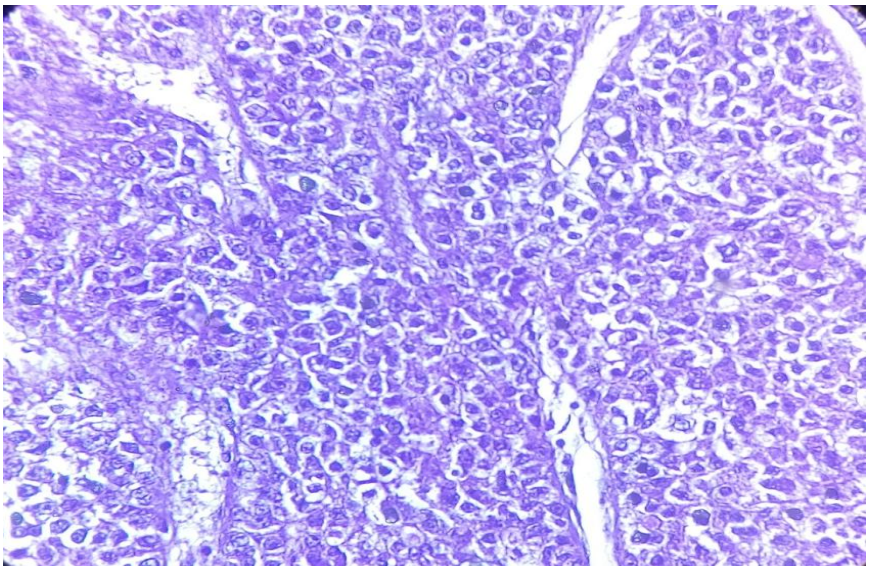


Fig 12 H & E picture of Moderately Differentiated Hepatocellular Carcinoma(40 X View)

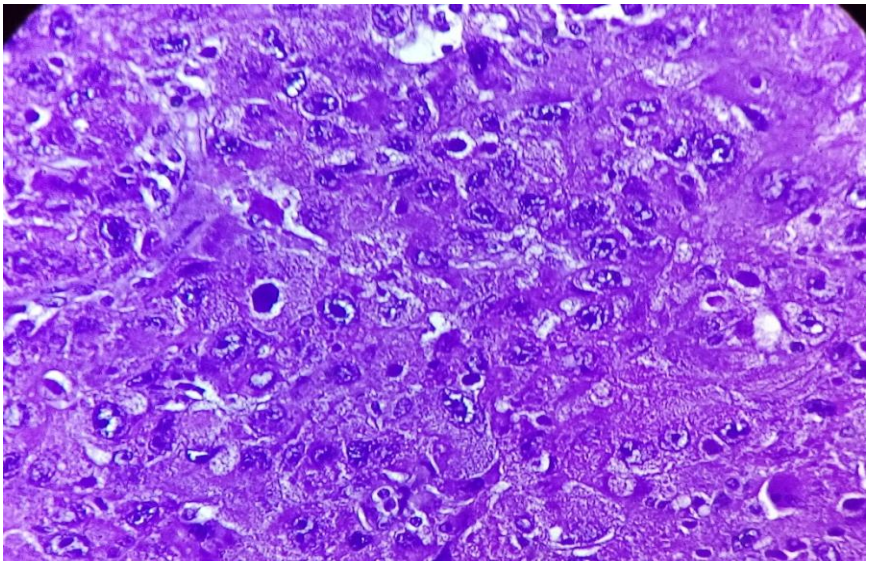


Fig 13 H & E picture of Poorly Differentiated Hepatocellular Carcinoma(40 X View)

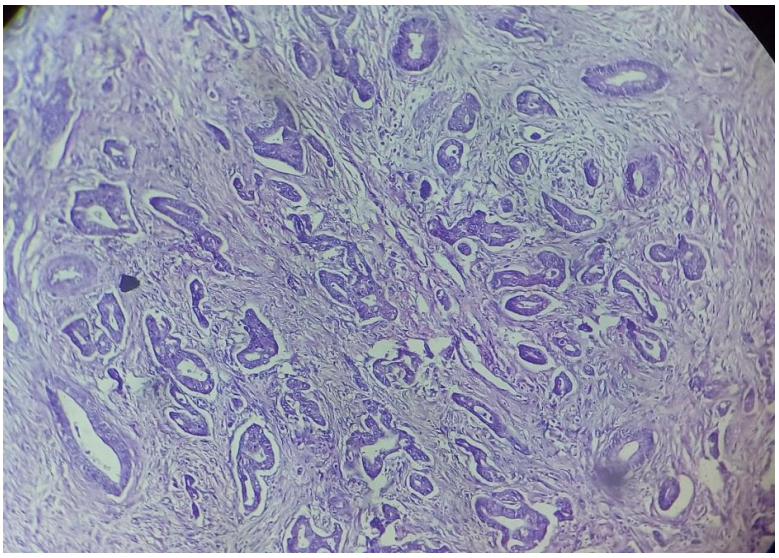


Fig 14 H & E picture of Cholangiocarcinoma(40 X View)

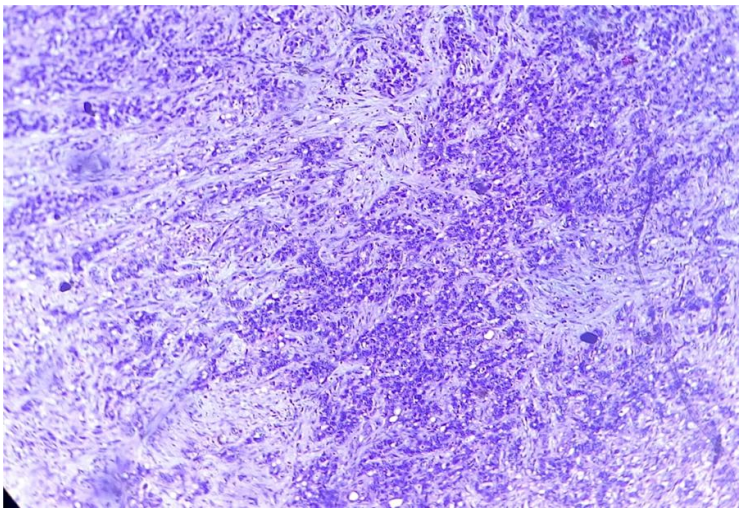


Fig 15 H & E picture of Metastatic Adenocarcinoma deposit in Liver (10 X View)

Fig 16 Bone marrow aspiration needle (16 gauge used) for taking core from donor block

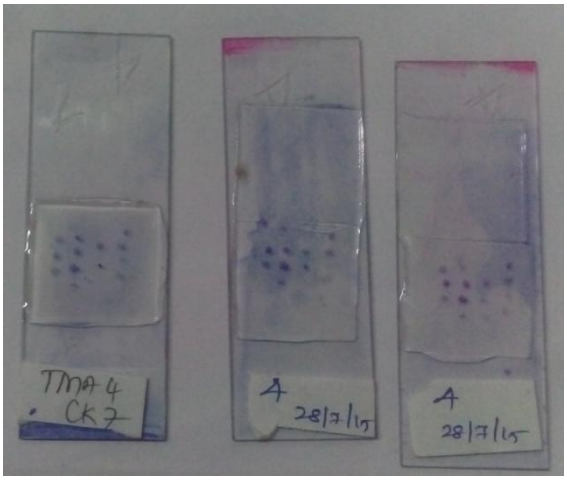


Fig 17 TMA Slides(H & E, IHC)

Fig 18 TMA BLOCK 1

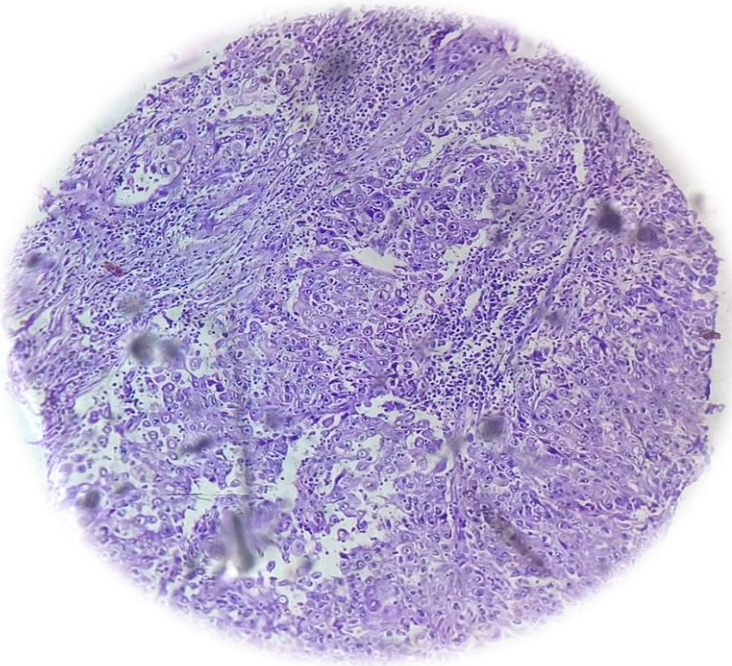


Fig 19 H & E picture of TMA Core (10 X View)

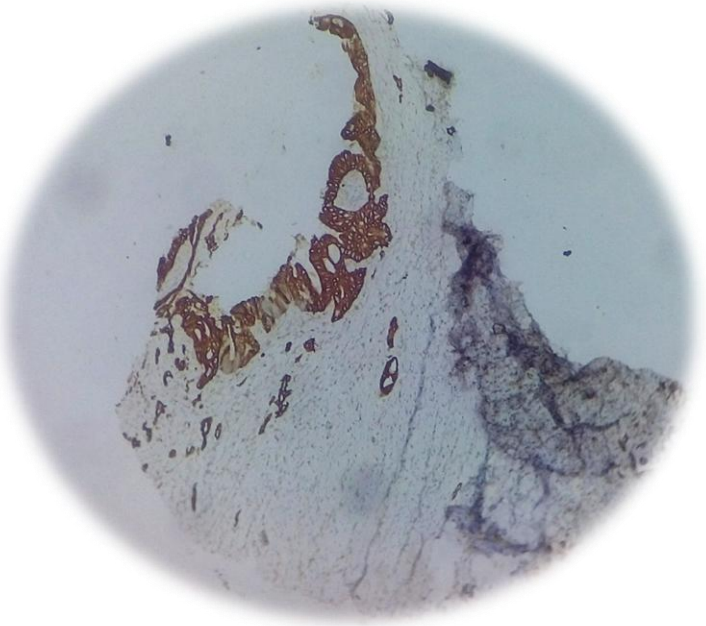


Fig 20 CK 20 control positivity in Colonic Adenocarcinoma(10 X View)

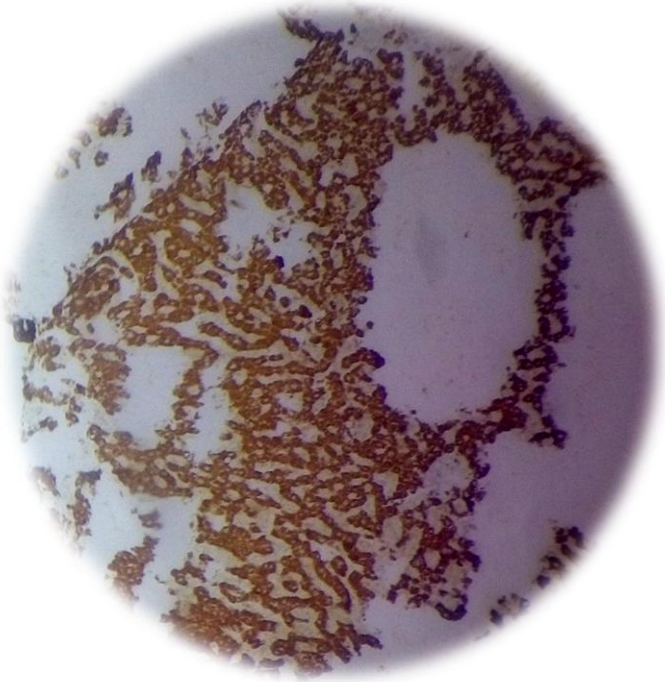


Fig 21 Hep Par1 positivity in Well differentiated HCC(6 + positivity)

- **10 X View**

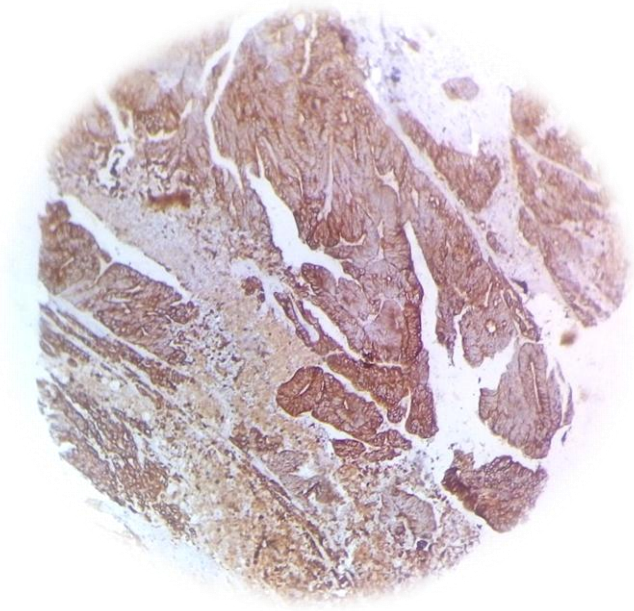


Fig 22 CK 19 Positivity in Metastatic Adenocarcinomatous deposit (10 X View)

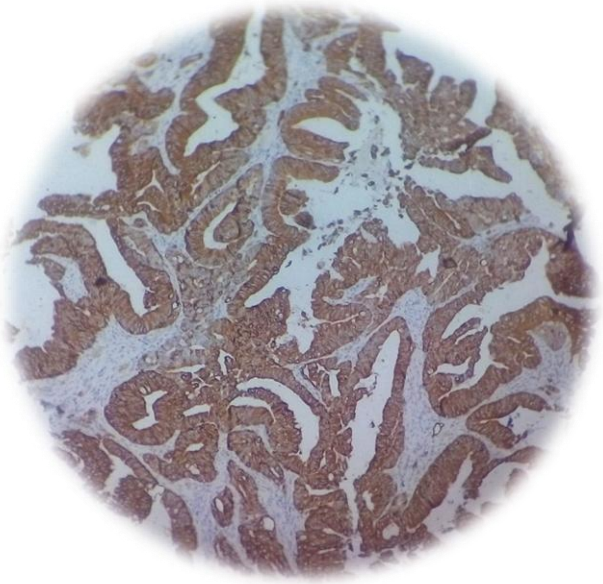


Fig 23 CK 20 Positivity in Metastatic Adenocarcinomatous deposit (10 X View)

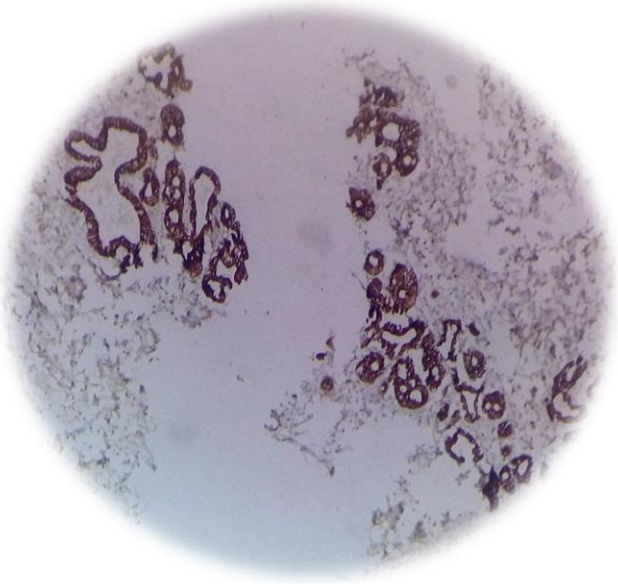


Fig 24 CK 7 Positivity in Intrahepatic Cholangiocarcinoma (10 X View)

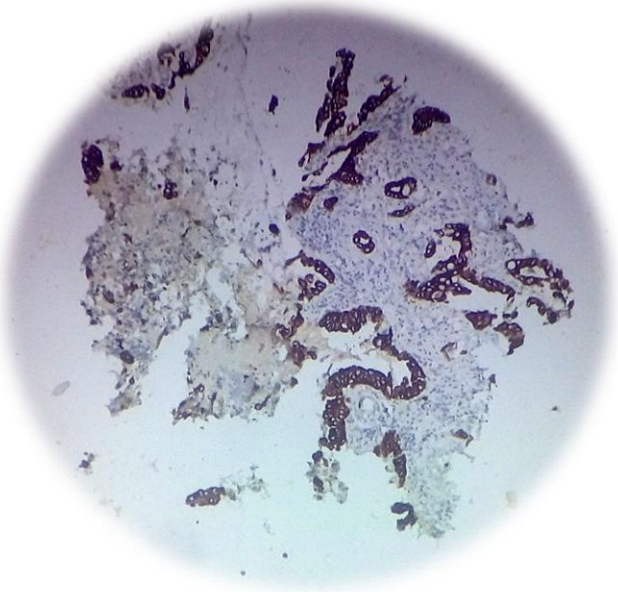


Fig 25- CK 19 Positivity in Intrahepatic Cholangiocarcinoma (10 X View)

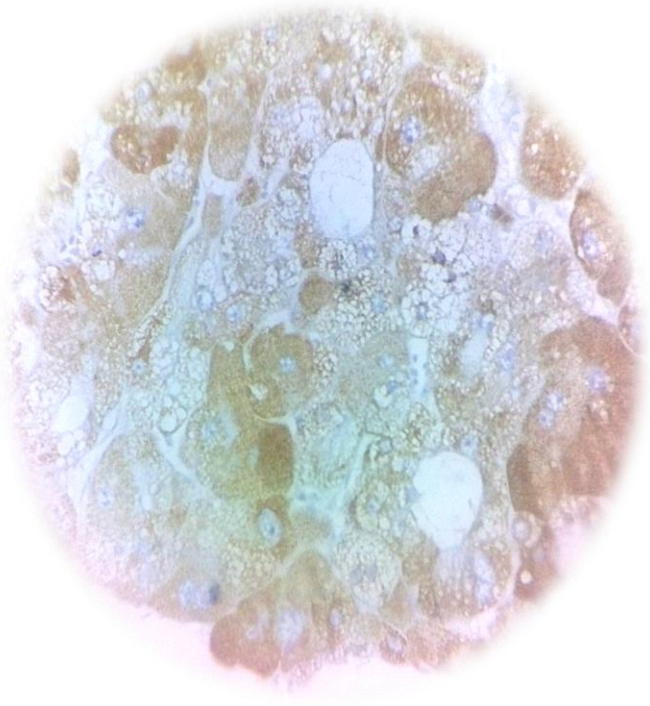


Fig 26 Hep Par1 Positivity(5+) in Well differentiated Hepatocellular Carcinoma(40 X View)

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MASTER CHART

S.No	Biopsy Number	Age	Sex	Histological Diagnosis	Grade	Hep Par1	CK 7	CK 19	CK 20
1	6708/14	55	Male	Hilar Cholangio Carcinoma		Negative	Positive	Positive	Negative
2	6616/14	65	Male	Hepatocellular Carcinoma	PD	2+	Positive	Negative	Negative
3	5952/14	60	Female	Hepatocellular Carcinoma	MD	5+	Negative	Negative	Negative
4	5324/14	75	Male	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Positive	Negative
5	4779/14	60	Female	Hepatocellular Carcinoma	PD	2+	Negative	Negative	Negative
6	4561/14	62	Male	Hilar Cholangio Carcinoma		Negative	Positive	Negative	Negative
7	3889/14	43	Female	Hilar Cholangio Carcinoma		Negative	Positive	Positive	Negative
8	3756/14	73	Male	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Positive	Positive
9	3408/14	41	Female	Hepatocellular Carcinoma	PD	1+	Negative	Negative	Negative
10	3355/14	35	Male	Hilar Cholangio Carcinoma		Negative	Positive	Negative	Negative
11	3025/14	62	Male	Hepatocellular Carcinoma	PD	2+	Negative	Negative	Negative
12	2915/14	78	Male	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Negative	Positive
13	2815/14	65	Male	Hilar Cholangio Carcinoma		Negative	Positive	Negative	Negative
14	2341/14	52	Male	Hilar Cholangio Carcinoma		Negative	Positive	Positive	Negative
15	2209/14	43	Male	Hilar Cholangio Carcinoma		Negative	Positive	Negative	Negative
16	2199/14	60	Female	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Negative	Positive
17	2166/14	27	Female	Hepatocellular Carcinoma	PD	Negative	Negative	Negative	Negative
18	1979/14	57	Male	Hilar Cholangio Carcinoma		Negative	Positive	Positive	Negative
19	954/14	50	Male	Hepatocellular Carcinoma	WD	5+	Negative	Negative	Negative
20	903/14	80	Male	Hilar Cholangio Carcinoma		Negative	Positive	Negative	Negative
21	773/14	35	Female	Hepatocellular Carcinoma	PD	Negative	Negative	Negative	Negative
22	754/14	63	Male	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Negative	Positive
23	482/14	60	Male	Hepatocellular Carcinoma	WD	6+	Negative	Negative	Negative
24	6586/13	60	Female	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Positive	Positive
25	4628/13	50	Female	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Positive	Positive
26	4610/13	80	Male	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Positive	Positive
27	4446/13	53	Male	Hilar Cholangio Carcinoma		Negative	Positive	Positive	Negative
28	4010/13	45	Female	Hepatocellular Carcinoma	PD	1+	Negative	Negative	Negative
29	3799/13	55	Male	Hepatocellular Carcinoma	WD	6+	Negative	Negative	Negative
30	3200/13	42	Male	Hepatocellular Carcinoma	PD	Negative	Negative	Negative	Negative
31	3017/13	57	Male	Hepatocellular Carcinoma	MD	6+	Negative	Negative	Negative
32	2820/13	57	Male	Hepatocellular Carcinoma	WD	6+	Negative	Negative	Negative
33	2488/13	60	Female	Hepatocellular Carcinoma	WD	6+	Negative	Negative	Negative
34	2643/13	55	Male	Metastatic		Negative	Negative	Positive	Positive

				Adenocarcinomatous Deposit					
35	2278/13	60	Female	Hilar Cholangio Carcinoma		Negative	Positive	Negative	Negative
36	1376/13	70	Female	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Positive	Positive
37	1323/13	68	Female	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Negative	Positive
38	1182/13	66	Male	Hepatocellular Carcinoma	PD	2+	Negative	Negative	Negative
39	703/15	89	Male	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Positive	Positive
40	1091/15	75	Male	Hilar Cholangio Carcinoma		Negative	Positive	Negative	Negative
41	6553/13	45	Female	Hepatocellular Carcinoma	MD	3+	Negative	Negative	Negative
42	1081/13	59	Female	Hepatocellular Carcinoma	PD	Negative	Negative	Negative	Negative
43	868/13	55	Male	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Positive	Positive
44	673/13	73	Male	Hepatocellular Carcinoma	PD	2+	Negative	Negative	Negative
45	477/13	46	Male	Hepatocellular Carcinoma	WD	5+	Negative	Negative	Negative
46	6105/12	61	Male	Hilar Cholangio Carcinoma		Negative	Positive	Negative	Negative
47	6017/12	58	Male	Hepatocellular Carcinoma	PD	Negative	Negative	Negative	Negative
48	4063/12	52	Male	Metastatic Adenocarcinomatous Deposit		Negative	Positive	Positive	Negative
49	3784/12	50	Male	Hepatocellular Carcinoma	PD	2+	Negative	Negative	Negative
50	3602/12	53	Male	Hepatocellular Carcinoma	WD	6+	Negative	Negative	Negative
51	2829/12	65	Male	Hepatocellular Carcinoma	WD	4+	Negative	Negative	Negative
52	1475/12	53	Male	Hepatocellular Carcinoma	PD	Negative	Negative	Negative	Negative
53	1207/12	40	Female	Hepatocellular Carcinoma	PD	2+	Negative	Negative	Negative
54	1166/12	37	Male	Hepatocellular Carcinoma	PD	1+	Negative	Negative	Negative
55	1052/12	45	Female	Hepatocellular Carcinoma	WD	5+	Negative	Negative	Negative
56	564/12	38	Male	Hepatocellular Carcinoma	WD	5+	Negative	Negative	Negative
57	339/12	51	Male	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Positive	Positive
58	6084/13	32	Female	Metastatic Adenocarcinomatous Deposit		Negative	Negative	Negative	Positive
59	6553/13	45	Female	Hepatocellular Carcinoma	WD	6+	Negative	Negative	Negative
60	5282/13	63	Male	Hilar Cholangio Carcinoma		Negative	Positive	Negative	Negative