DISSERTATION ON

PROGNOSTIC IMPACT OF CATHEPSIN D EXPRESSION IN PANCREATIC CARCINOMA A STUDY OF 50 CASES

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CHENNAI-TAMILNADU

DECLARATION BY THE CANDIDATE

I solemnly declare that this dissertation titled

"PROGNOSTIC IMPACT OF CATHEPSIN D EXPRESSION IN PANCREATIC CANCER– A STUDY OF 50 CASES" is the original and bonafide work done by me under the guidance of

Dr. V . RAMAMOORTHY , M.D., Professor, Department of Pathology at the Government Stanley Medical College & Hospital, Chennai – 600 001, during the tenure of my course in M.D. Pathology from May-2010 to April-2013 held under the regulation of the Tamilnadu Dr. M.G.R. Medical University, Guindy,

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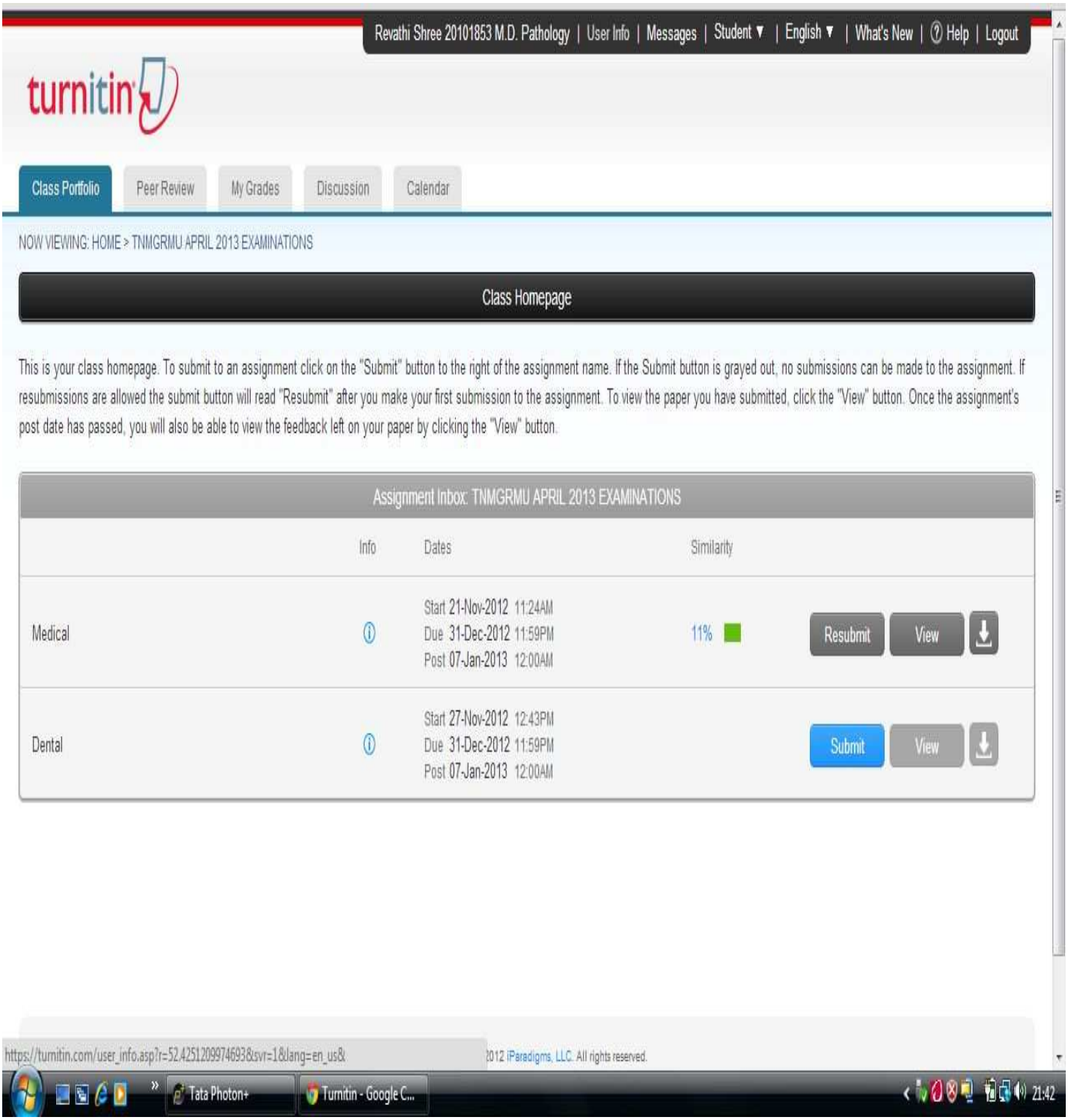
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ABBREVATIONS

CA 19.9 –Carbohydrate Antigen

CT – Computerised Topography

Cath – Cathepsin

5 –FU- 5- Fluorouracil

HPC- Human Pancreatic cells

IPMN- Intraductal Papillary Mucinous Neoplasm

LFT- Liver Function Test

Mac 2 BP- Macrophage 2 Binding Protein

MAP- Mitogen Activated Pathway

MD- Moderately Differentiated

MUC- Mucin

MVD- Micro vessel Density

NIR – Near Infra red fluorescence

Pan IN- Pancreatic Intraepithelial Neoplasm

PCR- Polymerase Chain Reaction

PD- Poorly Differentiated

Ro – no residual tumor

WD- Well Differentiated

VEGF- Vascular Endothelial Growth Factor

INTRODUCTION

One of the leading causes of mortality in case of malignancies is carcinoma of the pancreas. Worldwide, in death due to malignancies , adenocarcinoma of the pancreas , has the overall five year survival rate of 3% .

In India, the pancreatic cancer incidence is 1 to 2.5 / 1 lakh men. In the United states it is the fifth leading cause of death.

Most of the cases presents in advanced stage because of subtle clinical manifestation. The percentage of those patients presenting with a resectable tumor is only 13 -22 % [1] . With a curative resection the five year survival rate reaches to 25-30%. [2,3,4].

The causes for the poor prognosis of the tumor is attributed to:

- Subtle clinical presentation.
- Invasion of the adjacent blood vessels-Hematogenous metastasis [5]
- Neovascularization [6]
- High Micro Vessel Density (MVD) of the tumor [7]

- Angiogenesis promoting factor – Vascular Epidermal Growth Factor (VEGF) [8]

Besides angiogenesis, the local invasion of the tumor cells through detachment from the primary lesion is another important critical step in tumor progression and metastasis. Local tumor invasion and metastasis is also caused by the family of lysosomal cysteine proteases namely the Cathepsins. These proteases plays critical role in distinct tumorigenic processes such as proliferation, angiogenesis, apoptosis and invasion into adjacent organs and metastasis . They degrade the extracellular matrix and give neovascularization and tumor cell migration space [9,10,11]

In case of solid tumors , local tumor invasion has been promoted by lysosomal proteases cathepsin enzymes. Of which highly expressed Cathepsin plays critical role in invasion and metastases of pancreatic adenocarcinoma [12].

In a multi variate analysis, curative resection i.e no residual tumor (Ro) was the only clinical parameter related to favourable prognosis. Some cases have poor outcome despite curative resection [13] . In recent years, the expression of various factors like MVD, VEGF and cathepsins

were assessed using biological markers in terms of prognostic evaluation in pancreatic adenocarcinoma. Studies have shown that after curative resection numerous predictor factors UICC stage, , tumor grading and TNM classification for survival , but cathepsin is believed to be essential predictor for early tumor recurrence [14,15]. When compared to other organ tumors , pancreatic cancer has poor prognosis.

When all stages are combined together :

- 5-year relative survival rates is approximately 6%.
- 1 year survival rate is 22 – 26 %.

The 5-year survival rate is approximately 20% for local disease [16] .

In terms of months, the median survival rate:

- For locally advanced disease is 10 -12 months
- For metastatic disease, it is about 6 - 8months respectively [17,18].

This study focuses at assessing the correlation between the lysosomal proteases cathepsin D and pancreatic adenocarcinoma grade, perineural and lymphovascular invasion , serum Carbohydrate antigen 19.9 level in terms of prognosis. And thereby studying the histopathology of lesions with a note on incidence of the tumor during the study period.

AIMS AND OBJECTIVES

Primary Objective:

- To assess the diagnosis of pancreatic lesions by conventional histopathology technique .
- To assess the positivity of cathepsin D and its correlation with nuclear grade, perineural invasion, lymphovascular invasion and serum carbohydrate antigen 19.9 levels.

Secondary Objective:

- To study the clinicopathological correlation in pancreatic carcinoma .
- To find the incidence of pancreatic carcinoma during the study period.
- To analyse pancreatic carcinoma with respect to age and sex of patients.
- To analyse the correlation between alcoholism and pancreatic carcinoma

REVIEW OF LITERATURE

Pancreas adenocarcinoma is fifth cause of cancer related death .

In the global scale , Pancreatic carcinoma accounts for eighth cause of cancer-related deaths. fourth In the United states it is the fifth leading cause of death. [16].

The incidence of pancreatic cancer in India is 1 to 2.5 / 1 lakh men .

The 5-yr overall survival rate is only 3% [17].

Pancreatic carcinoma originates from the transformed cells arising in pancreatic tissues. Adenocarcinoma accounts for 95% of the pancreatic tumors , which arise from the pancreatic exocrine component. Neuroendocrine tumors , which constitutes minority of pancreatic tumor , arise from islet cells .

Diagnosis depends on the location, size, and clinical features and the histopathological type of the tumor.

Early pancreatic cancer do not cause any clinical symptoms. Advanced carcinoma shows non specific symptoms. This results in pancreatic cancer diagnosing at an advanced stage [17].

The word pancreas was derived from the Greek word , means all flesh . It is a long, J-shaped organ measuring 10-16 cm in length , 2 -4 cm broad and 1-2 cm thick, weighing 70- 90 gms . It is a soft, lobulated organ located retroperitoneally .

In the posterior abdominal wall , it lies transversely, lying behind the stomach, across the lumbar spine from L1 to L2 [19,20].

EMBROLOGY

The development of pancreas is by two endodermal outpouchings.It arises at the midline of the foregut and midgut , from the primitive duodenum ,as smaller ventral bud and larger dorsal bud .Both soon fuses to form the pancreas.

Small ventral bud gives rise to the head . Large dorsal bud gives origin to the body and uncinate process and tail of pancreas .

The main pancreatic duct of Wirsung develops as a result of fusion of distal part of dorsal bud with entire ventral bud. Ventral bud also gives rise to common bile duct and ultimately common channel is produced after fusion with the duct of wirsung . Rest of the dorsal bud duct gives rise to accessory pancreatic duct of Santorini . At the site of duodenal major papillae , bile duct and the main pancreatic duct enters .

Islets of langerhans develops in the third month from the pancreatic tissue [19].

Molecular Regulation of Pancreas

Pancreatic and duodenal homeobox gene is upregulated . Notochord synthesis Fibroblast growth factor 2 and activin , which helps in the development of pancreatic bud.

Gross Anatomy

Shape of the pancreas is prismoid . It has three surfaces -anterosuperior, posterior, and anteroinferior surfaces. It also has three borders- superior,anterior and inferior.

Pancreas lies transversely at the level of first and second lumbar vertebrae.

Grossly , it is divided into head, neck , body and tail .

Head of pancreas – lies within curve of duodenum. It has :

- Three borders - superior , right lateral and inferior border
- Two surfaces – anterior and posterior

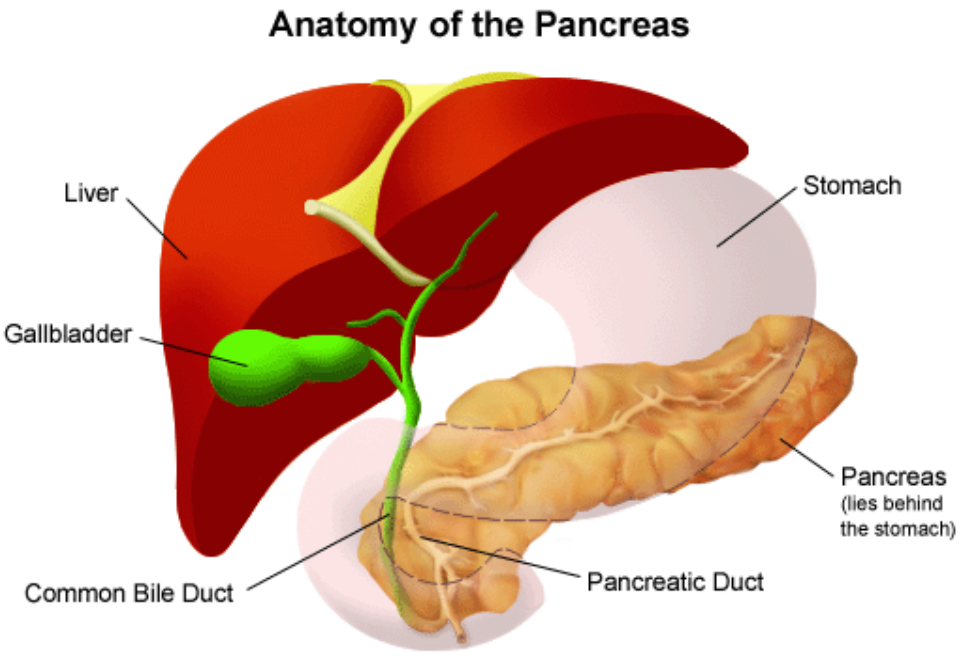
Uncinate process – related to aorta posteriorly and superior mesenteric vessels anteriorly.

Neck of the pancreas- is a constricted part , that is present between head and body. Two surfaces present – anterior and posterior.

Body –is elongated part , extending from neck to tail. It has :

- Three borders – superior , anterior and inferior
- Three surfaces – anterior , inferior and posterior

Tail of pancreas – narrow left out end, lies in contact with lower portion of spleen.



Endoscopic anatomy of pancreas

The pancreatic duct of Wirsung runs throughout the pancreas , and ultimately enters into the head in its inferior part . Common bile duct lower part and uncinate process duct, joins with wirsung duct in the inferior part of the head.

Ultimately results in the development of hepatopancreatic ampulla, a common channel . It opens into the dome of the major duodenal papilla , after running through the medial duodenal wall .

Sphincter of Oddi , smooth muscle that is present around the common bile duct and the pancreatic duct helps in preventing refluxion of duodenal juices.

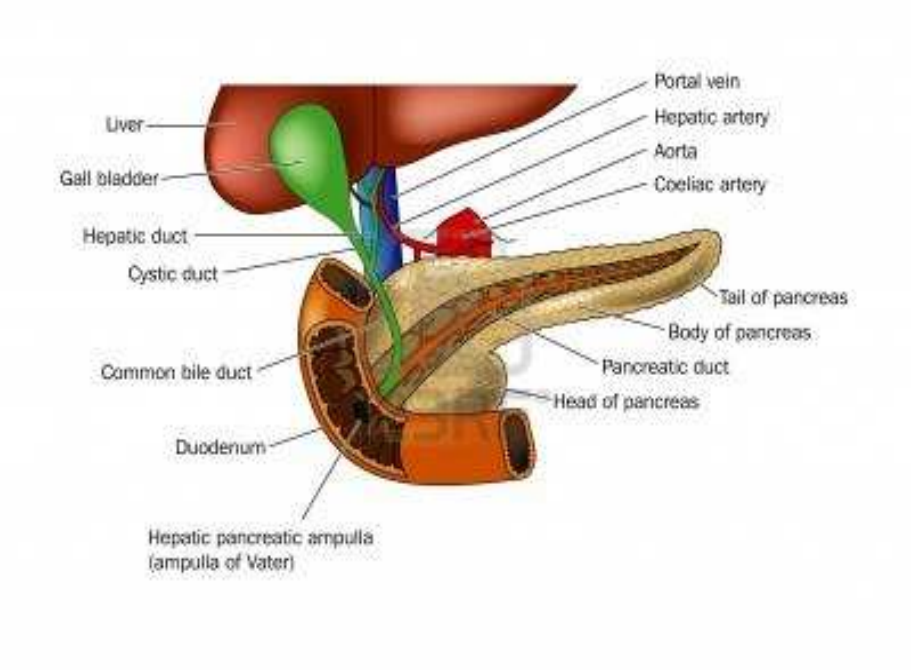
Refluxion of bile into main pancreatic duct is prevented by another muscle sphincter that is present around its terminal part .

Refluxion of pancreatic juices into the common bile duct is prevented by another smooth muscle sphincter , that surrounds the common bile duct in its lower part.

Drainage of the head of the pancreas is done by accessory pancreatic duct, which at the level of minor duodenal papilla opens into the duodenum .

Communication between accessory pancreatic ducts and main duct occurs [19,20].

Relationship of pancreatic duct



Endoscopic ultrasound anatomy

Endoscopic ultrasonography is the technical tool that is developed recently to evaluate the pancreas . An ultrasonographic probe was mounted on endoscope , which is then passed into duodenal C loop, the second part .

By using it well visualized portions are the head of pancreas , common bile duct- intrapancreatic part , both main and accessory ducts terminal parts, and pancreaticoduodenal lymph nodes.

Nerve supply

Thoracic splanchnic nerves and the celiac plexus gives origin to sympathetic supply .

Via celiac branch , posterior vagal trunk gives rise to parasympathetic nerve supply .

Arterial supply

At the level of T12 –L 1, from the aorta , in its anterior surface , celiac axis arises , which divides into splenic artery, hepatic artery and pancretico duodenal arteries and left gastric artery .

Splenic artery , by giving rise to multiple pancreatic branches , supplies body and tail .

Venous Drainage

Pancreato duodenal veins - superior and inferior , are accompanied by the respective arteries. The portal vein drains the superior pancreaticoduodenal veins and the superior mesenteric vein drains the

inferior pancreaticoduodenal veins. Gastrocolic trunk receives venous drainage from the pancreatic head .

From the body and tail , numerous small veins , drains directly into the splenic vein.

Lymphatic drainage

Pancreaticoduodenal and hepatoduodenal ligament lymph nodes - drains head of the pancreas .

Superior mesenteric and para aortic nodes - drains body and tail .

Functions

- Digestive - by secretions of enzymes such as trypsin , lipase and amylase from pancreatic juice.
- Endocrine – secretion of insulin and other hormones
- Pancreatic juice – provides alkaline pH for other enzymes to perform its function.

Histology

Both exocrine and endocrine components are present in this lobulated gland . Exocrine pancreas constitutes most of the organ. It is separated by thin capsule which divides the pancreas into numerous lobules by

thin fibrous septae. Serous acini, composed of zymogenic cells around central lumen , are arranged in lobules.

Islets of langerhans are found within serous acini masses. Each lobule has its own ductules. Ductules from the acini drains into intralobular ducts, which further drains into interlobular ducts and finally drains into the main pancreatic duct branches .

Serous zymogenic cells secrete a variety of enzymes such as trypsin , lipase and amylase that helps in digestion of protein , fats and carbohydrates . [21].

Alkalinity of the pancreatic juice was due to bicarbonate secretion by ductular cells

Pancreatic islets of Langerhans – contains alpha cells , beta cells and delta cells that secretes glucagon , insulin, and somatostatin.

Risk factors for pancreatic cancer may include:

- Age- pancreatic cancer development increases with age. Rises to peak after 60 years, but before 40 years are rare.
- Smoking
- Alcoholism
- History of familial pancreatitis.
- Low fibre diet

- Obesity
- Red meat high diet
- Diabetes mellitus
- Soft drinks
- Pancreatitis – especially Chronic.
- Helicobacter pylori infection
- Family history of pancreatic cancer – 5 -10% increased risk [17].

Genetic alterations associated with Pancreatic cancer :

- Mutation in oncogenes – such as Her 2 neu , KRAS , MYB ,AKT 2
- Tumor suppressor gene mutation –P 16 , P53, DPC 4 , BRCA 2 ,ALK 5 and LKB1 /STK11
- DNA mismatch repair mutations – MSH 2, MLH 1[22,23].

Syndromes associated with Pancreatic cancer are :

- Familial atypical multiple mole melanoma- cancer syndrome
- Autosomal dominant- BRCA 2 gene mutation
- Peutz-Jeghers syndrome
- Familial pancreatic cancer

- Hereditary non-polyposis colon cancer
- Ataxia Telangiectasia
- Familial adenomatous polyposis
- Hereditary pancreatitis
- Autosomal recessive ataxia-telangiectasia [22,23].

Pathophysiology

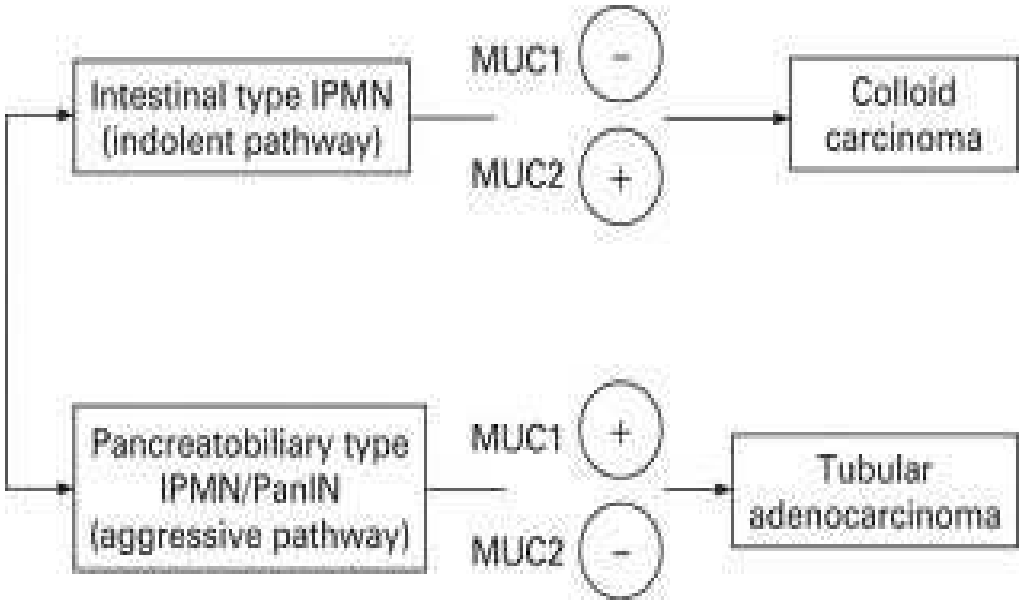
Acceleration by genetic or environmental factors gives rise to pancreatic cancer development by:

- Inactivation of tumor suppressor genes
- Over expression of oncogenes.

Precancerous lesion are:

- Intraductal papillary mucinous neoplasms
- Mucinous cystic neoplasms
- Pancreatic intraepithelial neoplasia

Flow chart depicting pancreatic cancer development :



Clinical features

Common symptoms include:

- Upper abdominal pain
- Nausea
- Vomiting
- Anorexia
- weight loss
- Jaundice.

- Itching – due to excess bile secretion
- Trousseau sign- in which thrombosis occurs in the deep veins of the extremities and in the portal blood vessels .
- New onset diabetes in an elderly individual
- Symptoms of metastasis
- Clinical depression

Diagnosis:

According to location of the cancer , clinical presentation varies :

1. Malignancies in the pancreatic body and tail- causes weight loss and pain

2. Malignancies in head –causes jaundice and steatorrhea .

Laboratory diagnosis:

Serum markers :

- Liver function tests -raised conjugated bilirubin , alkaline phosphatase levels and gamma glutamyl transpeptidase .
- DUPAN 2
- Carbohydrate antigen 19.9 level.

- SPAN 1
- Carcino embryonic antigen

Immunohistochemistry

- Mucin 1,3 and 5/6
- Cytokeratin 8 ,18 ,19
- Carbohydrate antigen 19.9
- DUPAN 2
- CA 125
- SPAN 1
- Sialyl SSEA
- TAG 72

Imaging studies

1. CT scan-used for staging

2. Endoscopic ultrasound -latest technical tool to evaluate the pancreas

3.Endoscopic Retrograde Cholangio Pancreatograpy

3. Endoscopic needle biopsy or surgical excision -gives definitive diagnosis .[44]

Most of the pancreatic ductal adenocarcinoma are moderate to poorly differentiated.

The major draw back is that immunohistochemical profile of pancreatic cancers and hepatobiliary cancers and some stomach cancers are similar. Hence it is very difficult to conclude that the tumour's primary site is pancreas .

Mucinous tumors , which ranks second , next to adenocarcinoma has slightly better prognosis.

Incidence of pancreatic neuroendocrine tumors is very low .

Role of cysteine cathepsins:

Cysteine cathepsins , lysosomal proteases , that are often upregulated in various cancers . During cancer progression, cathepsins are translocated from the cytoplasm and are secreted into the extracellular milieu, where they causes proliferation , angiogenesis, apoptosis and invasion .

These enzymes promote tumor invasion through several mechanisms:

- First, they cleaves the basement membrane and the extracellular matrix components , which helps in the invasion of tumor cells away from the primary tumor mass.
- Second, cathepsins can cause proteolysis by activating other proteases such as matrix metalloproteinases and urokinase plasminogen activator, which in turn promote invasion.
- Finally, cell adhesion protein, such as E-cadherin, gets cleaved at the cell surface, which causes disruption of adherens junctions and thus facilitate cancer cell migration and invasion.[24]

Classification of Cathepsins done according to their active site and functional status.That are used as prognostic markers in cancer are Cathepsin D, cathepsin B and other cathepsins. Measurement of Cathepsin D in pancreatic tissue is significant in predicting disease free and overall survival.

General belief is that elevated concentrations of cathepsin D in pancreatic cancer is highly significant indicators of the potential for recurrence. Cathepsin B, plays important role in pancreatic and colorectal because it causes rupture of basement membrane , by causing degradation of laminin, . Cathepsin D in addition to proteolytic activity ,also has mitogenic activity .

In a study by, the Methodist Hospital Research Institute, they studied proteolytic enzymes Cathepsin E and Cathepsin D (Cath D) expression in malignancy. Cath E has a limited cellular localization and tissue distribution , whereas Cath D is present ubiquitously . This results in less frequent association of Cath E with malignancy , when compared to cath D . It is selectively expressed in certain diseases such as pancreatic cancer. However, both Cath E and Cath D have very similar substrate selectivity. Recently, Cath E expression was found to be extremely low in normal pancreas and foci of chronic pancreatitis , upregulated highly in early dysplastic cells and in pancreatic cancer tissues.So they have shown that Cath E could be an early marker for pancreatic cancer detection.[25].

Study by nakata et al , showed that ,Lysosomal enzymes, cathepsin B and D,plays vital role in their possible relationship to the ability of malignant cells to invade and metastasize. In our current investigation, cathepsin B and D were detected immunohistochemically using avidin-biotin-peroxidase complex method in the 21 patients pancreatic cancer cells. Positive rate of detection of cathepsin B and D were found to be 43% and 81% respectively.In their study it was found that Cathepsin D stained more strongly than cathepsin B.The plasma membrane stained

intense strongly in both instances. They have found that in pancreatic cancer cells , cathepsin B and cathepsin D expression doesn't correlated with the degree of metastasis to lymph nodes, liver, or lung .[26].

In a study by Mads Gronberg and Troels zakarias et al , they have done analysis on proteins that were up-regulated in pancreatic cancer such as cathepsin D, cathepsin B , integrin β1 , macrophage colony stimulation factor and fibronectin receptor . They were also identified to be up-regulated in their proteomic study. After obtaining the Human Protein Reference Database they provided a list of protein functions and subcellular localization and annotations regarding presence of signal peptide . They listed information about all proteins identified in the study such as post-translational modifications, and peptide sequences, heavy/light intensity ratios. Mac-2BP belongs to scavenger receptor cysteine-rich domain family of proteins.

Mac-2BP function is helps in binding to galectins and fibronectin, collagen and β1 integrins. It has been increased in serum of patients with different solid tumors, *like* breast and pancreas , etc.It is associated with metastatic spread in these malignancies and resulting in poor survival . In their study, when compared with normal cells , it was found to be increased to 5-folds in pancreatic cancer cells . Cathepsin

B and cathepsin D were also detected in their study. Cathepsins are nothing but lysosomal proteolytic enzymes which is present in most of the mammalian cells. In different solid cancers , Cathepsins B and D , both are involved and were found to be up-regulated 2- and 10-fold times, respectively. Up-regulation of these proteins were also confirmed by Western blotting techniques.It was also found in their screening test that Prosaposin, a protein which is interacting with cathepsin D. They are found to be up-regulated upto 13 folds in pancreatic cancer when compared with normal pancreatic tissue [27].

In a study by Wostbrock B, Sturm JW, Willeke F , They obtained samples from seventy patients with pancreatic ductal adenocarcinoma , with a 3 years period follow-up. Cathepsin B and Cathepsin L expression was performed by immunohistochemical method using corresponding polyclonal anti-Cathepsin B and Cathepsin L antibodies. By performing nonisotopic in situ hybridization method they detected Cathepsin B and Cathepsin L expressing cells . It was found that the immunoreactivity for cathepsin B was 96% and for Cathepsin L 90% . Cathepsin B and Cathepsin L expression were correlated with clinical parameters and pathological grading , and survival rate and results were tabulated .

They also detected cytoplasmic localization of specific mRNA sequences. It was found that tumor cells cytoplasm , fibroblasts and macrophages, expresses positive mRNA signals were obtained in 77% of Cathepsin B and 81% of Cathepsin L respectively. Significant correlation was found between Cathepsin B and Cathepsin L expression and tumor grading ($P < 0.05$) by statistical analysis. Significant correlation ($P = 0.05$) was found between Cathepsin B and lymphatic invasion in cancer cases. Survival rate after curative surgical resection and expression of Cathepsin B and Cathepsin L was found to be statistically significant by Kaplan-Meier analyses ($P < 0.05$) .

Besides tumor size, and grading ($P < 0.05$) and nodal status and staging , in multivariate analysis it was found that both cathepsin B and L are strong prognostic markers ($P = 0.0001$) . For recurrence of pancreatic cancer after curative surgery , it was found in a multivariate analysis that Cathepsin B expression can be used as single prognostic marker and found to be statistically significant ($P = 0.0001$).

It was found that both Cathepsin B and Cathepsin L are strong and independent prognostic markers in resectable adenocarcinoma rather than staging and tumor grading . Furthermore, it was found that after surgical curative resection Cathepsin B is a single prognostic marker for early recurrence.

Hence these data highlights the role of proteolytic enzymes in invasion of tumor cells to adjacent sites and to distant sites metastasis , so helps in defining the groups of patients at risk for developing recurrence and metastasis [28].

In a study by Ohta and Terada et al, they have analysed in 23 pancreatic ductal adenocarcinomas surgically resected cases immunohistochemical expression of a polyclonal antibody against cathepsin B and monoclonal antibody against Pancreatic trypsinogen. These proteolytic enzymes are invoved extra cellular matrix digestion . In invasive adenocarcinomas pancreatic trypsinogen was expressed in the carcinoma cells cytoplasm , in a coarse granular pattern in fifteen of 20 (75%) cases . Lymph nodes metastasis also expressed pancreatic trypsinogen . Cathepsin B expression was found diffusely in the carcinoma cells cytoplasm in a fine granular pattern in 14 of 20 invasive adenocarcinomas (70%) . Whereas , three intraductal non-invasive adenocarcinomas did not express trypsinogen as well as cathepsin B. Hence these findings suggests that pancreatic trypsinogen and cathepsin B expression occurs in invasive ductal adenocarcinomas but not in non invasive ductal carcinoma . In the process of pancreatic carcinoma

progression , invasion and metastasis, cathepsin B and trypsinogen acts independently of each other.[29]

In a study by Hannah J.Whiteman et al , Overexpression of S100A6, and cathepsin D was due to increased expression of S 100 P . Both proteases , S 100 A 6 and cathepsin D are associated with cellular invasion. Increase in cathepsin D expression causes increased invasive potential of S100P.

Conclusion of their study data suggests that poor prognosis of pancreatic carcinoma was due to the invasive and metastatic potential created by these proteases [30].

In a study by Mark E. Weeks, Sally E , Dowen et al , they have found that increased expression of S100P results in significant changes in cathepsin D expression ,resulting in cytoskeletal changes. Identification of Cathepsin D on the gel in a row of four spots at several pH units, which indicates post-translational modifications. In S100P-overexpressing cell lines three cathepsin D spots seems to present in abundance , which indicates post translational modification. When there was no post-translational modifications , down regulation spot was identified. Cathepsin D believed to exists in three forms:- unprocessed

pre pro cathepsin D, partially processed pro cathepsin D, and mature enzymatically active cathepsin D. Western blot analysis showed the active form of cathepsin D showed mild increased expression in S100P-overexpressing clones but there is marked increase in expression of other two isoforms of cathepsin D . The invasive potential of cancer cells was believed to be due to preproisoforms and proisoforms of cathepsin D . In S100P-over expressing cells , they have carried out invasion assays mainly to look for the cathepsin D contribution to the invasive potential in these cells . They have observed a significant increase in the invasion of S100P-overexpressing clones, with more than 2- 4 fold increase in the number of invading cells when compared with controls. So to know the cathepsin D role in the invasive potential of S100P-overexpressing cells, they have examined its activity by using a fluorogenic assay. There they have showed significantly higher cathepsin D activity in association with S100P-overexpressing cells when compared with control . Following treatment with 100 μmol/L of pepstatin A for 48 hrs, the cathepsin D inhibitor , activity of cathepsin D in S100P-overexpressing cells was decreased significantly to those levels as observed in control cells. Hence they repeated the cell invasion assays after treating with pepstatin A. After inhibition of cathepsin D activity, they found a significant decrease in the number of invading , similar to that observed

for the control cell . They found that the increase in cathepsin D expression contributes to the increased invasive potential of S100P-overexpressing cells. They showed that S100P overexpression results in significant changes in the expression of preproforms and proforms of cathepsin D with a milder increase in the active form. Since cathepsin D plays a critical role in extracellular matrix proteolytic degradation , up regulation of its expression is seen in several cancer types, including pancreatic ductal adenocarcinoma . The procathepsin D secretion and its conversion into enzymatically active cathepsin D , which has the propensity to invade the cells , and hence associated with poor prognosis in pancreatic cancer. They have observed increased expression of both S100P and cathepsin D levels in PaTu 8988s cells when compared with PaTu 8988t. These cells were isolated from the same human primary pancreatic adenocarcinoma . PaTu 8988s was isolated from poorly differentiated and highly invasive and was associated with metastasis. PaTu 8988t cells are well differentiated and does not metastasize. Using a paired Student's *t* test ,statistical analysis of the data revealed significance P value of < 0.05.

Their results suggests that the increased cathepsin D expression has the ability to degrade the extra cellular matrix resulting in invasion and metastasis [31]

In a study by Leto G et al, Lysosomal cathepsins B, L and D, they estimated the serum levels by immunoassays in patients with acute or chronic pancreatitis and with ductal pancreatic carcinoma patients. Correlation has been done with clinical parameters and some biological parameters of the tumor. Expression of Cathepsin B was found to be statistically significantly ($p < 0.01$) higher in pancreatitis and ductal adeno carcionoma when compared with healthy subjects. But there is no difference between these two groups. . Cathepsin D expression were increased in pancreatitis patients when compared with cancer patients or healthy subjects ($p < 0.01$). Elevation of Serum Cathepsin L levels were seen in cancer patients only when compared with pancreatitis and normal subjects ($p < 0.05$), but there is no difference between these two groups. In their study, no correlation was found between Cathepsin D, B and L levels and tumor size and clinical stage. But, significant correlations were found between serum Cathepsin D and serum CA50 levels and between Cathepsin D and Cathepsin L , which was found to be ($p < 0.02$) and ($p < 0.05$) respectively [32].

In a study by Brentnall TA, Crispin DA, Goodlett DR, Aebersold R et al they used tandem mass spectrometry and ICAT technology -based proteomics to study systematically in chronic pancreatitis ,the different

types of protein expression . They identified 116 differentially expressed proteins in chronic pancreatitis . Both in pancreatic cancer and in chronic pancreatitis 40% of these proteins which were expressed differentially were detected in common . This indicates that in between these two disease , some proteins are expressed in common. Biological network analysis indicates that c-MYC is a common regulatory protein , involved in both chronic pancreatitis and pancreatic carcinoma . For validation by immunohistochemistry and Western blot analysis , five proteins were chosen . Insulin-like growth factor-binding protein 2 and Annexin A2 were found to be overexpressed in pancreatic cancer but not in chronic pancreatitis. Hence they can be used as promising biomarker detection of pancreatic cancer. In both pancreatic cancer and chronic pancreatitis , cathepsin D, were found to be overexpressed . In both these conditions it was also found that there was expression of integrin beta1, and plasminogen . These proteins were expressed in both conditions , hence these proteins expression will lower the specificity in pancreatic carcinoma .[33]

In a study by Yamaguchi N, Chung SM et al, analysis in pancreatic carcinoma cell line HPC-YP, spent culture medium , was done for the presence of the secreted cysteine protease, cathepsin L. The lysosomal

form was distinguished from secreted form of cathepsin L by its decreased stability at alkaline pH, by its reduced thermostability, and by its smaller molecular size. HPC-YP cathepsin L was found to be stable at 56 degrees C after 60-min preincubation, at pH 7.4. Molecular weight of pancreatic cancer enzyme was found to be 68,000, when compared with a molecular weight of 29,000 for normal liver cathepsin L. HPC-YP enzyme when analysed by western blot , was found to be composed of two components, one with a molecular weight of 31,000 and the other with a mol.wt of 37,000. HPC-YP enzyme in the spent medium analysis suggests that it is a complex of the proenzyme and the mature enzyme. In their study they have found that cathepsin L which is secreted from cancer cell lines plays a major role in the destruction of basal lamina, and helps in the invasion of tissue, and metastasis to distant sites.[34]

In a study by Losch et al,they studied in 103 invasive ductal carcinomas of the breast , the immunohistochemical expression of cathepsin D , for the prognostic value at stages pT1 and 2. They also analysed the cathepsin D expression and its association between histomorphological tumour subtypes, multifocal tumour and invasive ductal carcinoma with extensive intraductal component . Expression of cathepsin D was examined both within the epithelial and stromal component of all

tumours , separately at two cut-off levels -positive and highly positive . Epithelial expression was detected in 20 (19.4%) as highly positive and as positive in 32 (31.1%) patients . Stromal expression was found in 19 (18.4 %) and 35 (34%) cases.It was found that epithelial cathepsin D expression was associated with nuclear grade and staging , but not with lymph node status. In their study , they have found statistically significant prognostic value for overall survival rate in highly positive (p value = 0.003) and positive (p = 0.001) cathepsin D expressing epithelial cells respectively and there was no tumor recurrence , P value was 0.04 and 0.02. But in stromal cells there was no associated with either recurrence or survival rate and expression of cathepsin D. Their study revealed the association of epithelial component of breast cancer cases survival rate and recurrence and cathepsin D immunohistochemical expression . [35]

In a study by Shen et al, they studied differentially expressed proteins in 7 cases of pancreatitis, and 6 normal pancreatic tissues, and in 6 cases of pancreatic carcinoma and 2 adjacent normal tissues by using mass spectrometry and proteomic approach of two-dimensional gel electrophoresis . Extraction of protein of individual sample and pooled samples of each tissue type were separated by using two different pH ranges , on 2D gels. After gel digestion differentially expressed protein

spots were identified by using mass spectrometry. In that they have found overexpression of peroxiredoxin I , cathepsin D, galectin-1, annexin A4, cyclophilin A, alpha-enolase, and S100A8 in pancreatic carcinoma when compared with normal pancreatic tissues and pancreatitis . These identified proteins were confirmed by immunohistochemical analysis and western blot analysis.[36].

In a study by Losch et al, they investigated the immunohistochemically detected cathepsin D expression and its prognostic value in endometrial adenocarcinoma. Patients included in the study were Endometrial adenocarcinoma cases of all FIGO stages and those who were receiving consecutive irradiation therapy. In 115 tissue specimens , they performed immunohistochemical expression of cathepsin D, out of which 35 cases were found to be positive . In the univariate analysis , they have showed statistically significant association between expression of Cathepsin D and overall survival rate (P-value = 0.007). In the multivariate analysis they found association between recurrence free period and cathepsin D expression (P-value = 0.002). Cathepsin D expression by immunohistochemical detection helps in planning treatment for patients with endometrial adenocarcinoma and survival rate. [37]

In a recent clinical research discipline ,they have found that peptic ulcer disease is one of the risk factor for the development of pancreatic cancer with the odds ratio of 3.9. Intraductal papillary mucinous neoplasms , a pancreatic precursor lesion ,is associated with development of invasive pancreatic adenocarcinoma . Methylation of a gene promotor region causes altered gene expression resulting in inactivation of tumor suppressor gene. In pancreatic adenocarcinoma more than 400 alterations in gene expression transcription have been identified. In situ neoplasia is associated with common molecular markers over expression such as p53 and HER-2/neu . Via autocrine or paracrine mechanisms molecules such as epidermal growth factor ,TGF beta causes modulation in gene transcription. Neoplastic cells causes release of several cytokines which causes cachexia , peripheral insulin resistance, ineffective utilization of glucose . In patients taking chemotherapeutic regimens , Positron emission tomography scanning is used for evaluation of early tumor response . Neoadjuvant 5-fluorouracil (5-FU) based chemoradiation improved local tumor control in 39 resectable patients with average survival rate of 19 months . 4-year average survival rate in these cases was 19% . Gemcitabine has shown better clinical response than single agent 5-flurouracil in alleviating tumor-related symptoms (23.8 vs. 4.8%, p = 0.0022.[38]

In a study by Bramhall SR, Allum WH,they studied treatment trends and outcome of 13,560 pancreatic cancer patients .The incidence of the pancreatic carcinoma were determined between 1957 and 1986 , using data collected from the health registry , West Midlands . Patients were divided into two categories . Those diagnosed in the first 20 years (1957-76) and the next 10 years (1977- 86). In their study they have found pancreatic adenocarcinoma were more common in men. When compared with bypass surgery and laparotomy , the 30-day mortality rates between the two periods were improved after surgical resection . When compared with survival rate after bypass , there was significant improvement in the 5-year survival rate after resection (P value was < 0.015) .[39]

By both direct and indirect mechanisms VEGF , a cytokine that accentuates angiogenesis. VEGF stimulates nearby microvessels endothelial cells to proliferate,and to migrate by causing gene expression pattern alteration and thereby causing these cells hyperpermeable which results in spillage of plasma proteins into the extravascular space ,which further causes the clotting of extravasated fibrinogen resulting in deposition of a fibrin gel. Extravasated fibrin functions as a provisional matrix which favors the ingrowth of new blood vessels and other mesenchymal cells. TGF-alpha is a potent

angiogenic factor, but it does not by itself causes increase microvascular permeability.[40]

In a study by Wael Abd et al, they studied Cathepsin E expression in pancreatic cancer . Cathepsin E activity was detected by using specific fluorogenic probes in both non-invasively image mouse xenografts from human pancreatic cancer cells (MPanc96) and in vitro. Cath E selective peptide substrate, was synthesized and conjugated with a grafted copolymer to yield a fluorescently quenched probe. Fluorometerically , probe specificity was identified in vitro. Near infrared fluorescent imaging was performed before and after probe administration, in MPanc96 xenografts in nude mouse, at 18, 24, 48 and 72 hrs respectively. For negative controls, control imaging probe were administrated into tumor-bearing nude mice. Using NIR fluorescence , ex vivo imaging was done for tissues of interest . Native fluorescently quenched probe was converted to highly fluorescent state upon specific Cath E cleavage . Within 24 hr post-injection, the probe detected Mpanc96 tumors. High tumor ratio (~ 6) was observed when compared ro non tumor tissue . There was no signal was derived from other tissues examined . The selectivity of the probe in detecting upregulation of Cathepsin E in pancreatic cancer cells was confirmed [41] .

In a study by Gopinathan and Denicola et al, they have found that upregulation of cathepsin B in ductal adenocarcinoma of pancreas . Hence this lysosomal protease represents a potential therapeutic target . Cathepsin B loss causes decreased invasion and metastasis in mouse models of intestinal , islet, and breast carcinoma , due to delay in tumour progression . The aim of their study was to know the role of cathepsin B in the invasion and metastasis of human pancreatic ductal adenocarcinoma. The role of cathepsin B in vitro Cell lines derived from tumours were investigated. Using subcutaneous allografts , role of cathepsin B in pancreatic adenocarcinoma were tested in both autonomous and non- autonomous cells . The delayed progression of both pancreatic intraepithelial neoplasm and ductal carcinoma were due to constitutive cathepsin B loss and a improved survival in mice. Reduced proliferation and MAP signalling was due to Cathepsin B-loss in Pancreatic intraepithelial neoplasm and adenocarcinoma cells.[42].

Study by Gukovskaya et al, they have done study on alcohol consumption and risk of development of pancreatic cancer. It has been found that there is some interaction present between the pancreatic exocrine and endocrine parts and also in inflammatory processes and

development of malignancy . Heavy alcohol consumption for long period results in the development of chronic pancreatitis and type 2 diabetes mellitus, both are risk factors for the development of pancreatic cancer. Alcohol and its metabolites will alter the mechanisms involved in the development of inflammatory response and carcinogenesis. The following mechanisms are involved : (1) activation of zymogens prematurely (2) activation of nuclear factor-kappa which results in activation of the inflammatory response (3) oxidative DNA damage because of increased synthesis of reactive oxygen species (4) fibrosis because of pancreatic stellate cells activation (5) gene mutation in enzymes related to cytochrome P450, trypsinogen, and secretory trypsin inhibitor , glutathione S-transferase, aldehyde dehydrogenase (6) additive effects of carcinogen produced by ethanol and tobacco on nitrosamine 4-1-butanone metabolism and (7) dysregulation of cell division and (8) apoptosis. Diabetes mellitus and acute and chronic pancreatitis develops as a result of interaction of metabolic effects of alcohol with various risk factors such as genetic, dietary, environmental and, ultimately, induces the steps involved in the process of carcinogenesis which results in the pancreatic cancer development .[43]

In a study by William et al, they assessed the expression of cathepsin E (Cath E) enzymatic activity , which is strongly and specifically expressed in pancreatic intraepithelial neoplasia lesions and ductal adenocarcinoma. By applying immunohistochemistry and quantitative real-time PCR method , cathepsin E expression was analysed for normal pancreatic tissue, chronic pancreatitis and adenocarcinoma patients . Cathepsin E activity selective fluorescent probe was injected into human pancreatic adenocarcinoma xenografts and mouse models of pancreatic adenocarcinoma which were genetically engineered and imaging was done using an optical imaging system.

By applying immunohistochemistry and quantitative real-time PCR , Cath E expression specificity in adenocarcinoma patients and in mouse model pancreatic cancer was confirmed.

It was also found that , by applying cathepsin E sensitive probe, detection of pancreatic intraepithelial lesions in mouse model before tumour formation was also possible .[44]

WHO histological classification of exocrine pancreatic tumours

Epithelial tumours

Benign

Serous cystadenoma

Mucinous cystadenoma

Intraductal papillary-mucinous cystadenoma

Borderline

Mucinous cystic neoplasm with moderate dysplasia .

Solid-pseudopapillary tumor

Intraductal papillary-mucinous neoplasm with moderate dysplasia .

Malignant

Ductal adenocarcinoma

- Noncystic mucinous carcinoma
- Adenosquamous carcinoma
- Signet ring cell carcinoma
- Anaplastic carcinoma
- Undifferentiated carcinoma with osteoclast-like giant cells
- Acinar cell carcinoma

Mixed ductal-endocrine carcinoma

Serous cystadenocarcinoma

Mucinous cystadenocarcinoma – non-invasive and invasive

Intraductal papillary-mucinous carcinoma – non-invasive and invasive

Acinar cell cystadenocarcinoma

Solid-pseudopapillary carcinoma

Mixed acinar-endocrine carcinoma

Pancreatoblastoma

Others

Non-epithelial tumours

Secondary tumours [23]

TNM CLASSIFICATION

Primary tumor (T)

TX	Tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor confined to the pancreas , measuring less than 2 cm in diameter
T2	Tumor confined to the pancreas, measuring more than 2 cm in diameter
T3	Tumor extension beyond the pancreas , but there is no involvement of the superior mesenteric artery or celiac axis
T4	Unresectable primary tumor. ie . tumor involving the superior mesenteric artery or the celiac axis

Regional lymph nodes (N)

NX	Regional lymph nodes status cannot be assessed
N0	No evidence of regional lymph node metastasis

N1 Regional lymph node metastasis present.

Distant metastasis (M)

M0 No evidence of distant metastasis

M1 Metastasis to distant sites present , ie. To the liver , lung ,etc

Tumor staging

Staging of the cancer is done by combining the T, N, and M classification.

Stage 0: (Tis, N0, M0) - indicates carcinoma in situ, in which the tumor has not yet extended outside the duct in which it originated.

Stage IA: (T1, N0, M0) - tumor measuring less than 2 cm in the pancreas. Tumor has not spread to distant sites or regional lymph nodes .

Stage IB: (T2, N0, M0) - tumor measuring larger than 2 cm in the pancreas. Tumor has not spread to other parts of the body or regional lymph nodes.

Stage IIA: (T3, N0, M0) - tumor extending beyond the pancreas, but the tumor has not spread to other body parts , arteries or veins or to the regional lymph nodes .

Stage IIB: (T1, T2, or T3; N1; M0) - tumor of any size that has spread to regional lymph nodes , but has not spread to nearby arteries or veins but not to other body parts .

Stage III: (T4, N1, M0) - tumor has spread to nearby regional lymph nodes or arteries, veins, and/ but has not spread to other body parts .

Stage IV: (any T, any N, M1) - Any tumor size that has spread to other body parts such as liver , lung , brain , etc

Surgical Classification

This method is more simpler and found to be more descriptive than the American Joint Committee Cancer system . Here tumor classification done based on whether the tumor can be surgically removed or not.

Resectable: This tumor type can be removed surgically . These tumors lies within the pancreas or extends to the adjacent organs, but do not extend into local blood vessels. Tumor can be surgically resectable , only when there is no evidence of spread of the tumor outside the pancreas.

Locally Advanced: Using traditional methods , these tumors cannot be removed surgically , because at the time of diagnosis tumor has invaded nearby organs or blood vessels . But, there is no evidence of spread to distant sites of the body.

Metastatic: This tumor type cannot be removed surgically because at the time of diagnosis the tumor has spread to other body parts. Unfortunately, most of the pancreatic tumor were detected at this unresectable stage .[45].

Treatment for pancreatic cancer depends on its stage:

If pancreatic cancer is resectable, surgery followed by chemotherapy or radiotherapy improves survival rate.

Resectable Pancreatic Cancer Treatment

Patients with resectable pancreatic cancer can undergo any of the following surgeries- depending on the location :

1. Whipple pancreaticoduodenectomy :- removal of parts of the stomach and small intestine , and the head of the pancreas, the gallbladder, and the common bile duct, and some lymph nodes .

Rest of the organs are connected for favour further digestion.

2 . Distal pancreatectomy : removal of body and / or the tail of the pancreas , but not the head. This surgery is done for tumors located in locations in pancreas without involvement of head.

3 . Total pancreatectomy : Entire pancreas along with the spleen is surgically removed completely.

Chemotherapy or radio therapy

Both can also be used in combination with surgery for both resectable and unresectable pancreatic cancer :

- Neoadjuvant therapy -given before surgery , shrinks pancreatic tumor thereby improving the chances of complete resection.

- Adjuvant therapy – given after surgery, delays pancreatic tumor recurrence and thereby improves survival rate .

Chemotherapeutic drugs used in pancreatic cancer are the following :

- 5-fluorouracil (5-FU)
- Gemcitabine

In radiation therapy , beams of high-energy X-rays were passed to kill the tumor cells. Radiotherapy is given during a series of daily treatments, usually over a period of weeks.

Treating Locally Advanced (Unresectable) Pancreatic Cancer

Treatment consists of nonsurgical therapies such as chemotherapy with or without radio therapy. For patients with locally advanced pancreatic tumor either gemcitabine or 5 fluorouracil can be used to

extend the life .

Metastatic Pancreatic Cancer Treatment

Here, surgery is done mainly to control symptoms, such as for jaundice, or gastric outlet obstruction..

Radiotherapy / chemotherapy-can also be used to relieve symptoms .

Chemotherapy- Gemcitabine is the single most effective drug for treating metastatic pancreas cancer.

Alternative - 4 drug regimen used , known as FOLFIRINOX (combination of 5-FU/leucovorin/oxaliplatin/irinotecan), found to be superior to gemcitabine. Other combinations are:

gemcitabine with erlotinib,

gemcitabine with capecitabine

gemcitabine with cisplatin,

gemcitabine with nab-paclitaxel. .

If an individual progresses on gemcitabine, usual regimens include oxaliplatin with 5-FU or capecitabine or cisplatin with 5-FU .

Palliative Treatment

- Bile duct stents procedure - relieves jaundice
- Opiod analgesics - relieves pain.
- Antidepressants – to treat depression

Prognosis of Pancreatic Cancer

For all stages combined - 1-year relative survival rate is 25%

5-year survival is less than 5% to 6%

For local disease, the 5-year survival is 20%.

. In terms of months, the median survival rate for locally advanced and for metastatic disease, is about 10 and 6 months respectively [17,18].

Without active treatment, metastatic pancreatic cancer has a median survival of 3–5 months.

Complete remission is rare.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry plays role in both immunology and histology. Immunohistochemistry is used to determine the expression of particular antigen and its microanatomical location in the tissue.IHC uses antibody to distinguish the antigenic differences between the cells. These differences can specifically identify the lineage of cell population and define biologically the distinct population of cells within the same lineage.

Immunohistochemistry was started in 1940,when coons developed an immunoflourescence technique to detect corresponding antigen in frozen sections.

Taylor and colleagues in 1974 showed it was possible to demonstrate antigens in routinely processed tissues. Antigen retrival technique was introduced by shi and associates in 1991. antigen retrival technique is a simple method that involves heating paraffin processed sections at a high temperature before IHC staining.

Enzyme digestion was introduced by Huang and colleagues as a pretreatment to immunohistochemistry staining to unmask some antigens that had been altered by formalin fixation . However leong and

colleagues found that enzyme digestion did not improve the immunohistochemistry staining.

Another drawback of enzyme digestion was that it is proved to be difficult to control the optional digestion condition for individual tissue when stained with different antibodies. These difficulties in standardization , provided a powerful incentive for the development of a new technique.

The antigen retrival technique was developed by sln and his associates in 1991.In contrast to enzyme digestion this technique is a simple method that involves heating routinely processed paraffin sections at high temperatures before the immunohistochemistry staining. An alternative method that did not use heating was applied in celloidin sections.

Various articles demonstrated that the immunohistochemistry staining intensity was dramatically increased after antigen retrieval pretreatment.

One of the critical issues in the development of immuno peroxidase techniques was related to the need to achieve greater sensitivity from the one step direct conjugate method to multi step techniques such as the peroxidase and anti peroxidase method , avidin biotin conjugate and

biotin sterptavidin method , together with amplification methods and highly sensitive polymer based labeling techniques.

The development of hybridoma techniques facilitated the improvement of immunohistochemistry and results in the production of highly specific monoclonal antibodies. Many of these antibodies were initially applied in staining of tissues , later they have found to be applied successfully in formalin fixed paraffin embedded tissue sections.

The use of antibody in IHC depends on the sensitivity and specificity of the antigen and antibody reaction and the hybridoma technique provides limitless source of highly specific antibody.

Basic Principles

The object of all stains is to recognize the existence and distribution of substances micro chemically .

Basic principle applied here is that sharp localization of target compounds in cells and tissues , based on signal to noise ratio. The major strategy to achieve a satisfactory result is by amplifying signals that reduces non specific background staining (noise).

An antibody is a molecule that combines specifically with the antigen. Antigen – antibody recognition is based on revealing protein structure , in which antigen retrieval plays crucial role.

Comparison of sensitivity and specificity between polyclonal and monoclonal antibodies indicate that polyclonal antibodies are more sensitive but less specific than monoclonal antibody . The reason is that polyclonal antibody recognize several epitopes on a single antigen , whereas monoclonal antibody recognizes only particular type of epitope on a single antigen.

Fixative used – 10 % Neutral Buffered Formalin

Antigen Retrieval

The disadvantage of masking of antigens during fixation can be overcome by antigen retrieval technique . Techniques used to unmask the antigens are the following :

- Proteolytic enzyme digestion
- Microwave antigen retrieval
- Pressure cooker antigen retrieval

Blocking non-specific background staining

Background staining is due to either non specific binding or the presence of endogenous enzymes. Non specific binding with polyclonal primary antibody is minimized by preincubating sections with serum from same species on optimal working dilution.

Endogenous enzymes such as peroxidase seen in normal and neoplastic tissues are abolished by peroxidase blocking or by using alternate system such as Immunogold technique.

Methods suggested to overcome endogenous activity include incubation in methanol containing 0.5% hydrogen peroxide for 10 minutes at room temperature . By addition 0.1M concentration of levamisole solution to the enzyme substrate solution , endogenous alkaline phosphatase is blocked .

Detection system

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

Methods of IHC

Direct labeling method

Antibody is attached with a label by chemical means and directly applied to tissue sections. It is rapid and easy procedure and carries the disadvantage of multiple antigens which requires separate incubation with respective antibodies.

Indirect labeling technique

Enzymes are labeled with the secondary antibody, which is produced against primary antibody. This method is more sensitive and easy to handle. The advantages also include increased versality, higher working dilution of primary antibody, secondary antibodies against primary antibodies of different species and easy to prepare.

Avidin biotin techniques

High affinity binding between biotin and avidin is used in this procedure. Biotin is chemically linked to primary antibody thus localizing the peroxidase moiety at the site of antigen.

Disadvantage of this technique is that the endogenous biotin produces non specific background staining.

Avidin biotin conjugate procedure

In this technique primary antibody is added followed by biotinylated secondary antibody and next by preformed complexed of avidin and biotin horse radish peroxidase conjugate.This is a more sensitive method.

Biotin streptavidin system

Streptavidin is used in place of avidin. Streptavidin is more stable than avidin.

Immunogold silver staining technique

This is used in ultrastructural immunolocalisation. Gold particles are enhanced by the addition of several layers of metallic silver. The fine silver deposits in the background creates confusion when small amount of antigen are identified.

MATERIALS & METHODS

This study was done after obtaining the approval from Institutional Human Ethical Committee (IHEC) of Govt. Stanley Medical College, Chennai.

The study was carried out in the Department of Pathology, Govt.Stanley medical college, in collaboration with the Department of Surgical Gastroenterology,

from February 2010 to August 2012.

Total of 50 whipples pancreato duodenectomy specimens were taken for this study.

Eligibility Criteria:

Inclusion Criteria-

All specimens of pancreatic cancer by pancreatectomy and whipple's pancreatico duodenectomy were processed routinely and stained with hematoxylin and eosin.

Exclusion Criteria-

Biopsy proven cases of benign pancreatic lesions, cysts, carcinoma in situ cases

METHOD:

For all the 50 cases,details of age,sex and other relevant clinical data were recorded.

Site of growth,size,gross appearance and three dimensional measurement taken.

Multiple bits from the growth and the adjacent areas were taken and processed routinely for paraffin embedding and sections were cut at 5 microns.

Hematoxylin and eosin staining of sections were done.Histopathological examination of these sections were done.

Necessary photographs were taken.

Sections from pancreatic carcinoma are graded according to their degree of differentiation into well,moderate and poorly differentiated.Perineural invasion and lymphovascular invasion were noted.

Immunohistochemical study using cathepsin D antibody was done in 50 cases and degree of antibody expression was scored in each case.

Method applied for tissue preparation of IHC

10 % buffered neutral formalin were used for specimen fixation. The tissues were processed in automated histokinette through various grades of alcohol and xylene . Paraffin blocks were prepared . Sections were cut using semi automated microtome with disposable blade and

stained with hematoxylin and eosin . Suitable sections were chosen for IHC .

Slides were coated with chrome alum . Sections were subjected to antigen retrieval using microwave technique with TRIS –EDTA buffer solution.

PROCEDURE

1.Deparaffinise sections using xylene – 2 changes –each 15 minutes and 90% alcohol -1 min and 70 % alcohol -1 minute

2. Running tap water wash -10 minutes

3. Rinse in distilled water – 2 minutes

4. Antigen retrieval by microwave method in citrate buffer – 15 minutes

5. Cool to room temperature for 10 -20 minutes

6. Rinse in distilled water – 5 minutes

7. Rinse in TBS buffer – 2 changes – each 5 minutes

8. Peroxidase block -15 minutes

9. Rinse in TBS buffer – 2 changes – each 5 minutes

10. Power block -15 minutes

11. Drain and cover with primary antibody -1 hour

12. Rinse in TBS buffer – 2 changes – each 5 minutes

13. Super enhancer- 30 minutes

14. Rinse in TBS buffer – 2 changes – each 5 minutes

15. Secondary antibody label with horse raddish peroxidase- 30 minutes

16. Rinse in TBS buffer – 2 changes – each 5 minutes

17. DAB and substrate solution – 5- 10 minutes

18. Wash in distilled water- 5 minutes

19. Counterstain with hematoxylin – 1 minute

20. Air dry and mount in xylene

Evaluation of Immunostaining:

Cathepsin D expression was scored by counting the number of all positive cells per x 100 field, which includes carcinoma cells and stromal cells such as fibroblast, and lymphocytes.

Tumor cells were compared in all samples.

SCORING OF CATHEPSIN D EXPRESSION:

0 - no staining .

1- If the tumor tissue consists of less than 10% immunoreactive cells

2 - 10–50% immunoreactive cells

3- more than 50% immunoreactive cells

RESULTS AND OBSERVATION

The collected details of cases have been recorded in the master chart. All collected data were analyzed and following results were obtained

Table-1:Age distribution of pancreatic carcinoma

Age(yrs)	No. of patients	% of cases
30-40	1	2
40-50	19	38
50-60	22	44
>60	8	16

Total number of patients included in this study was 50.Age group ranges from 30-70 years, with mean age of 53 years.Pancreatic carcinoma peaks in the age group of 50-60 years.

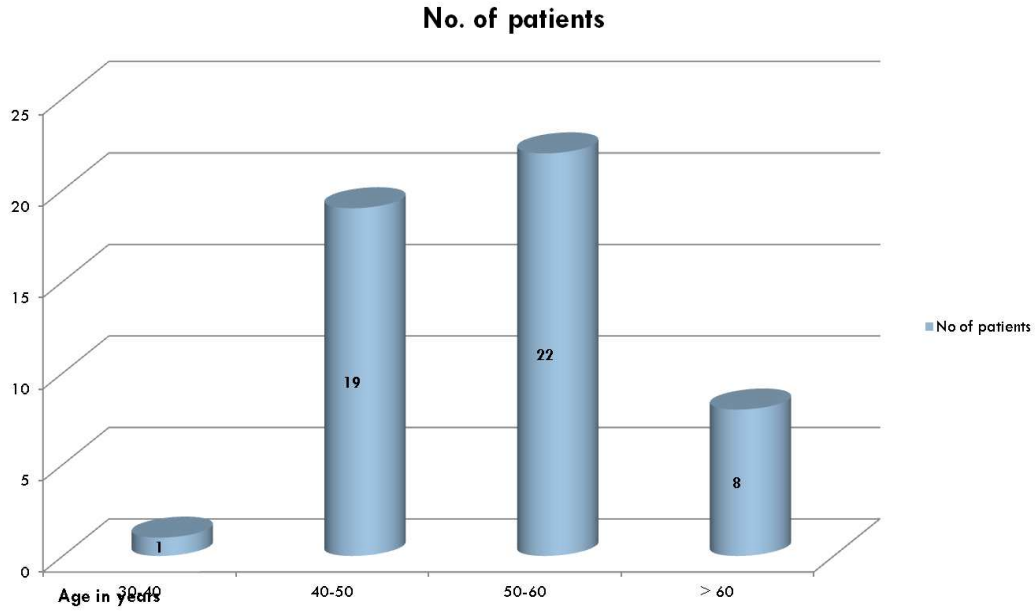


Table-2: Sex distribution of pancreatic carcinoma

Sex	No. of patients	% of cases
Male	34	68
Female	16	32

Out of 50 cases of pancreatic adenocarcinoma , males were 34 (68%) and females were 16 (32 %) with a M : F of 2.1:1.

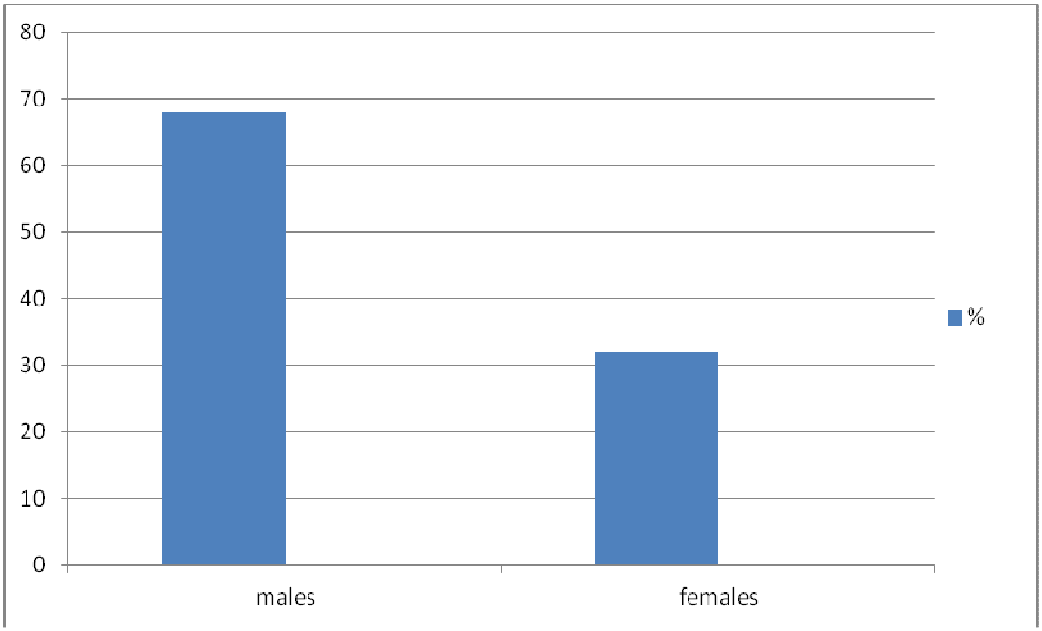


Table-3: Size of the tumor

Size	No. of cases	% of cases
<2 cm	18	36
>2cm	32	64

Out of 50 cases of pancreatic adenocarcinoma , 18(36%) were less than 2 cm and 32(64%) were more than 2 cm.

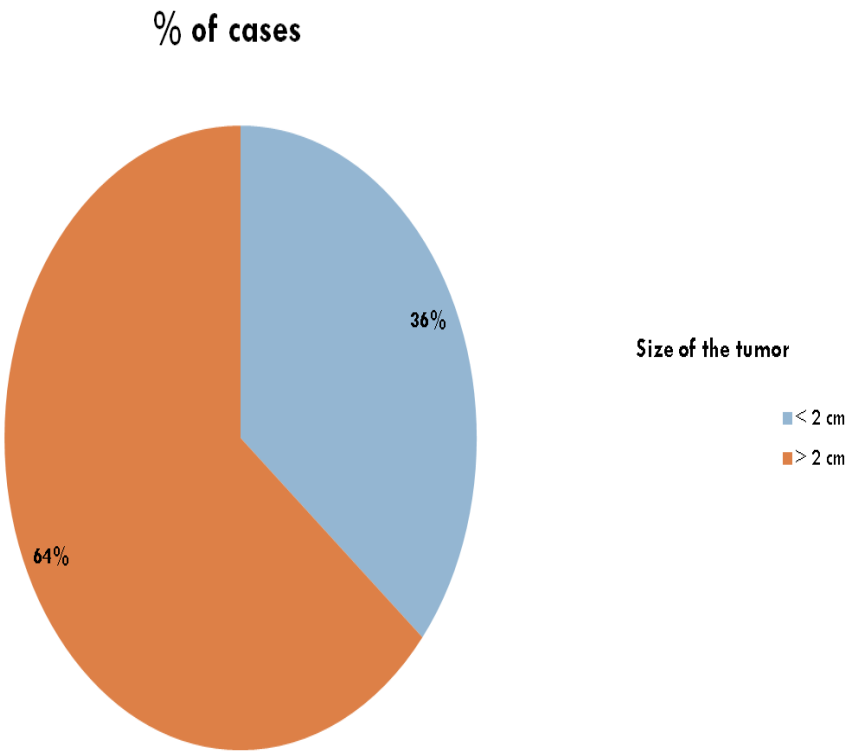


Table-4: Tumor grading

Grade	No. of cases	% of cases
WD	6	12
MD	21	42
PD	23	46

Out of 50 cases of pancreatic adenocarcinoma,6(12%) cases were well differentiated,21(42%) were moderately differentiated and 23(46%) cases were poorly differentiated carcinoma.

In our study, most of the cases were moderately to poorly differentiated.

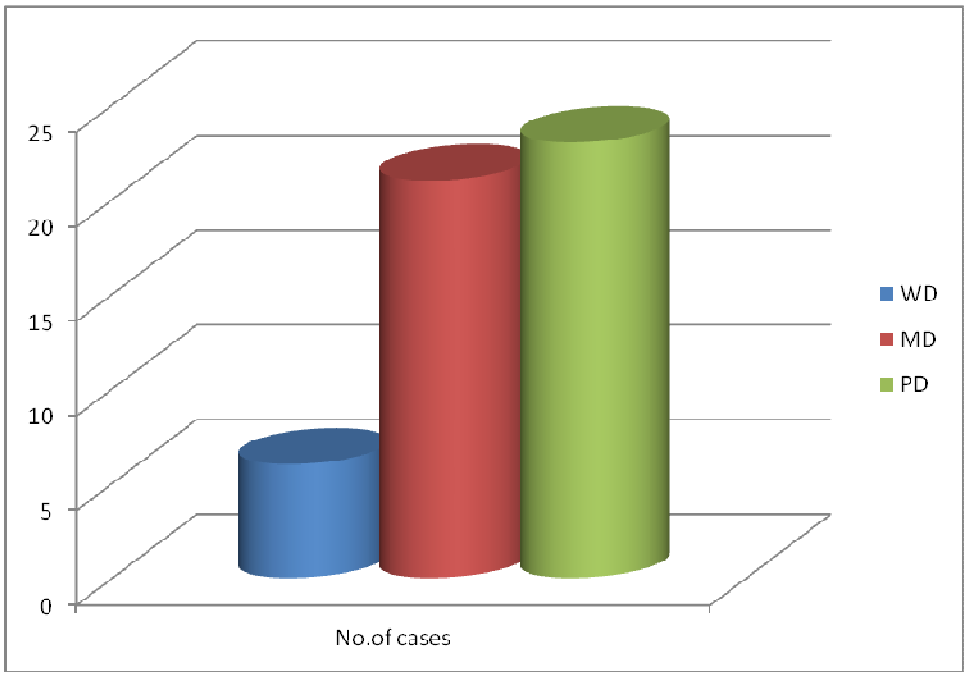


Table-5: Site of growth

Site of tumor	No.of cases	% of cases
Head	41	82
Body and tail	9	18

Out of 50 cases of pancreatic carcinoma,41(82%) were located in head of the pancreas and 9(18%) were located in body and tail of pancreas.

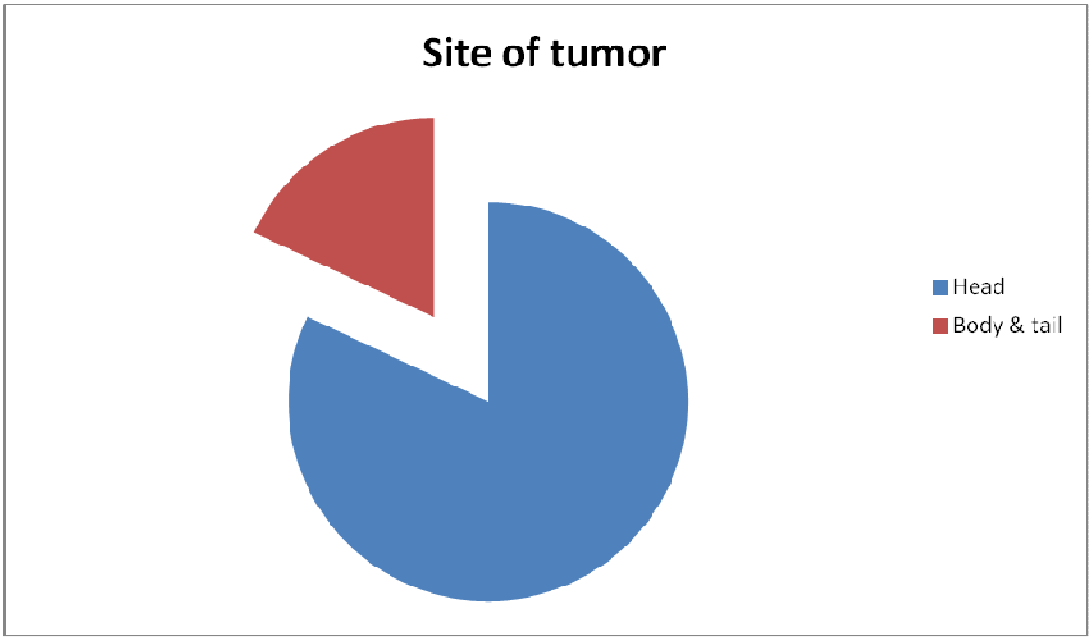


Table-6:Risk factors such as smoking, alcohol intake in 50 patients of pancreatic carcinoma

Risk factor	Males	Females	% of cases
Alcohol	25	-	80.6
Smoking	20	-	64.5

Out of 50 cases,25 (80.6%) were alcoholic and 20 (64.5%) were smokers.

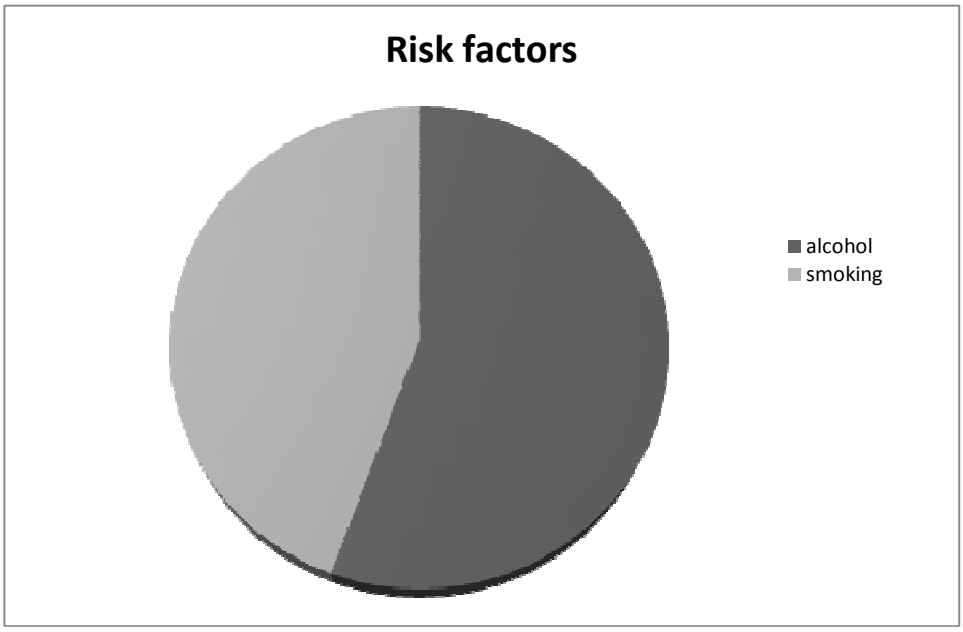


Table-7: Association between alcoholism and pancreatic carcinoma in male cases - Chi square test

Alcoholism	Pancreatic carcinoma		Total
	present	absent	
present	19	6	25
absent	2	7	9
Total	21	13	34

Out of 50 cases of Pancreatic adenocarcinoma,25 (80.6%) were alcoholic. out of 25 alcoholics 19(76%) were associated with pancreatic adenocarcinoma.

By applying chi square test using SPS software version 16.0 , p value is found to be 0.035 which is significant

Table-8:Association between smoking and pancreatic carcinoma in male cases - Chi square test

Smoking	Pancreatic carcinoma		Total
	present	absent	
present	16	4	20
absent	3	8	11
Total	19	12	31

Out of 50 cases of pancreatic adenocarcinoma,20 (64.5%) cases were smokers.Out of 20 smokers,16 (80%) cases were associated with pancreatic adeno carcinoma.

By applying chi square test using SPS software version 16.0 , p value was 0.035 , which is significant.

Table-9: Pancreatic carcinoma cases associated with pancreatitis

H/o pancreatitis	No.of patients	% of cases
Present	34	68
Absent	16	32

Out of 50 cases of pancreatic adenocarcinoma, 34 (68%) were associated with previous history of pancreatitis,and in 16 (32%) of cases there was no previous history of pancreatitis.

Table-10:Lymph node metastasis

Lymph node mets	No. of cases	% of cases
Present	35	70
absent	15	30

Out of 50 cases of pancreatic adenocarcinoma, lymph node metastasis were present in 35 (70%) of cases and absent in 15 (30%) of cases.

Table- 11:Perineural invasion

Perineural invasion	No.of cases	% of cases
Present	28	56
Absent	22	44

Out of 50 cases of pancreatic adenocarcinoma, Perineural invasion were present in 28 (56%) of cases and absent in 22 (44%) of cases.

Table-12:Pancreatic carcinoma association with LFT

LFT	No.of cases	% of cases
Increased	36	72
Normal	14	28

Out of 50 cases of pancreatic adenocarcinoma,LFT is increased in 36 (72%) of cases and normal in 14 (28%) of cases.

Table-14 : Association between perineural invasion and LN mets in all cases - Chi square test

LN mets	Perineural invasion		Total
	present	absent	
present	25	5	30
absent	5	15	20
Total	30	20	50

Association between perineural invasion and lymph node metastasis was done by applying chi square test.

By applying chi square test using SPS software version 16.0 , p value was found to be 0.000 , which is highly significant.

Table-15:Tumor grading based on HPE and cathepsin expression

Grading	No.of cases in HPE	No.of cases expressing cathepsin D
1	6	4
2	21	22
3	23	24

Tumor grading based on Histopathological examination and cathepsin D expression was done .Out of 50 cases of pancreatic adenocarcinoma , 6 cases were well differentiated pancreatic adenocarcinoma , cathepsin D expression score of 1 was given for 4 cases. 21 cases were moderately differentiated adenocarcinoma . cathepsin D expression score of 2 was given for 22 cases. . 23 cases were poorly differentiated pancreatic adenocarcinoma. cathepsin D expression score of 3 was given for 24 cases.

Our study shows that increased expression of cathepsin D was associated with increased grading of pancreatic adeno carcinoma .

Table 16:

Association between cathepsin expression grading with Serum Carbohydrate antigen 19.9 levels in all cases:

Sr CA 19.9 LEVELS	GRADE 1	GRADE2	GRADE 3
>50	1	3	22
35 -50	1	18	2
<35	2	1	-
TOTAL	4	22	24

By applying SPS software version 16.0 , One way ANOVA test for cathepsin D expression and Serum carbohydrate 19.9 levels done.

It showed significant increase in Carbohydrate antigen 19.9 levels in association with cathepsin D expression progression.

DISCUSSION

This retrospective study was carried out in the Department of Pathology, Govt. Stanley medical college, in collaboration with the Department of Surgical Gastroenterology.Total of 50 whipples pancreatoduodenectomy specimens were taken for this study and were evaluated.

In our study pancreatic adenocarcinoma was most common in 40 to 60 years.

Males were more frequently affected than females. Most of the cases were moderately to poorly differentiated , associated with perineural invasion and lymphatic invasion. Serum carbohydrate antigen 19.9 level was found to be elevated in association with moderate to poorly differentiated carcinoma.

Histological grade and stage influences the prognosis of pancreatic adenocarcinoma. Well differentiated adenocarcinoma and those measuring less than 2 cm , without lymphatic and perineural invasion were associated with better survival, whereas poorly differentiated adenocarcinoma and locally advanced carcinoma and those associated with perineural invasion and lymphatic invasion had poor survival rate.

The main aim of this study is to know the association between pancreatic adeno carcinoma grading and expression of cathepsin D and its relationship with perineural and lymphatic invasion and its impact on survival rate .Secondary objective is to know the association of pancreatic adenocarcinoma grading with biochemical parameters such as serum carbohydrate antigen 19.9 levels and liver function test.

In our study, moderate to poorly differentiated pancreatic adenocarcinoma shows increased intensity of expression of cathepsin D as well as increased number of pancreatic cancer cells showing positivity when compared with well differentiated adenocarcinoma . This is correlating with the study done by Wost brock B and Strum JW where they have found that significant statistical correlation between pancreatic adeno carcinoma grading and expression of cathepsin B and cathepsin D.

Pancreatic carcinoma peaks in the age group of 50-60 years , with mean age of 53 years in our study.It is rare below 40 years and above 70 years. This is correlating with the study done by Ohta and Terada where they have shown that pancreatic adeno carcinoma peaks in the age group of 40 to 60 years.

The male to female ratio of pancreatic adenocarcinoma in our study is

2.1 :1. Losch et al says that in their study male to female ratio was 1.8:1 with male preponderance.

Most of the cases (64%) in our study were measuring more than 2 cm . Most of these cases were located in the head of the pancreas. This is correlating with the study done by Yamaguchi N and Chung SM et al.

Those cases that are located in the head of the pancreas presented in advanced stage extending into adjacent organs and they are associated with lymphatic and perineural invasion.This is similar to the study done by Hannah J Whiteman and Sally E. Doewn et al where they have found that cases that are located in the head of the pancreas presented in advanced stage and have increased probability of lymphatic and perineural invasion.

In our study Out of 50 cases of Pancreatic adenocarcinoma , 25 (80.6%) were alcoholic. out of 25 alcoholics 19(76%) were associated with pancreatic adenocarcinoma and the p value was found to be 0.035 which is significant.

Out of 50 cases of pancreatic adenocarcinoma,20 (64.5%) cases were smokers. Out of 20 smokers,16 (80%) cases were associated with pancreatic adeno carcinoma. p value was found to be 0.035 which is significant.

Out of 50 cases of pancreatic adenocarcinoma, lymph node metastasis were present in 35 (70%) of cases and absent in 15 (30%) of cases. Perineural invasion were present in 28 (56%) of cases and absent in 22 (44%) of cases. Lymph node metastasis and perineural invasion were seen especially in cases of moderately to poorly differentiated adeno carcinoma in high percentage when compared with well differentiated adeno carcinoma. By applying chi square test for its correlation with pancreatic adenocarcinoma p value was found to be 0.000 which is highly significant. This finding is correlating with the study done by Niedegethmann M and Willeke and Hildenbrand et al .In their study they have observed that perineural and lymphatic invasion were associated with increased grading of pancreatic adeno carcinoma.

Out of 50 cases of pancreatic adenocarcinoma,LFT is increased in 36 (72%) of cases and normal in 14 (28%) of cases serum Carbohydrate

antigen 19.9 level is increased above 37 U/ml in 42(84%) of cases and less than 37U/ml in 8(16%) of cases.

Tumor grading based on Histopathological examination and cathepsin D expression was done in our study. Out of 50 cases of pancreatic adenocarcinoma 6 cases were well differentiated pancreatic adenocarcinoma , cathepsin D expression score of 1 was given for 4 cases. 21 cases were moderately differentiated adenocarcinoma . Cathepsin D expression score of 2 was given for 22 cases. . 23 cases were poorly differentiated pancreatic adenocarcinoma. cathepsin D expression score of 3 was given for 24 cases. Our study shows that increased expression of cathepsin D was associated with increased grading of pancreatic adenocarcinoma. This finding is similar to study done by Ohta and Terada et al,where they have found that cathepsin expression is increased with increased grade of pancreatic adeno carcinoma.

One way ANOVA test for cathepsin D expression and Sr. carbohydrate antigen 19.9 levels done . It showed significant increase in CA 19.9 levels in association with cathepsin D expression progression.

SUMMARY & CONCLUSION

Out of the 50 whipples specimen studied, we found that pancreatic adenocarcinoma was most common in the age group of 40 to 60 years. Of which males were more frequently affected than females with a male to female preponderance ratio of 2.1 :1. Most of the cases were moderately to poorly differentiated carcinoma, associated with perineural invasion and lymphatic invasion. Serum CA 19.9 level was found to be elevated in association with moderate to poorly differentiated carcinoma.

In our study, moderate to poorly differentiated pancreatic adenocarcinoma showed increased intensity of expression of cathepsin D as well as increased number of pancreatic cancer cells showing positivity when compared with well differentiated adenocarcinoma . Most of the tumour size (64%) in our study measured more than 2 cm. They were mostly located in the head of the pancreas and presented in advanced stage extending into adjacent organs and were associated with lymphatic and perineural invasion.

In our study, out of the 50 cases of pancreatic adenocarcinoma, 25 (80.6%) were alcoholic and there was a significant association between pancreatic adeno carcinoma and alcoholics with a p value of 0.035. 20 (64.5%) cases were smokers and 16 (80%) cases were associated with pancreatic adeno carcinoma. The association between both these variables showed a p value of 0.035 which was significant.

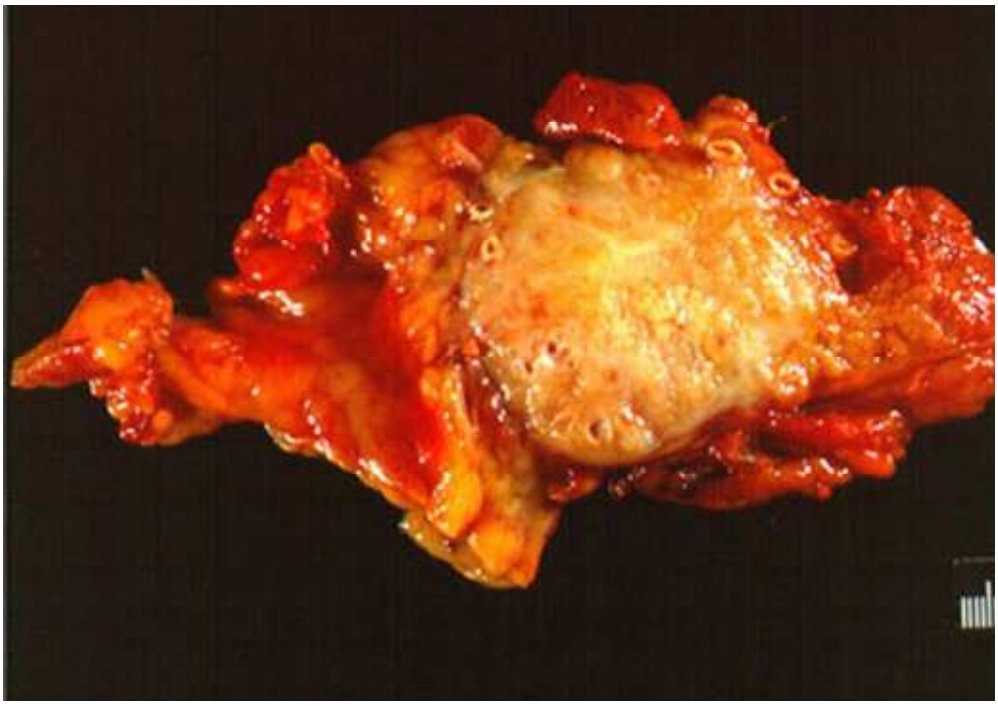
Lymph node metastasis were present in 35 (70%) of cases and perineural invasion were present in 28 (56%) of cases and absent in 22 (44%) of cases. Perineural invasion and lymph node metastasis were especially seen in cases of moderately to poorly differentiated adenocarcinoma in high percentage when being compared with well differentiated adenocarcinoma. By applying chi square test for its correlation with pancreatic adenocarcinoma, p value was found to be 0.000 which was highly significant.

LFT was increased in 36 (72%) of cases and serum CA 19.9 level was increased above 37 U/ml in 42(84%) of cases.

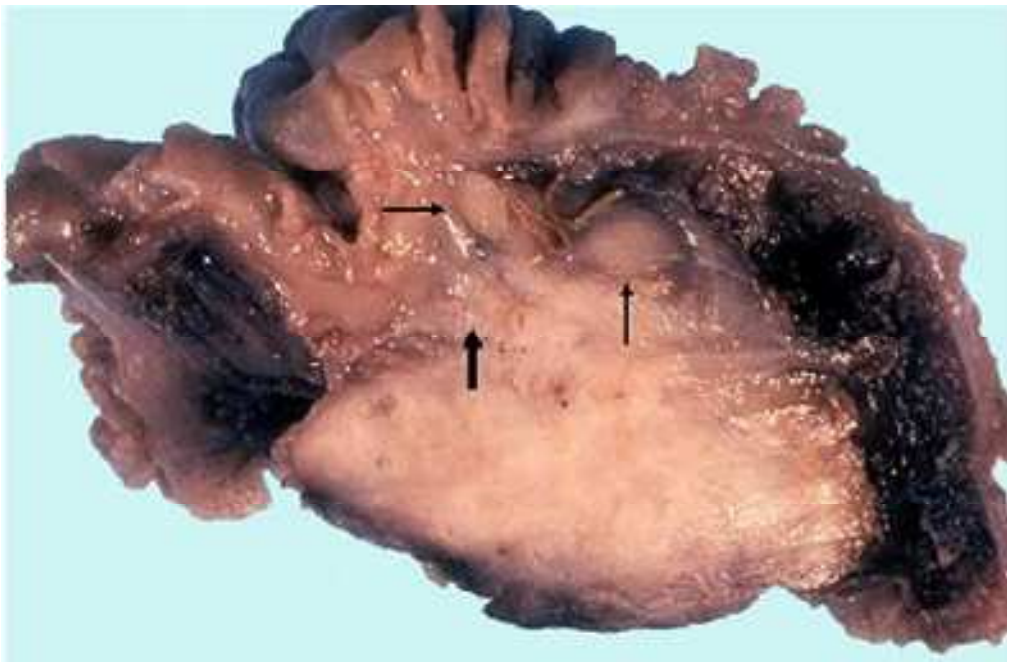
One way ANOVA test for cathepsin D expression and Sr. CA 19.9 levels was done and it showed significant increase in CA 19.9 levels in association with cathepsin D expression progression.

Tumor grading based on histopathological examination and cathepsin expression were done in our study. Out of the 50 cases of pancreatic adeno carcinoma 6 cases were well differentiated adeno carcinoma of which the cathepsin D expression score of 1 was given for 4 cases. 21 cases were moderately differentiated, of which cathepsin D expression score of 2 was given for 22 cases. 23 cases were poorly differentiated, of which cathepsin D expression score of 3 was given for 24 cases.

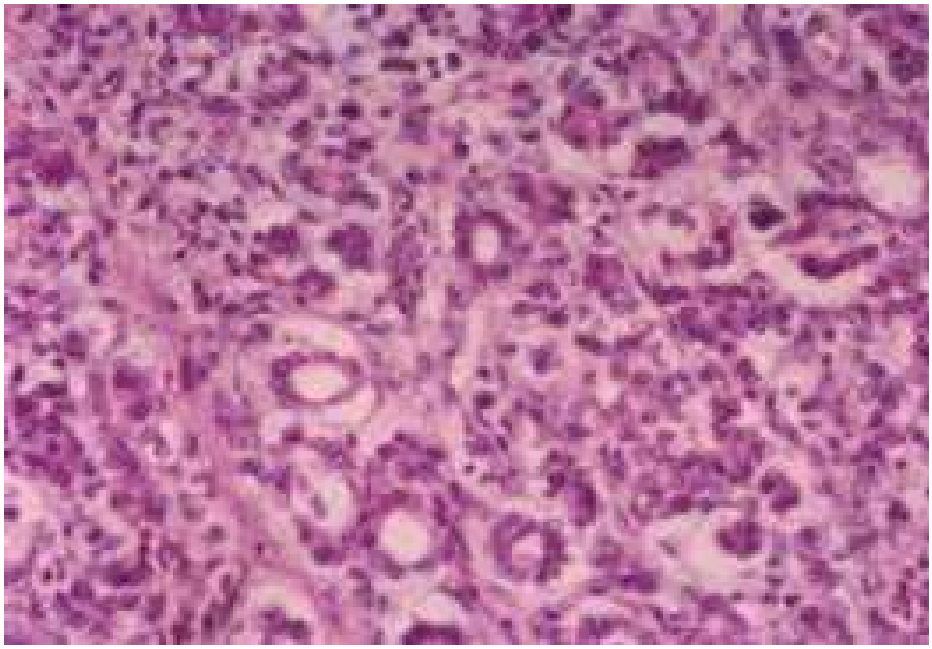
Hence our study showed that increased expression of cathepsin D was associated with increased grading of pancreatic adeno carcinoma , results in poor survival.



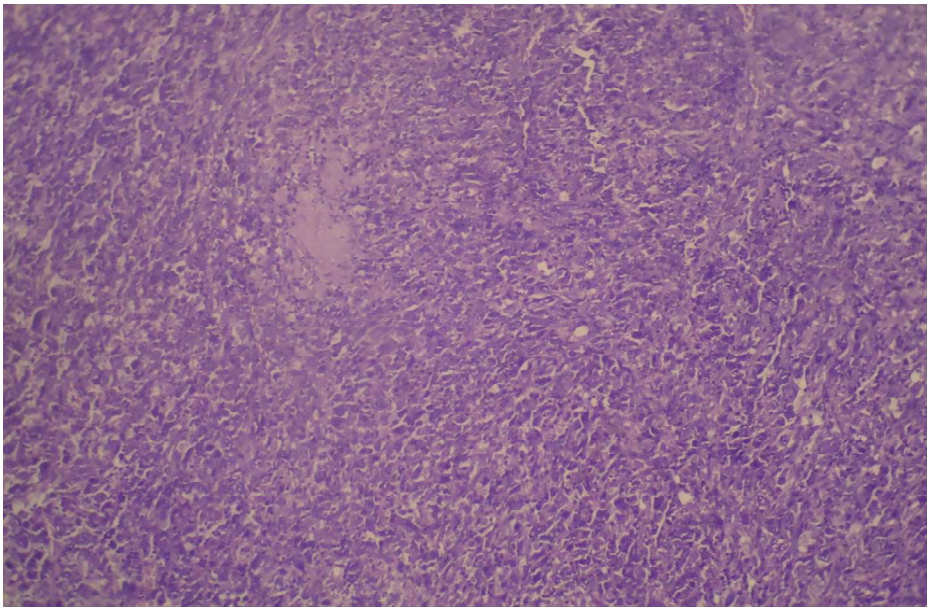
GROSS – Whipples pancreatoduodenectomy showing pancreatic growth



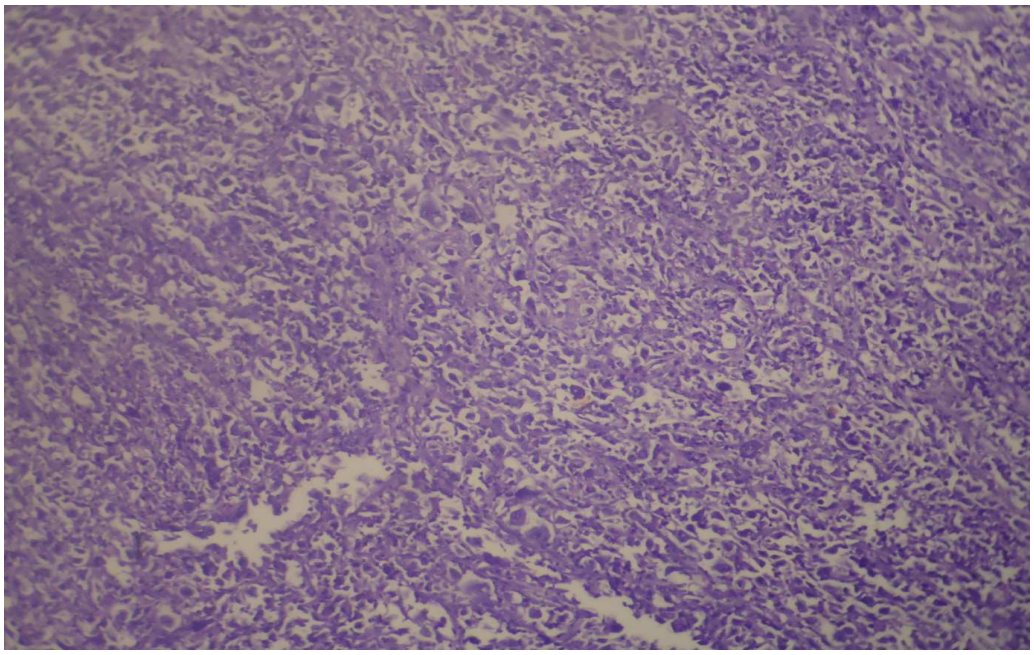
GROSS – Whipples specimen with pancreatic growth and peripancreatic lymphnodes



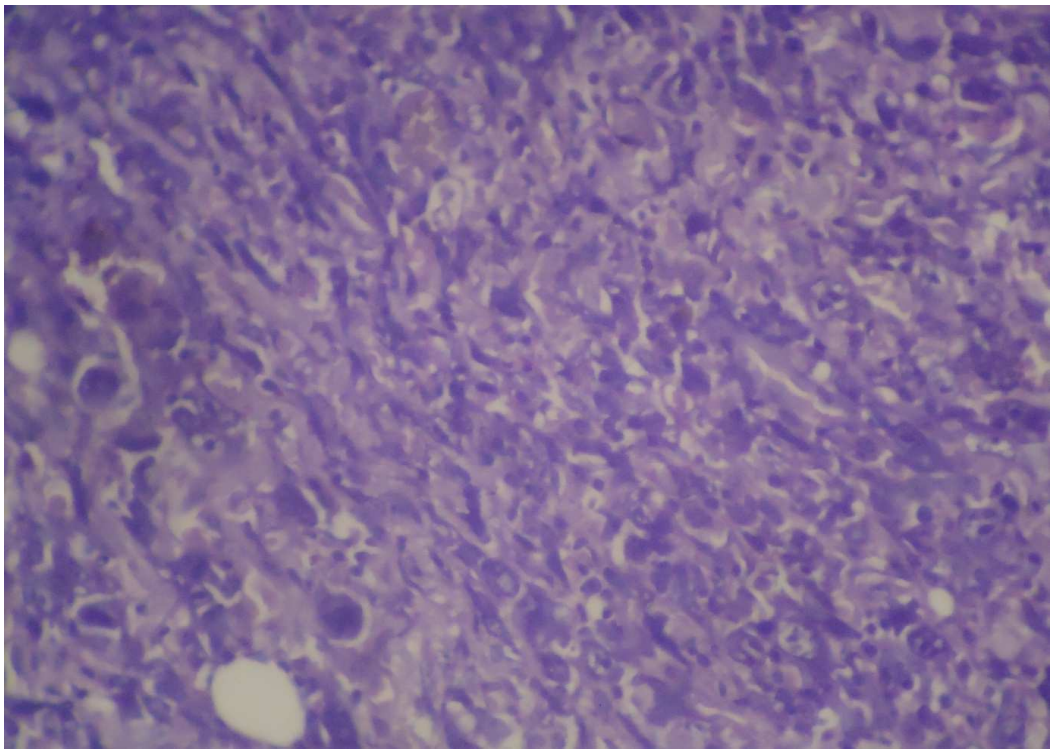
10 X view- Welldifferentiated pancreatic adenocarcinoma



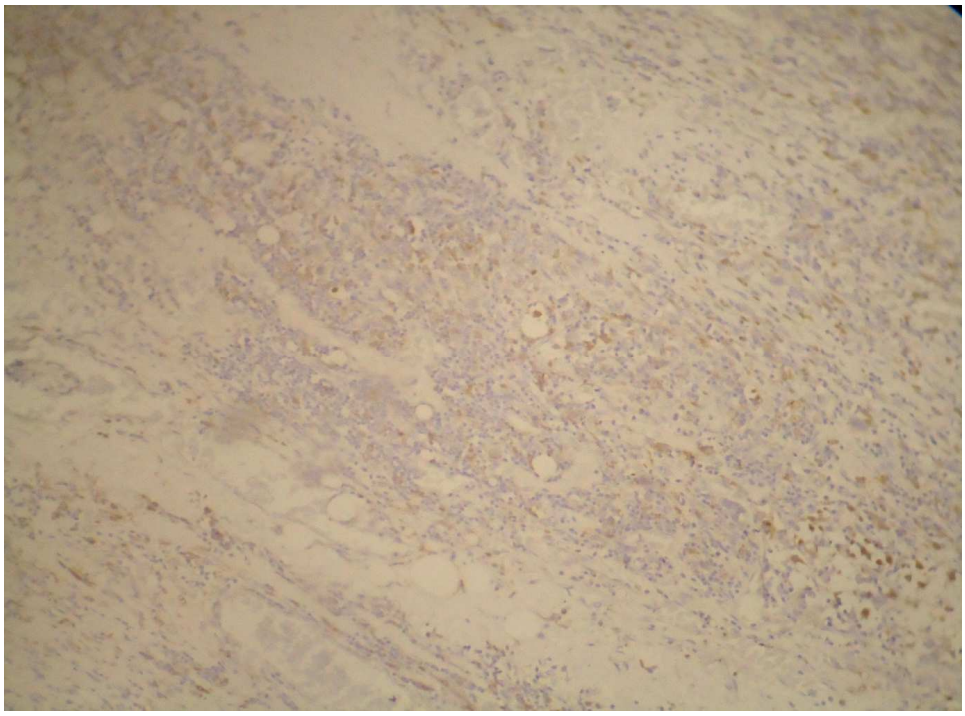
10 X VIEW – Moderately differentiated adenocarcinoma



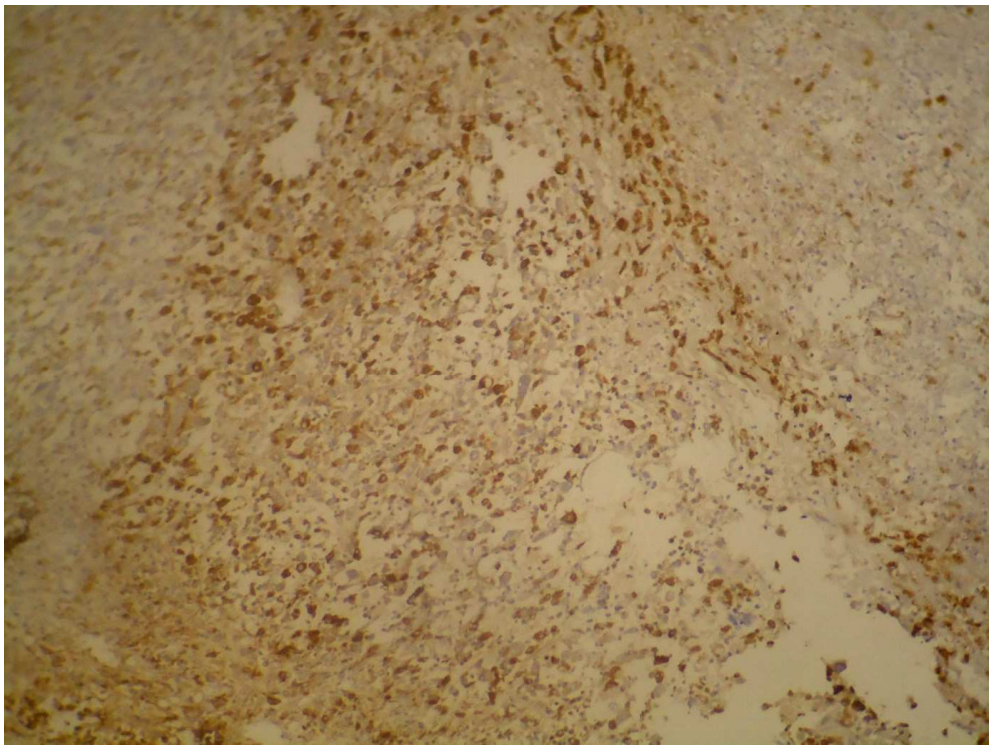
10 x VIEW –Poorly differentiated adenocarcinoma



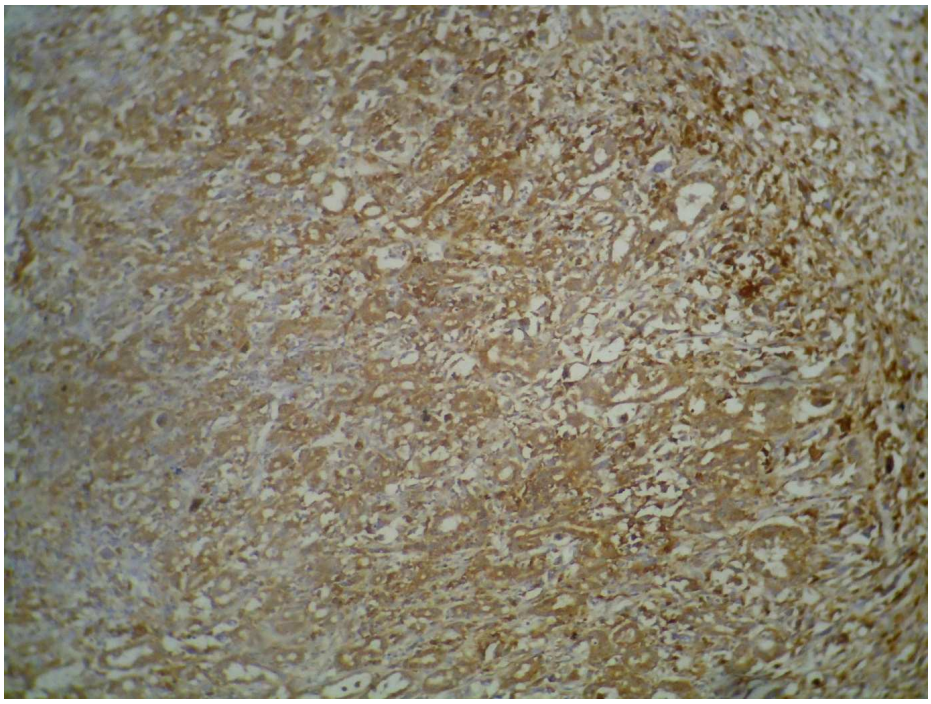
45 X VIEW – showing perineural invasion



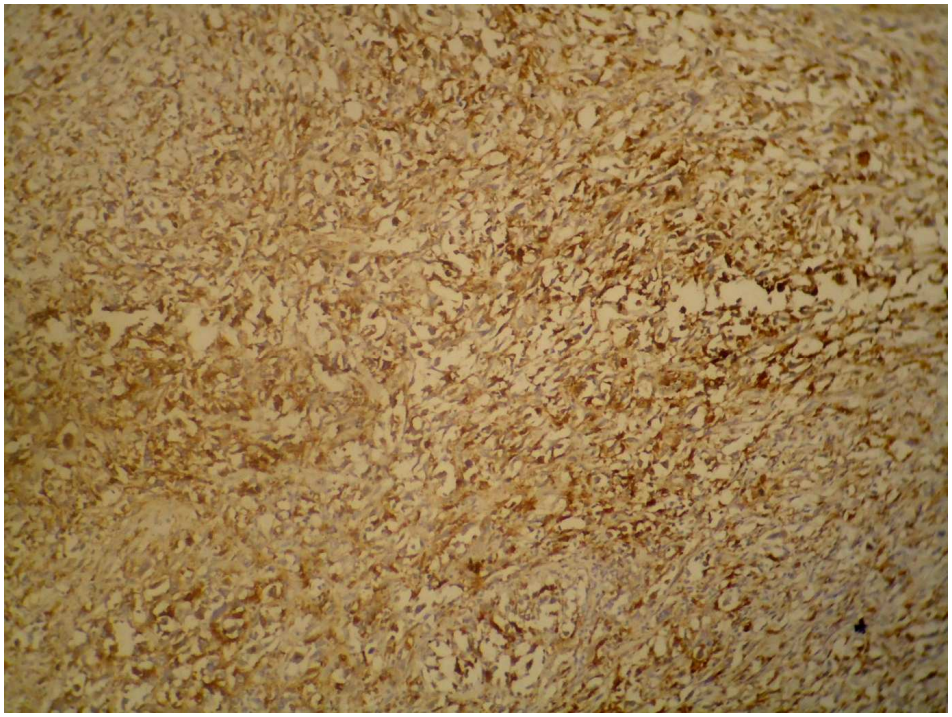
10 X VIEW – Cathepsin D Expression Score 1



10 X VIEW – Cathepsin D Expression –Score 2



10 X VIEW –Cathepsin D Expression –Score3



10 X VIEW- Perineural Invasion expressing cathepsin D

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