PREVALENCE OF K-RAS AND P53 MUTATION IN PERIAMPULLARY CARCINOMA AND ITS IMPACT ON THE PATHOLOGICAL STAGING AT A TERTIARY REFERRAL HOSPITAL IN CHENNAI

THESIS

Submitted to

THE TAMILNADU DR.M.G.R. MEDICAL UNIVERSITY
CHENNAI

For the award of the degree of

DOCTOR OF PHILOSOPHY

IN

SURGERY AND SURGICAL SPECIALITIES

(SURGICAL GASTROENTEROLOGY AND PROCTOLOGY)

by

DR.L.ANAND

M.S.,MCh (SGE).,F.R.C.S (Ed).,DNB.,

JULY 2016

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DR.L.ANAND M.S.,MCh (SGE).,F.R.C.S (Ed).,DNB.,

Under the Guidance of

PROF.DR.S.JESWANTH M.S., MCh.,(SGE)
Professor Of Surgical Gastroenterology
Institute Of Surgical Gastroenterology and Liver Transplantation
Stanley Medical College Hospital,Chennai-600001.

JULY 2016

DECLARATION

I **Dr.L.ANAND** hereby solemnly declare that this Thesis entitled **"Prevalence of Kras and P53 mutation in Periampullary carcinoma and its impact on the pathological staging at a tertiary referral hospital in Chennai"** is an original research work done by me in the Institute of Surgical Gastroenterology and Department of General surgery, Stanley Medical College Hospital,Chennai and it was not used previously either partly or fully for the award of any other Degree, Diploma, Associateship, Fellowship or any other similar title.

Signature of the candidate

Date:

Place:

CERTIFICATE

This is to certify that the Thesis entitled "**"Prevalence of Kras and P53 mutation in Periampullary carcinoma and its impact on the pathological staging at a tertiary referral hospital in Chennai"** submitted by **Dr.L.ANAND MS.,Mch.,FRCS(Ed).,DNB.,** for the award of the Doctor of Philosophy in Surgery and Surgical specialities(Surgical Gastroenterology and Proctology) is a bonafide record of research done by him during the period of study,under my supervision and guidance and that it has not formed the basis for the award of any other Degree, Diploma, Associateship,Fellowship or other similar title.I also certify that this thesis is his original independent work.I recommend this thesis should be placed before the examiners for their consideration for the award of Ph.D Degree.

RESEARCH GUIDE

Prof.S.Jeswanth MS.,Mch.,
Professor of Surgical Gastroenterology
Institute Of Surgical Gastroenterology
Stanley Medical College Hospital
Chennai.

CO-GUIDE

Prof.P.Ravichandran MS.,Mch.,
Director
Institute Of Surgical Gastroenterology
Stanley Medical College Hospital
Chennai.

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Dr.L.ANAND
MS.,Mch.,FRCS(Ed).,DNB.,

CONTENTS

CHAPTER I

INTRODUCTION

Periampullary cancers are relatively uncommon malignancy. They arise from the region which is less than 2 centimeters from the Ampulla of Vater. The common bile duct merges with the pancreatic duct of Wirsung to form a common channel which traverses through the ampulla into the duodenum. The distal most portion of the common bile duct appears dilated (i.e., forms the ampulla of Vater) and is also surrounded by the sphincter of Oddi,the sphincter muscle described by Boyden, which spirals upward around the terminal part of the duct.

Periampullary Carcinoma tends to manifest early due to biliary outflow obstruction. This is in contrast to to pancreatic neoplasms which are often advanced at the time of diagnosis. Periampullary carcinoma includes cancers arising from Ampulla of Vater, Intrapancreatic distal end of common bile duct, Mucosa of 2nd part Duodenum close to Ampulla of vater and tumors of pancreatic head involving Ampulla[1,2]. It is a relatively uncommon malignancy accounting for 6% of all gastrointestinal malignancies[3].When compared to pancreatic cancers periampullary cancers present early, since it blocks the flow of bile into the intestine early in tumorogenesis.

Patients affected with periampullary cancers present with symptoms of painless progressive jaundice, pruritus (itching of skin),passing high coloured urine

and clay coloured stools.Diagnosis is by clinical examination of hepatomegaly and palpable gallbladder. The diagnosis is confirmed by utrasonogram of abdomen and Multidetector CT scan of abdomen. CT scan will reveal either a tumor mass in the periampullary region or dilated intrahepatic and extrahepatic biliary tree with abrupt cut-off at the lower end of common bile duct. A double duct sign is seen in the CT scan with tumors arising from the Ampulla of vater.

An upper gastrointestinal endoscopy will either reveal an ulcerated growth in the Ampulla, in which case biopsy can be obtained or a just a prominent ampulla. The diagnosis can further be substantiated by the tumor marker CA19-9.Multidetector CT scan of the abdomen can help in the staging work-up like the size of the tumor(T stage) with or without adjacent organ involvement and vascular involvement, regional and non-regional nodal involvement, presence of liver metastasis and ascitis. The role of EUS(Endoultrasonography) is indispensible for predicting the nodal status and superior mesenteric vein and superior mesenteric artery involvement[4].

Compared to pancreatic head cancers, where the resectability rate is around 15-20%, the resectability rate for periampullary carcinoma is around 80%[5].This is because of its early presentation, diagnosis and surgical resection before it becomes unresectable, due to local vascular infiltration or distant metastatic spread. Though various etiological factors like cigarette smoking, obesity and hereditary factors has been linked to pancreatic cancer, the precise cause for periampullary cancers has not been defined. Mutation of the p53,the tumor suppressor gene, is the most common genetic alteration in most of the human

malignancies. Overexpression of this p53 protein has been widely reported in the literature for various neoplasms, including those of the periampullary region [6,7,8] .

The K-ras oncogene belongs to the ras gene family (H-ras, N-ras and K-ras)[9]. It is located on chromosome 12p12 and is about 45 Kb in size . The proteins encoded by these genes adopts a structural conformation with a weight of 21 Kd which is located in the plasma membrane and is involved in the signal transduction pathway and cell differentiation[10] . Mutation of this gene is the most common genetic event in human carcinogenesis[11]. Over 90% of the mutations are found in codon 12 and less frequently in codons 13 and 61 [12,13] . K-ras mutations has been reported in 50% of colorectal carcinoma [14] ,50% of lung carcinoma [15] and 60% of thyroid cancers [16] . In pancreatic cancer k-ras mutation frequencies ranges between 70 and 100% [17,18,19] ,while in Ampullary cancers it is between 19 and 70% [20,21,22,23,24,25,26] .In extrahepatic bile duct and gallbladder carcinoma k-ras mutation widely ranges from 0 to 100% [27,28] .

Many research studies has been published in the literature linking k-ras and p-53 mutation in pancreatic cancers which is around 80-90% [29,30,31].But there is lot of geographical variation linking k-ras and p53 mutation with the development of periampullary carcinoma [32]. Also the data available in the literature on the subject is limited. Hence this study is done to look for k-ras and p53 mutation status in periampullary carcinoma in a regional referral centre at Chennai. Furthermore analysis was carried out to assess the association of mutation status with demographic characteristics (age and sex) and clinicopathological variables such as size of the tumor at presentation (Tstage),Lymph nodal metastasis (N stage), lympho-vascular invasion, Grade of the tumor and liver metastasis(M).

CHAPTER II

AIM

To study the prevalence of K-ras and P53 mutation in periampullary carcinoma and to analyze the impact of mutation on the pathological staging.

OBJECTIVES

1. To evaluate the prevalence of kras and P53 mutation in periampullary carcinoma.
2. To identify the association between mutation and higher stage of the disease with pathological correlation.
3. To suggest ways to obtain tissues either during the pre-operative work up or to subject post-operative tumor specimen for mandatory genetic testing, if association is found. This will enable to start biologicals or gene therapy along with chemotherapy in the adjuvant setting to prolong survival.

CHAPTER III

REVIEW OF LITRATURE

CANCER

Cancers are a very vast group of diseases that involves uncontrollable cell growth with the tendency to spread to other parts of the body. There are many types of cancers ,each of which are grouped according to the cell which is first affected. Cancer or neoplasm is a change and multiplication which occurs in a group of cells ending up in growth. There are five features of cancer they are;

- Uncontrollable cell divison
- Cell division without proper signalling mechanism
- Angiogenesis
- Inhibition of programmed cell death

CANCER SCENARIO WORLDWIDE

Cancer is a common worldwide. Cancer prevalence has increased through out the world because of changes in lifestyle, environmental factors and food habits. New cases of cancer of 14.1 million in 2012 occurred worldwide[33]. More than half of cancers occur in less developed countries of the world. . The

corresponding estimates for total cancer deaths in 2010 were 8.6 million (about 28,000 cancer deaths a day), 3.1 million in economically developed countries and 5.2 million in economically developing countries. By 2050, the global burden is expected to grow to 41.5 million new cancer cases and 15.4 million cancer deaths simply due to the increase of the aging population. The future burden would increase in economically developing countries due to smoking, poor diet, physical inactivity, and reproductive factors. Cancers related to these factors such as lung, breast, and colorectal cancers are increasing in developing countries. If preventive measures are not widely applied the rate of cancers may arise even in western countries. Most common cancers occurring worldwide are lung, breast, bowel and prostate cancer. Lung cancer is the most common cancer in men across the globe.

GENDER, INFECTION AND GEOGRAPHICAL VARIATIONS IN CANCERS WORLDWIDE:

In developed countries, prostate, lung, and colorectal cancers are the three most commonly diagnosed cancers among men. Cancers of the breast, colorectum and lung are the most common among women. Lung ,stomach and liver cancers occupy the first three ranks respectively in male population and the common cancers of women are breast ,cervix uteri and lung cancers in the developing regions. Depending on the risk factors and means of screening ,regional variations occur in cancers. Infection plays a fairly important role in the development of cancers. In men stomach and liver cancers and in women cervix and stomach cancer and were found to have an infective base. Stomach cancer continued to be the most common infection-related cancer worldwide[34], followed by liver and cervix.

Approximately 18% of cancers wide are attributable to infections. This percentage is about four times higher in developing countries than in developed countries . The frequency of occurrence of the most common cancers differs by geographical locations. Variations in the most frequently diagnosed cancers are observed by examining individual countries worldwide. In 2010, the common cancer site among males in most economically developed countries was prostate. Lung cancer dominated as the top cancer site in most of Eastern Europe and Asia. The greatest variation among males was observed in Africa, where the most common cancers included prostate, lung, liver, oesophagus, bladder, Kaposi sarcoma, and Non-Hodgkin lymphoma[35]. Except Mongolia, China and Vietnam (liver)[36].the most common site for women was found to be either breast or cervical cancer.

PHYSICAL AND CHEMICAL AGENTS IN CARCINOGENESIS:

The majority of cancers are due to factors occurring in environment and lifestyle changes. The commonest causes being tobacco, infections, diet and nutritional causes.

ONCOGENES AND TUMOR SUPRESSOR GENES

Human cancers are associated with either over expression of oncogenes or Mutation in tumor suppressor genes. These mutations can be gamete mutation or somatic mutation.

Tumor suppressor genes involved in certain human cancers are depicted in **Table 1.**

Table 1

Tumor Suppressor	Chromosome Location	Protein Function	Cancer Type
APC	5q21	Regulates Beta-Catenin, Cell-cell Recognition and Role in Chromosome instability	Colorectal
P53	17p13	DNA repair,apoptisis	Approximately 50% of all human cancers
RB1	13q14	E2F Binding, Cell Cycle Control	Retinoblastoma,lung,prostate and Breast
SMAD4	18q21	Intracellular Signalling in Transforming Growth factor beta(TGF-beta) Pathway	Pancreatic
WT1	11p13	Transcription Factor	Wilms' Tumor
VHL	3p25	Role in RNA Stablity	Kidney, Phaeochromocytoma

DNA AND GENETIC CODE

Genetic information is transferred from one cell to another cell in the form of deoxyribonucleic acid (DNA).DNA Encodes m-RNA i.e. Messenger Ribonucleic acid. m-RNA is transcribed into Proteins. The DNA is made of 4nitrogenous bases, a sugar residue and a phosphate bond, the bases are. Adenine (A), Guanine(G) Cytosine(C) and Thymidine (T). The transcribed Proteins are made of 20 Amino acids. There are about three billion nucleotides in the human Genome. Only 5% of these three billion nucleotides are transcribed into Proteins. A gene has Exon and Intron. Exon contains the actual information for coding of synthesis of

Proteins. Introns are situated between exons. They do not have any coding information. They are removed before a protein gets translated. Codons make up nucleotides, single aminoacid is encoded by a single codon.

The entire set of 64 three letter codes is the Genetic code which converts codons into amino acids. The stop Codon terminates the aminoacid chain. Mutation is alteration in the normal pattern of DNA. Mutations that arise in cancer cells are called somatic mutation;

TYPES OF MUTATION AND PROTEIN ALTERATIONS

Point Mutation

This results from single nucleotide substitution. Point mutations are detected by Mass Spectrometry or Capillary electrophoresis.

Insertion and Deletion

As the name denotes both results from nucleotides that are inserted or deleted in exon of the genomes. They can be detected by direct sequencing of DNA or by PCR-based sizing assays;

Gene amplication and Fusion

DNA that encode genes can become replicated into many copies .Other than this , Regions of DNA not normally present next to each other become fused together. These amplification and fusion can be detected by FISH (Fluorescence in situ Hybridisation).The other method used is RT-PCR i.e. Reverse transcriptase Polymerase chain reaction.

m-RNA-based alternation

m-RNAs may or may not be present in cancers. Though mutations of overexpressed m-RNA's can be detected by RT-PCR, since they are less stable compared to DNA, they are degraded in samples for analysis. Hence generally DNA is used to detect Mutations.

Protein-Based Alteration

Overexpression of protein, when present is cancer cells can be detected using IHC (Immunohistochemistry).This consists of staining sample tissues with antibodies to detect specific proteins that is overexpressed. But levels of proteins are not clinically relevant. Pister et al 2012, has shown that EGFR expression when present in cancer, will respond to cetuximab (anti EGFR Antibody) plus chemotherapy where compared to chemotherapy alone.(ref pg 8 written A4 sheet)[39].

DEVELOPMENT AND SPREAD OF CANCER

Malignancy involves tumor growth by cell division, angiogenesis and metastasis. The process by which new blood vessels develop in malignancy is called angiogenesis. This results is tumor growth and local extension of tumor.Many factors are involved in angiogenic process like growth factors which influence it. These are secreted by the cancer cells within the microenvironment of the tumor. Metastasis is the process by which tumor spreads from the site of origin to a different site, either close to the primary tumor or to a distant organ. Metastasis is facilitated by increased cell motility from the primary tumor and invasiveness.

Angiogenesis

Unlike vasculogenesis, where formation of endothelium and blood vessel wall occurs from mesoderm cell precursors, angiogenesis develops from pre-existing blood vessels. Blood vessel formation is vital for growth and development. Its the main process in wound healing after trauma and surgery by way of forming granulation tissue[40]. But in a neoplastic scenario, angiogenesis is vital for transformation of benign into malignant tissue. This is the basis for using inhibitors of angiogenesis in the treatment of malignant conditions. It was Judah Folkman, who in 1971 proposed the role of angiogenesis in the development and progression of tumor [41,42].

Various types of Angiogenesis

Sprouting angiogenesis and Intussusceptive angiogenesis are the two types

Sprouting angiogenesis

Sprouting angiogenesis was the first to be discovered. The process occurs in stages. endothelial receptors of already existing blood vessels are activated by angiogenic growth factors .Proteases are released from these cells which dissolve the basement membrane and endothelial cells escape from parent vessel wall. The escaped endothelial cells multiply in the surrounding matrix and form solid sprouts. The sproutsd extend towards the angiogenic stimuli and form loops and become full fledged vessel lumen. Sprouting angiogenesis occurs as several mm per day and enables the new vessels to grow across gaps. Its different from intussusceptive

angiogenesis in that it forms new vessels as opposed to splitting of existing ones from latter.[43] .

Intussusceptive angiogenesis:

Intussusceptive angiogenesis is also called splitting angiogenesis, its formed by the splitting of the existing blood vessel into two .This happens in four stages .The first stage starts with first two opposing walls gain contact. As a second step the endothelial junctions are recognized and the bilayer is perforated to allow growth factors and cells to penetrate into the lumen .In the third stage a core is formed between new vesels which is filled with pericytes and myofibroblasts, which start laying collagen fibres into the core for providing extracellular matrix for the growing vessels. Finally core is flushed without basic alteration. Intussusception is important during embryo development since there wont be enough resources to create a rich vasculature ,everytime a new vessel sprouts.

Angiogensis occurs through numerous stimulating factors mainly mechanical and chemical factors. Apart from mechanical factors, the chemical factors are Fibroblast growth factors ,Vascular endothelial factors ,Matrix metalloproteinase and Angioproteins.

Fibroblast growth factor

The fibroblast growth factor (FGF) are a family with mainly two types FGF-1 (acidic FGF) and FGF-2 (basic FGF).and 22 members upto date .They stimulate many finctions by binding with FGF receptors in cell surface in the presence of Heparin proteoglycans. The FGF receptors are made of seven members

which are all protein in nature they are protein tyrosine kinases activated by auto phosphorylation induced by FGF mediated receptor dimerization. This leads to signal transduction cascade and gene activation thus bringing out biological responses this initiates the process of mitogenic activity which is critical for the growth of endothelial cells.FGF-1 is unique in that it can bind to all seven subtypes of FGF receptors making it the potent mitogen leading to angiogenic reponse in damaged tissues.FGF-1 stimulates the differentiation and proliferation of all cell types necessary for building an areterial vessel wall .This fact differentiates it from other pro-angiogenic factors.. They are single chain peptides of 16-18 kDa.

So far three clinical trials with FGF-1 has been done and successfully completed with FGF-1, First in which the angiogenic protein was injected directly into the damaged cardiac muscle. The other trial has been completed to promote wound healing in diabetics with chronic wounds[44]. FGF-2 also plays an important role in endothelial cell proliferation and lumen organisation ,though potent than VEGF or PDGF it is less potent than FGF-1.Both FGF-1 and FGF-2, are important in wound healing apart from angiogenesis

Vascular endothelial growth factor

VEGF is one another important angiogenic factor with action and mechanism similar to FGF-1 and FGF-2.Muscle contraction due to increased blood flow is found to be the single most factor for upregulation of VEGF receptors. The increased blood flow increases the production of mRNA which in turn increases the production of VEGF receptors 1 and 2.The increase in receptor production means

muscle contraction could cause upgrade of all activities in the cascade which signals angiogenesis[45].

Angiopoietins

Angiopoietins, Ang1 and Ang2, are necessary for the function of blood vessels .required for the formation of mature blood vessels[46]. Ang1 and Ang2 are protein growth factors they bind with receptors Tie-1 and Tie-2 to impart the necessary functions. The receptors are tyrosine kinaseses which have the mechanism of action similar to FGF-1 and FGF-2which act by binding with their receptors, Tie-1 and Tie-2.

Matrix metalloproteinase

Matrix metalloproteinase (MMP)[47]. Is another important contribtion of angiogenesis ,It plays a nleading role in dissolution of the protein of the basement of the vessel wall promoting the escape of endothelial cells into the matrix. Inhibition of MMP prevent sprouting angiogenesis and further of new capillaries. These enzymes are highly regulated during angiogenesis since their unchecked activity may decrease the integrity of the microvasculature.

Tumor angiogenesis

As was previously discussed, angiogenesis is vital for formation and metastasis of malignant tumor. During this process cancer cells accrues mutations. It is the same tumor cells which secretes growth factors like VEGF(Vascular endothelial growth factor) and fibroblast growth factor beta to induce capillary

overgrowth in cancer mass. These newly formed blood vessels supply essential nutrients and oxygen supply for the growth of cancer cells. There is mounting evidence to suggest that tumor mass contains mosaic vessels, bearing both endothelial cells and tumor cells. This mosaic pattern has been attributed for shedding of tumor cells from the primary tumor and its circulation in the peripheral blood [48].This knowledge has helped in initiating the usage of Angiogenesis-based tumor therapy like Endostatin, Tumistatin and angiostatin [49].Targeted therapies directed against endothelial cells are now used in many cancers like colon cancers.But on the other hand tumor vessels can develop acquired resistance to drugs and active research is in progress on this topic [50].

Tumor Metastasis

Metastasis, are tumour implants which are discontinuous with primary tumour. It marks a tumour as malignant since benign tumours do not metastasize. Of the four hall marks of malignancy, Anaplasia, Rapid growth, local invasiveness and metastasis, Metastasis occupies an important place. About 30% of newly diagnosed patients with solid tumours present with metastases. Strong metastases reduces the cure of a neoplasm, Spreading of cancer occurs through three main pathways seeding of body cavities, spread through lymphatics and Hematogenous spread. Metastases takes an important position in grading cancers where in TNM, the most common scale used for grading “M” denotes metastases and in stage grouping it occupies the 4rth grade where curative therapy is not a possibility. According to the organ system involved in metastasis, the patient displays signs and symptoms –when in lung, its shortness of breath and blood in sputum, while when its in bone its pain,

fracture, etc. Some patients, however, do not show any symptoms till the advanced stage of the disease.

Organ-specific metastasis

Each primary cancer has the tendency to spread to a particular organ ,this is organotropism. This was proposed by Stephen Paget in 1889 and called ;seed and soil' theory. For instance prostatic cancer metastazises only to bones and colon cancer metastasizes to liver. Apartt from its place of origin cancer cells find it difficult to survive in other regions .Hence is finds a region with similar charecteristics. This theory was challenged by James Ewing 1928 and proposed that spread occur purely by mechanical and anatomic routes. This theory is the base circulating tumour cell theory (CTCs theories)

Metastasis and primary cancer

Metastases always coincides with primary cancers, but in rare cases the "Primary" is not detected while the metastases is the presenting symptoms. About 10-15% of the population presenting to the oncology unit have these presentation and are called ;unknown' or 'occult' primary. The patient is said to have cancer of unknown primary origin[53]).Studies prove if simple questions on coughing or bone pain cannot trace the primary more complex imaging techniques too don't help much. It is estimated that 7% of all cancers are of unknown primary origin.. The use of immunohistochemistry has permitted pathologists to give an identity to many of these metastases[54]. In rare cases (e.g., of melanoma), no primary tumor is found, even on autopsy.

Diagnosis of Metastasis

Pathologists can clearly pinpoint the resemblance of metastatic tumor to the primary tumor by histopathological examination. Occasionally other tests like immunohistochemistry, FISH(fluorescent in situ hybridization) etc.,. are needed to further confirm the malignant nature of cancer cells. Metastasis may be synchronous(present at the same time as the primary tumor) or metachronous (appear months or years later), after the primary tumor has been treated. It was believed that tumor cells rarely metastasize unless they develop somatic mutation. But this osmatic mutation theory is not documented in human cancers. Genetic information from the primary tumor can refelect the metastatic potential of the primary tumor. A subset of genes were identified whose expression could identify primary cancer cells from metastatic cancer cells. This is called the "Metastatic signature". Genes like SNRPF, DHPS, Securin and HNRPAB are the up-regulated genes, and Actin, Myosin and MHC class II are the down-regulated genes associated with the "Metastatic signature" [55].The identification of such genes can differentiate the cells which are likely to metastasize from the primary tumor from those that do not have this potential. This has also been implicated in prognosticating the tumor. Further, identification of such gene expression can be the basis for potential targeted therapy to inhibit metastasis.

Cancer can be broadly classified into Carcinoma that develops from epithelial cells (e.g. cancers developing in the breast, prostate, lung, pancreas and colon are carcinomas) and Sarcoma that develops from connective tissues such as bone, cartilage and nerves. Likewise, Lymphoma and Leukaemia originate from

blood cells. Leukaemia most commonly occurs in children. But adults also develop lymphoma and leukaemia. Germ cell tumours are cancers arising from the testicles or Ovary. Blastomas arise from immature "precursor "cells i.e. embryonic tissue. Blastomas are very common in children than in adults[56].

THE GASTROINTESTINAL SYSTEM:

The organ system responsible for digestion, absorption and excretion of food is the Human Gastrointestinal Tract, its made of oesophagus, stomach, small intestine and Large intestine. Its divided into upper gastro intestinal tract and lower gastro intestinal tract. Embryologically into foregut, midgut and Hind gut. The entire digestive system comprises the entire Gastrointestinal Tract and accessory organs of digestion which include the Liver and Pancreas ,lingual and salivary glands.The Intestine maintains a constant mu8scle tone but also relaxes during peristalsis and wanted movements. The structures of the digestive tract release enzymes and harmones for targeted digestion of nutrients such as lipase, gastrin, pepsin, secretin and cholecystokinin, ghrelin.etc which are released either through duct system or autocrine system.

GASTROINTESTINAL CANCERS

These refer to the malignancies occurring in the gastrointestinal tract or the associated accessory digestive organs which are oesophagus, stomach, Intestines, Liver, Biliary system, Pancreas, rectum and anus. The presenting symptoms are related to the organs of occurrence or General features of Malignancy like obstruction and bleeding. The diagnosis is made through visualization of the tumour

through endoscopy and biopsies. Treatment is by location of the tumour, the cell type and the degree of invasion and metastasis. These are also the factors that determine the prognosis. Cancers of Gastrointestinal tract are responsible for the cancer deaths than any other cancer system throughout the world.

PERIAMPULLARY CARCINOMA

These malignancies refer to the cancers occurring in and around the ampulla of vater like the head of pancreas, distal CBD, mucosa of 2nd part of duodenum with 2cms of ampulla of vater. This term should be distinguished from ampulla of Vater, a dilated end of the common channel of bile duct and pancreatic duct which joins the small intestine. It is formed by the ampulla (common channel), the intraduodenal portion of the bile duct and the intraduodenal portion of the pancreatic duct, Which may show both intestinal and pancreatobiliary morphological characters..Ampullary carcinoma is a rare cancer arising in approximately 4.5% of all gastrointestinal malignancies. Periampullary Carcinoma though distinct clinically but presents similar to the head of pancreas leads to failure to distinguish the clinical and pathological behaviour of these tumours[57]. Many negative prognostic factors has also been investigated in carcinoma of Ampulla of Vater like positive resection margins, larger tumour size, lymph node involvement, histological differentiation, perineural and lymphatic invasion, the number of metastatis lymph node(LNN) and the ratio between metastatic lymph nodes to the nodes totally resected called the Lymph node ratio(LNR) [58] .Preinvasive lesions like adenoma and areas of dysplasia as well as intestinal type of carcinoma can also help in the distinction.Unlike the intestinal type, pancreatico-biliary type carcinoma lacks

adenoma componenets. But this distinction is also difficult in advanced invasive stage of the disease. Though the incidence of Kras mutation reported in the literature in much lower than pancreatic carcinoma (upto 60% vs upto 90%), investigating the molecular abnormalities may reveal if periampullary carcinoma resembles pancreatic, gallbladder and other GI tract malignancies. Telomerase activity in the tumor has been correlated with aggressiveness of periampullary carcinoma [59]. Similarly P53 mutations has ben associated with transformation of adenomas to more invasive carcinomas. Much research needs to be carried out in identifying the molecular abnormalities to ascertain the prognosis of the cancers within the same stage at the time of presentation.

HISTORY OF PERIAMPULLARY CARCINOMA

German anatomist, Abraham Vater (1684-1751), discovered ampulla of vater in 1720. It was Codivilla who first performed en block removal of the duodenum along with panceratic head for periampullary cancer. Halsted attempted the first successful local resection of periampullary carcinoma in1898 and Kausch performed the same resection by a two stage approach[60].Whipple and colleagues (1935)and Brunschwig (1937) independently described a one stage panceraticoduodenectomy[61]. Because of the high mortality and morbidity involved in this type of surgery, some surgeons questioned the role of resection for periampullary tumors and pancreatic carcinoma. This is based on the poor outcomes for patients after resection. This does not justify surgery as a modality of treatment. As of now, the marked advances in the anesthesia techniques, surgical expertise and

better acute care management has resulted in reduced post-operative morbidity and mortality for panceraticoduodenectomy in pancreatic and periampullary carcinomas.

INCIDENCE OF PERIAMPULLARY CARCINOMA

The overall incidence of periampullary carcinoma among digestive cancer is 0.8% in men and 0.9% in women. The partial annual incidence rate is 0.91 per 100000 inhabitants in men and 0.82 per 100000 inhabitants in women. The age-standardised incidence rate was 2.5. Overall incidence rates from 2000 to 2010 for periampullary carcinoma were 1.71, 1.33, 0.77, and 0.21 per 100 000 persons, respectively. (Donald earl Henson. et al., 2009) The frequency of has been increasing in the last 3 decades when compared to pancreatic ductal adenocarcinoma. Periampullary carcinomas have better overall survival rate than pancreatic ductal adenocarcinoma[62]**.** From 2000 through 2010, the incidence rates of EHBD and periampullary cancers remained relatively constant. Cancers of the pancreas are about 20 times more common than those in the periampullary region. In view of the observation that rate patterns for these cancers have remained stable for at least 35 years, and given that the diagnostic criteria have not changed no changes in disease etiology, in primary prevention, screening or detection rate, or in susceptibility to malignant transformation[63]**.**

Among the periampullary malignancies , Lymph node metastasis has been proposed as a major negative prognosis factor for periampullary carcinoma because it is associated with postoperative liver metastasis and poor overall survival.. Hurtuk *et al*., in 2010 were the first to review LNR and survival in patients with periampullary malignancies[64].

Survival status of Periampullary cancers:

The identification of prognostic factors is important to predict the survival probability and to draw conclusions for rational and pragmatic surgical therapy. The 5-year survival rates after resection is usually between 30% to 60%. When a patient had already survived five years after resection, the probability to survive another 5 years was 83%. Prognostic factors can be divided into patient related, tumour related and treatment related factors[65] **.** The respectability rate for ductal adenocarcinoma of the pancreas ranges between 5% and 15%.Morbidity and mortality rates are relatively high and the 5-year survival rate is 4% to 7%. Researchers from the Johns Hopkins hospital reported a 36% 5-year survival rate for 19 patients with periampullary carcinoma who had undergone Pancreaticoduodenectomy, whereas for 50 patients with pancreatic carcinoma who had undergone a similar procedure, the 5-year survival rate was 18% . In carcinomas from the biliary tract, location seems to be the most important prognostic factor. Lesions of the lower third are the most resectable. In a study of distal biliary tumours at the Lahey clinic, the resectability was 100%, the median survival time was 16 months and the 5-year survival rate was 20%. Primary duodenal carcinoma accounts for 0.3% of all gastrointestinal malignancies. Its rarity has made the understanding of the biology of this tumour difficult. The 10 year survival rate of 20 patients treated at Kansas medical center between 1975 and 1990 was 67%.

ETHIOLOGY AND PATHOGENSIS:

Unlike other cancers, the etiological role of diet habits, environmental factors and chemicals leading on to the pathogenesis of periampullary cancers is

questionable. Adenomas, which are precursor lesions to ampullary carcinomas has been widely studied and reported in the literature[66].Adenomas occur close to ampulla of vater in the duodenum. The decreased frequency of occurrence of adenomas in other sites of the duodenum and frequent occurrence in and around ampulla of vater has lead to the postulation that certain carcinogens present in the bile and pancreatic juice have a predominant role in the etio-pathogenesis of carcinoma of ampulla of vater[67]

RISK FACTORS FOR PERIAMPULLARY CARCINOMA:

The precise risk factors for Periampullary cancer are not clearly identified; however there is an association between this disease and **Familial Adenomatous Polyposis (FAP),Hereditary non-polyposis colonic carcinoma(HNPCC), Peutz-Jeghers syndrome and Juvenile Polyposis syndrome.**

- Cancer of the ampulla of vater is most important extracolonic manifestation and a pre-dominant cause of mortality in patients with FAP[68].
- FAP is an autosomal dominant genetic syndrome, caused by an inherited mutation in the APC gene, characterized by hundreds to thousands of polyps throughout the colon.
- Colon cancer occurs due to the genesis of adenoma to carcinoma with this mutation, but the lifetime risk of developing ampullary cancer in FAP is increased by 100-200% with a prevalence of 3-12%.

- The development of ampullary carcinoma is thought to be the same sequence as adenoma-carcinoma in colon cancers in patients with FAP.
- Adenomas have been identified in as many as 90% of surgical specimens of ampullary cancer, and it has been demonstrated that over half of adenoma specimens may include areas of invasive carcinoma.
- This suggests that genetic factosr play a an important role in the development of ampullary carcinoma.
- There is an association between cigarette smoking and the risk of developing ampullary carcinoma.

Evidences to suggest that ampullary adenomas develop into carcinomas are:

- Adenomatous foci are frequently in specimens of adenocarcinomas
- Adenomas are villous or tubulovillous in appearance, identical to those adenomas found in large bowel.
- There is increase in the grade of dysplasia on follow up.
- Genetic alterations are comparable to those lesions found in of other sites of the gastrointestinal tract.
- Age at presentation of patients with adenoma are younger than in patients with ampullary carcinomas.

Hence It is reasonable to postulate that the genesis of adenoma-carcinoma sequence in the ampullary region is as found in the colorecta1 cancers[69](ref 8 p71 sai-Offerhaus)

SIGN AND SYMPTOMS

The signs and symptoms of periampullary carcinoma include the following

- Anorexia
- Nausea
- Vomiting
- Jaundice (upto 90%)
- Pruritus
- Loss of appetite
- Weight loss
- Abdominal pain
- Diarrhoea or steatorrhea (due to bile duct or pancreatic duct obstruction)
- Anaemia with or without symptoms of bleeding from the digestive tract
- Courvoisier gallbladder (i.e., a distended, palpable gallbladder in a patient with jaundice)

- ❖ Fever, only when there is cholangitis.
- ❖ Presentation may be as acute pancreatitis
- ❖ Trousseau's sign(migratory thromboplebitis),is seen in patients with pancreatic cancer.

TNM STAGING OF AMPULLARY CARCINOMA

The American joint committee on cancer (AJCC) has designated Staging by TNM classification for periampullary carcinoma[70]. TNM stands for Tumour, Node, and Metastasis. This system describes the size of a primary tumour(T), lymph nodal involvement(N) and the cancer spread to other organ i.e., metastasis(M).

TNM staging of Ampullary tumors

Tumor stage (pT)	**Regional lymph nodes (N)**	**Distant Metastasis(M)**
Tx- Primary tumor cannot be assessed	**Nx-** Regional lymph nodes cannot be assessed.	**Mx-** Distant metastasis cannot be assessed .
To- No evidence of primary tumor	**N0-** No regional lymph node metastasis.	**M0-** No distant metastasis
Tis-Carcinoma in situ	**N1-** Regional lymph node metastasis	**M1**-Distant metastasis
T1-Tumor limited to ampulla of vater (not beyond sphincter of oddi)		
T2-Tumor invades duodenal wall		
T3-Tumor invades Pancreas		
T4- Tumor invades peri-panceratic soft tissues or other adjacent organs or structures		

DIAGNOSIS

Laboratory studies:

- Complete Hemogram
- Serum Electrolytes
- Liver function Tests
- Serum tumour marker CA 19-9(cut-off value 37U/ml) often elevated in pancreatic and periampullary malignancy, may have a role in assessing tumor burden with a low probability of resection,[71] to assess the response to therapy and/or predicting tumour recurrence.
- Carcinoembryonic antigen (CEA): A nonspecific tumour marker which is elevated in pancreatic malignancies; It assesses response to treatment or recurrence

RADIOLOGICAL WORK-UP

Accurate staging is needed to appropriately assess the primary tumor, local invasion and distant metastasis to decide on curative resection in the form of a Whipple's pancreaticoduodenectomy. The preoperative assessment should include investigations that are sensitive in detecting localized and potentially curable lesion and at the same time, specific enough to identify factors that render the tumour unresectable. Detection of locally advanced and metastatic disease by pre-operative

investigations avoids unnecessary laparotomy in favour of palliative endoscopic stenting to relieve the obstructive jaundice.

Various investigations like ultrasonogram of abdomen, multidetector contrast enhanced CT scan of abdomen, Magnetic Resonance Imaging with MRI cholangiography, Positron Emission tomography(PET-CT scan) and diagnostic Laparoscopy is being utilized to accurately stage the disease for curative resection.

The role of different methods of diagnosis for staging is to give information on:

- Location oaf theTumor and its size
- Lymph nodal involvement in peripancreatic,periportal and celiac axis.
- Tumor infiltration or encasement of superior mesenteric artery and venous involvement of portal-superior mesenteric veins
- Adjacent organ involvement
- Distant metastasis in liver and peritoneum.
- Presence or absence of ascitis

It is mainly because of the retroperitoneal situation of periampullary area, and its relationship with other adjacent viscera and major vascular structures like portal vein, SMV(Superior mesenteric vein) and SMA(Superior mesenteric artery), evaluation of tumor is difficult. With the recent improvement in the techniques of

ultrasonography, Multi detector CT scan of abdomen, MRI and CT angiogram in combination can accurately stage the disease. Pre incisional diagnostic laparoscopy can detect small metastasis <5mm in the surface of liver and in the peritoneum, as these are missed by MDCT scan. Laparoscopic ultrasonograhy can be combined with diagnostic laparoscopy to detect the TNM staging of periampullary carcinoma. Endoultrasonography is valuable in the pre-operative setting to stage small periampullary carcinoma as well as detects the vascular abutment and encasement of the carcinoma with precision. One additional advantage of EUS is that fine needle aspiration cytology can be done in case of suspicious nodal metastasis. MDCT, pre-incisional laparoscopy with laparoscopic ultrasonography is all that is needed to stage periampullary carcinoma. Once metastatic disease is detected, the treatment may altogether shift towards palliative care in the form of endoscopic stenting to relieve the jaundice and gastro-jejunal bypass to counteract the gastric obstruction.

Ultrasonography of the abdomen

- Abdominal ultrasonography is the initial study to evaluate patients with obstructive jaundice. Advantages are that it is easily available, can be done at the bedside, non-invasive and less expensive.
- Ultrasonography can pick up dilatation of gallbladder and extrahepatic bile duct upto the lower end of CBD(common bile duct) in periampullary carcinoma[72].
- Tumor mass will appear hypoechoic with irregular margins
- Ultrasonography can reveal metastatic disease in the liver and presence or absence of ascitis.

MDCT (Multidetector CT) scanning of the abdomen:(figure 1,2)

- MDCT scan of the abdomen is highly sensitive in upto 90% of cases in periampullary Carcinoma.
- CT scanning often demonstrates the size and extent of tumor spread including lymph node metastasis and vascular involvement.
- MDCT has a sensitivity of 100% for tumors with size > 1.5cms[73].
- MDCT is not helpful many a times to differentiate ampullary cancers from periampullary cancers.
- If the lesion is smaller than 1.5cms cm, duct dilatation both pancreatic or biliary may be the only presentation
- EUS(Endoscopic ultrasonography) is more sensitive(90-99%) in such cases with an added advantage of FNAC(Fine needle aspiration cytology)[74].

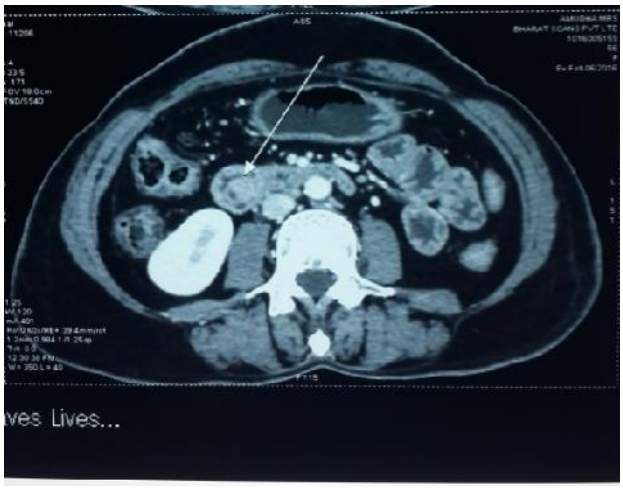


Figure 1. Axial MDCT scan of periampullary carcinoma

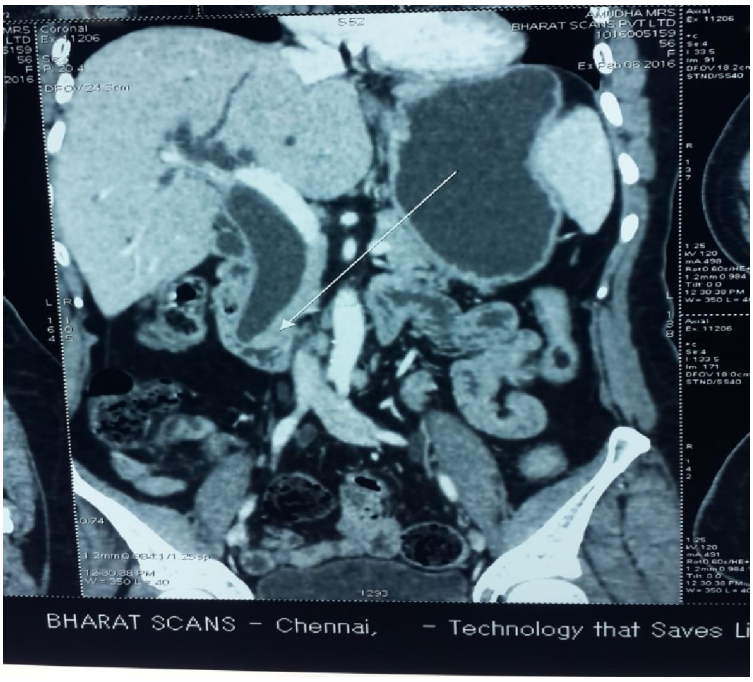


Figure 2. MDCT scan of periampullary carcinoma Showing Dilated CBD upto lower end of CBD

MRI(Magnetic Resonance Imaging) WITH MRCP:

1. The role of MRI in periampullary carcinoma is very limited now because of the excellent information obtained by the latest MDCT high resolution scans.

2. MRI is more sensitive in detecting very small liver metastasis when compared to CT scan.

3. Currently MRI has not replaced MDCT in the staging work-up of periampullary carcinoma.

EUS(Endoscopic ulrasonography):

1. It is considered to the optimal investigation to evaluate the periampullary region
2. It provides the size and extent of the primary tumor (accuracy of 83%), nodal involvement(accuracy of 100%) and vascular involvement[75].
3. EUS FNA provides tissue diagnosis in cases for which neo-adjuvant treatment is indicated[76].
4. Studies have reported that adding p53 immunostaining and k-ras mutational evaluation in EUS FNA specimens and pancreatic juice can add to the diagnosis of conventional cytology[77,78].

STAGING LAPAROSCOPY IN PERIAMPULLARY CARCINOMA:

With 98% of accuracy of detecting the resectability rate for periampullary carcinoma with MDCT[79] ,the role of staging laparoscopy is very limited. The yield of detecting missed out liver metastasis(Fig.3) and peritoneal metastasis by imaging modalities by staging laparoscopy for periampullary carcinoma is only 16%[80].Vollmer et al in 2002 has not shown any benefit of staging laparoscopy for ampullary and duodenal carcinoma[81].

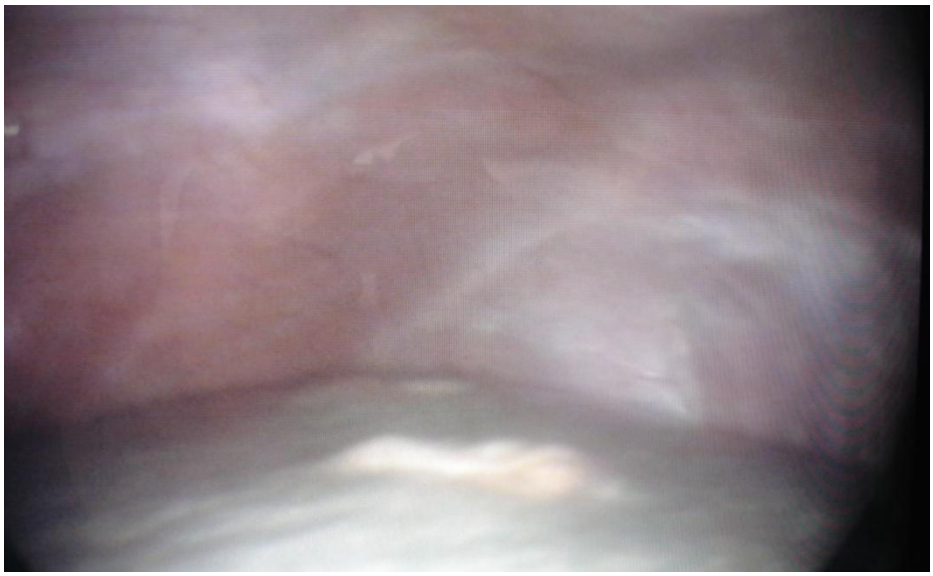


Figure 3. Staging Laparoscopy In Periampullary Carcinoma Showing Liver Metastasis

PREOPERATIVE BIOPSY

Endoscopy and biopsy play a very predominant role in obtaining an accurate diagnosis. Biopsy can be obtained by a side viewing endoscopy or during ERCP(Endoscopic retrograde cholangiopancreatography) done for palliative stenting procedure. Biopsy is mandatory in the neo-adjuvant setting also.Biopsy can be obtained in ulcerative lesion or nodular lesion involving the papilla of vater or the duodenal mucosa[82].But endoscopic tissue biopsy may have false negative result in 10-30% of patients[83].Hence even if endoscopic biopsy is inaccurate, surgery is indicated in patients with high suspicion of periampullary carcinoma on imaging.

Other imaging studies:

- **ERCP:** Has no role in the pre-operative evaluation except for obtaining biopsy in the neo-adjuvant treatment and for palliative stenting.

- **Positron emission tomography (PET) or PET-CT scanning:** PET or PET-CT scans can detect metastases that are too small to be reliably detected on a CT scan.But its role in pre-operative period is limited.It can pick up local recurrence and metastasis in the post-operative period.Combining PET and CT to diagnose the functional tumour tissue and its anatomical site in pancreatic and periampullary cancers,though proposed[84] currently there is no role for its routine use in staging pancreatic and periampullary carcinoma.

Carcinomas Metastastatic to Periampullary Region:

Periampullary carcinomas originate in and around 2cms of Ampulla of Vater. In that carcinomas arising from other organs metastatic to periampullary region forms a small percentageThe various types of carcinomas metastatic to periampullary region reported in the literature are squamous carcinoma of lung, Renal cell carcinoma, melanoma, choriocarcinoma and liposarcoma[85].There is a case report in which HCC(hepatocellular carcinoma) metastatic to periampullary region for which palliative treatment by deployment of endoloop through endoscope was done resulting in sloughing of the tumor. The pre-procedure endoscopic biopsy was

HCC tumor deposits[86].Involvement of the periampullary region by secondary tumors was by hematogenous route, as in a case of HCC with portal vein infiltration or by lymphatic embolisation of tumors.

TREATMENT OF PERIAMPULLARY CARCINOMA:

Surgery:

Enbloc resection by Surgery i.e. Whipple pancreaticoduodenectomy is the standard operation performed for the treatment of periampullary carcinoma.The resectability rate is 80% for patients with periampullary carcinoma and 20% for patients with pancreatic head carcinoma[87]**.**

Whipple Pancreatico duodenectomy (PD):

History of PD originated in the late 1800s. Halsted performed the first transduodenal local excision of an ampullary tumour in 1898. Allesandro Codivilla, in that same year, was the first to perform a PD in Imola, Italy. In 1912, Walter Kausch performed the first successful partial pancreaticoduodenectomy. Allen Oldfather Whipple (1881-1963),Clinical Director of MSKCC(Memorial Sloan Kettering cancer center), an American surgeon is credited with the developing of the first series of PDs in 1935 and the surgery earned the name as the "Whipple operation"[88]**.**The operation involves entering the abdomen by a laparotomy, either by a midline incision, in thin individuals or by a bilateral subcostal incision in others .Once the abdomen is entered, thorough search for secondary metastatic deposits in the liver surface or the peritoneum is done. If found, proceeding further with a planned Whipple PD is abdandoned and bypass surgery is performed to relieve the

obstructive jaundice. Whipple PD involves the enbloc removal removal of the gall bladder, distal common bile duct, whole length of duodenum and proximal 10cms of jejunum and distal half of stomach(as in a Classical Whipple operation) and the head of the pancreas involved with the tumor(Fig.4,Fig.5).PPPD(Pylorus preserving pancreaticoduodenectomy) was later introduced for periamupullary carcinoma to preserve the pylorus to retain gastrointestinal function like avoiding post gastrectomy syndromes[89].The reconstruction after resection involves a pancreatico-jejunostomy or a panceratico gastrostomy to restore the pancreatic secretion into the gastrointestinal system,, hepatico jejunostomy to restore the bile flow and gastrojejunostomy(as in a classical Whipple operation) or duodeno-jejunostomy(for a PPPD)[90].Though this is the standard procedure, several modifications of this procedure has been described.

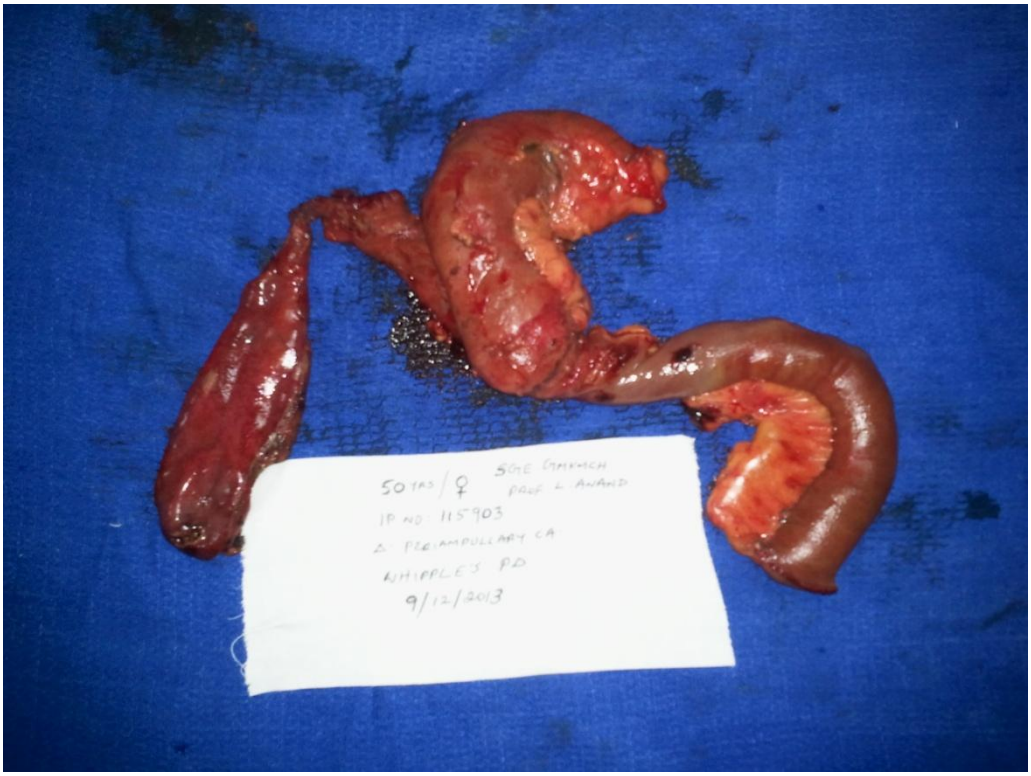


Figure 4. Whipple Pancreatico duodenectomy

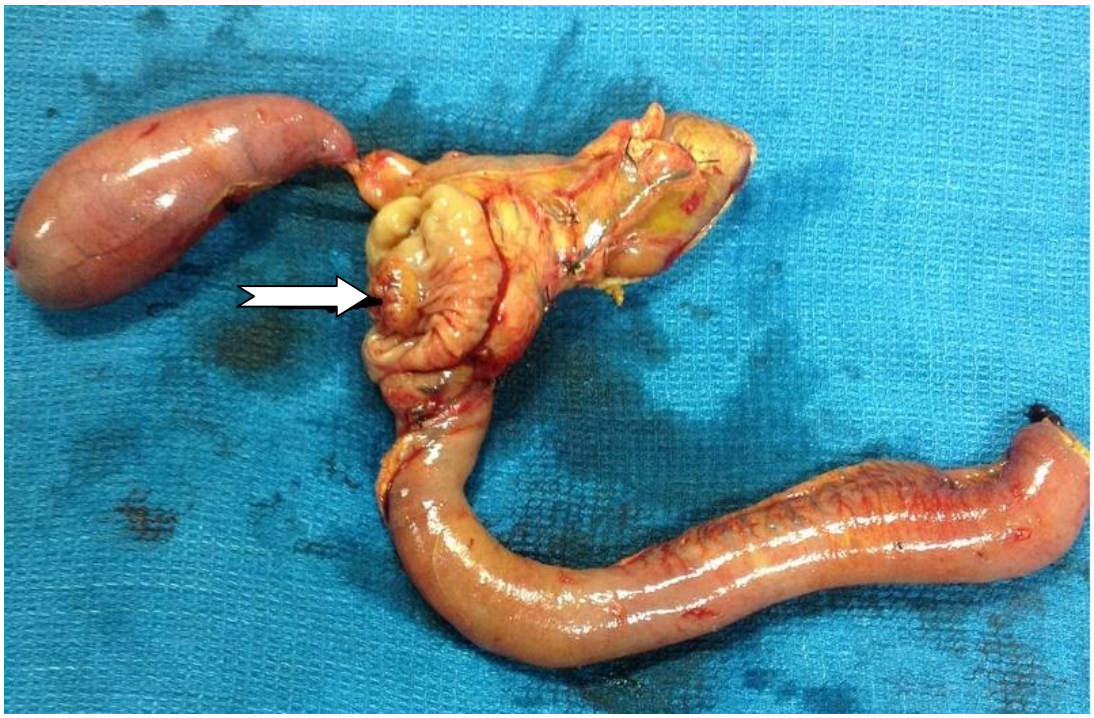


Figure 5. Whipple Pancreatico duodenectomy specimen showing Ampullary carcinoma

Trans Duodenal Excision or Ampullectomy:

The first ampullectomy resection procedure was performed in 1899 by William S. Halsted[91]**.** After many modifications, the technique is now refined and standardized over the years Ampullectomy for the treatment of ampullary carcinomas has long been debated for reasons that are quite obvious. Farnell et al in 2001[92] has described a recurrence rate of 32% at 5 years after TDE(Trans duodenal excision) of Ampulla. Further, local resection is applicable only for stage 0 and stage I tumours, small neuroendocrine tumours and benign lesions < 3 cms.Parmythiotis et al in 2004 has brought out some indications for TDE like < 2 cms villous or tubulovillous adenomas, adenomas with high grade dysplasia, and adenomas with in-situ carcinoma[93]**.**Local excision is also indicated in patients with

poor performance status and in patients refusing to undergo a radical procedure with its underlying complications. Arguments against ampullectomy are that mucosal spread with infiltration was frequently found even in early stage carcinoma, coexistent carcinoma is found in upto 35% of ampullary adenomas[94], final tumour staging is possible only after the final histopathological examination of the resected specimen and it is often associated with post-operative haemorrhage. Moreover frequent endoscopic surveillance is needed. Hence local resection, is not appropriate for duodenal (including ampullary) malignancies, early or late, because the recurrence rate is high

Complications of Pancreaticoduodenectomy:

As with any surgery, there are risks such as bleeding, infection, pulmonary complications and risk of deep venous thrombosis. The complications that are specific to pancreatico duodenectomy are

1. Early hemorrhage(< 24 hours after surgery)
2. Late hemorrhage (1-3 weeks after surgery)
3. POPF(Postoperative pancreatic fistula)
4. Delayed Gastric emptying
5. Intra-abdominal abscess.

Early hemorrhage is usually due to technical failure.It occurs in upto 10% of cases[95].Management initially is conservative using blood transfusion.Intraluminal bleed will warrant an early endoscopy. If bleeding occurs

from the jejunal limb,then early relaparotomy is done to control the source which is usually from the pancreatic stump. Delayed hemorrhage which occurs 1 to 3 weeks after surgery is usually due to pancreatic anatomotic leak.It needs endoscopy to rule out intraluminal source followed by CECT Scan to look for evidence of leak.Generally interventional radiology by way of embolisation of the bleeding source will control the bleeding[96].Patients who are hemodynamically unstable will require surgical intervention to arrest the bleeding.

Post-operative pancreatic fistula occurring in 2-24% of patients[97] is classified as per the ISGPF(International study group on pancreatic fistula) recommendations and managed accordingly[98].

Delayed gastric emptying(DGE) occurs in upto 14% of cases after pancreaticoduodenectomy.Pylorus preserving PD has an increased incidence of delayed gastric emptying.Intra-abdominal infections has been shown to play an important role in causing DGE[99]. Management is conservative with nasogastric tube and maintenance of nasogastric tube and correction of fluid and electrolyte imbalance. Studies have shown using low dose intravenous erythromycin has reduced DGE by upto 75%[100].

Adjuvant treatment after surgical resection

The concept of instituting Chemo-Radiotherapy after R0 resection for pancreatic carcinoma was due to the fact that upto 78% of patients had local recurrence and 61.5% of patients developed hepatic metastasis[101].With factors like decreased 'T' stage, node negative disease, No neural and Lymph Vascular invasion

and negative distant metastasis, favouring long-term survival, addition of adjuvant Chemo-Radiotherapy has taken up the survival even higher in pancreatic and periampullary carcinoma as per Gastrointestinal study group (GITSG) in 1985[102] and European organization for Research and Treatment of cancer (EORTC) trial in 1999[103]**.** In patients resected for pancreatic tumor, the mean duration of survival was 19 months in surgery alone group and 24.5 months in the group which undergone surgery and chemotherapy with Radiotherapy. Similarly after surgery for periampullary cancers, the two year survival was 63% in surgery alone group and 67% in the group with surgery and chemo-Radiotherapy, but there was no differenence in the loco-regional recurrence rate between surgery group and surgery with chemo-Radiotherapy group**.** These landmark trials gave an systematic and optimistic move towards starting adjuvant Radiochemotherapy for all resectable pancreatic and periampullary tumors as a standard treatment. On the other hand Sikora et al[104] published a retrospective study which represented the outcome from a large population of patients on the effect of adjuvant chemo-Radiotherapy after a Whipple pancreaticoduodenectomy for cancer of Ampulla of Vater. There was no survival advantage after R0 resection and no impact on the loco-regional recurrence pattern of the disease,But GISTG Trial and EORTC phase III trial clearly showed a overall survival advantage with adjuvant treatment. Lot of research is being carried out regarding genetic and molecular alteration at the cellular and subcellular level to study the carcinogenesis of pancreatic and periampullary cancers. These studies are aimed directly to use targeted drugs to block the receptors involved in the cell signalling pathway and Gene therapy to modify the altered genetic mutation in the causation of cancer.

Research on genetic mutation on adenocarcinoma of Pancreas has revealed molecular targets that may be the reason for the resistance of the cancer cells to cytotoxic chemotherapy and radiotherapy.One such target is the overexpression of C-myc and HER2/neu Oncogene in pancreatic cancer cells[105].The drug Herceptin has been added to the chemotherapy regimens to taget the gene with improved overall survival.Sarfan et al conducted a phase II trial of Trastuzumab, a monoclonal antibody against HER2 receptor along with Gemcitabine in 34 patients who were HER2/neu positive in advanced pancreatic cancer.Partial response was found in 2 of 32 patients(6%).Median survival was 7 months with a 1 year survival of 19%[106].Similarly EGFR overexpression and resistance to radiotherapy has been found in the pancreatic cancer cells.This has become the rationale behind using EGF Inhibitors to improve the effect of radiotherapy on cancer cells[107].Abburuzzese et al reported a phase II study of Gemcitabine plus cetuximab, a monoclonal antibody against EGFR in EGFR positive pancreatic cancer patients. The overall response rate was 12.55 with a median survival of 6.7months and I year survival of 33%[108] .

Erlotinib, which inhibits EGFR, which are small-molecule inhibitors of the receptor TK(Tyrosine kinase).In a phase III study Gemcitabine with Erlotinib was well tolerated, had a statistically better median survival of 6.37 months to median survival of 5.91months (p=0.025) in patients treated with gemcitabine and placebo[109].This trial was the basis of approval of Erlotinib by FDA to be used in combination with gemcitabine as a first line treatment of advanced pancreatic cancer. Ras Oncogene mutation has been detected in upto 90% of patients with pancreatic cancer.The enzyme farnesyl transferase mediates the farnesylation of Ras to make it active for oncogenesis. Hence farnesyltransferase inhibitors, tipifarnib

and lonafornib was introduced to be used with gemcitabine with a partial response rate in advanced pancreas cancer[110].

Research on overexpression of oncogenes, tumor dependent growth pathways, abnormal expression of tumor specific proteins has lead researchers to introduce drugs like marimastat, matrix metalloproteinase inhibitors(MMPs), Bevacizumab, a vascular endothelial growth factor inhibitor(VEGF Inhibitor), inhibitors of Ras oncogene expression and inhibitors of epidermal growth factor receptors to be used in combination with Gemcitabine in advanced pancreatic cancers. Much research is further needed on the genetic alteration like overexpression of oncogenes and mutation in tumor suppressor genes to introduce novel targeted therapy along with adjuvant chemo-radiotherapy to enhance the overall survival in periampullary cancer.

PROGNOSTIC FACTORS IN PERIAMPULLARY CARCINOMA:

Carcinoma inAmpulla of Vater:

Due to improvements in surgical expertise, anaesthetic techniques and critical care in the post-operative period, the mortality after Whipple PD has come below 5% in most of the centres.

The pathological factors that have been shown to affect the prognosis are

a. Size of the primary tumor

b. Nodal involvement

c. Stage of the disease at presentation

d. Grade of the tumor

e. Lympho-vascular and perineural invasion

Studies have shown that prognosis is good when the tumor to sphincter of Oddi (T0,Tis,T1) but poorer when the tumor infiltrates the pancreas[111].Similarly 5 year survival rate when nodal metastasis number is 0 to 2 when compared to more than 2 nodes[112].The 5 year survival rate falls from 85% for stage 1 Ampulla vater tumor to 0-24% in stage IV tumors.The 5 years survival rate is between 18-58.4% in poorly differentiated adenocarcinoma and 49-77.8% in well differentiated adenocarcinoma. Another vital factor which influences prognosis is the Pathological type of the tumor. The tendency for intestinal type of ampullary carcinoma to spread to lymph nodes is much less than the pancreatico-biliary type. Hence prognosis of intestinal type of ampulla of vater carcinoma is better[113]. Multivariate regression analysis of ampulla of vater cancers has shown that node negativity, infiltration of pancreas, lymphatic and perineural invasion are significant pathological factors[114].In a study by Yoshida et al[115],negative resection margins and curative resection were found to be good prognostic indicators in lower end CBD(Common bile duct carcinomas).Survival is longest for patients who undergo panceraticoduodenectomy for duodenal carcinoma(22%-53%) followed by carcinoma of ampulla of vater, distal bile duct tumor and finally for pancreatic adenocarcinoma. Even for patients in whom nodal metastasis is present aggressive en-bloc resection of the tumor along with the nodes will enhance survival in pariampullary cancers [116].The factors which has been shown to improve outcome after surgical resection are tumor size <2cms,node negative tumor, negative resection margins and lesser genetic alteration,

especially for bile duct carcinomas[117].The major issue in periampullary cancers is the difficulty for the pathologist to identify the exact site of origin of tumors, which will influence the determination of the survival outcomes.

Genetic mutation and alterations described in Pancreatic cancer:

Tumorogenesis means the process through which a cell acquires a transformation into a malignant cell. This particular section deals with genes with mutation and the pathways of this mutated gene in bringing out the changes. Though there are many tumours which can be distinguished histologically into many types,this particular section focuses on pancreatic ductal adenocarcinoma, which is the most common form[118].

Two types of genetic alterations , mainly genetic and epigenetic occur. Genetic alterations mainly occur in three types of genes: oncogenes, tumour suppressor genes and genome maintenance genes. Mutations can be further classified based on origin as somatic (acquired) or germline (inherited).In familial cancer syndrome of pancreatic cancer, it is usually through germline mutations but sporadic changes can also bring out a sporadic change.

The two most common mutagenic mechanisms involved in pancreatic cancers are CIN (chromosomal instability) leading to numerical changes or aneuploidy and MSI(microsatellite instability) which causes defects in replication and repair of DNA.

SMAD4 is a gene which is mutated in germline and causes familial cancer syndrome called Juvenile polyposis .It is also sporadically mutated in

pancreatic cancers. But the familial polyposis syndrome is in no way connected with development of pancreatic cancers. Among CIN and MSI,MSI causes defects in particular sequences of DNA which are repetitive sequences formed in microsatellites, while most of the pancreatic tumours of CIN type have mutations in the KRAS [119,120].

MSI changes are rare in pancreatic cancers, and occurs in a particular histologic type having a medullary pattern[119] and frequently has mutation in TGBR2 or ACVR2.

In epigenetic alterations, hypermethylation of gene promoters causes transcription, gene silencing and inactivation. Gene activation is caused by hypomethylation , loss of imprinting and chromatin modification. All these are all grouped as the mechanisms. Changes that occur in cells due to genetic alterations has a causal role as stated statistically, but epigenetic role of inactivation is not proved because it is a potentially reversible event.

Role of Oncogenes:

Oncogenes when they are mutated increase the activation of the encoded proteins. The mutations involved are subtle point mutations ,gene amplification and possible chromosome translocations.

KRAS is the most commonly mutated oncogene in pancreatic cancer with a rate of 90%.

In KRAS wild type BRAF mutations are also found.

Overexpression of protein through gene amplification has been found out for different genes in carcinoma of the pancreas. AKT2 gene in 10-20% [120,121] NCOA3(AIB1) gene in 30% and MYB gene in 10% [122].Though gene amplification is responsible for the oncogene activation, the two most important mechanisms involved are ,through gene amplification which involves one gene amplification and through huge DNA sequences. It may also include the culprit sequences. Secondly its not determined if the amplification areas are rather simply accumulated because of the structural reasoning and genetic drift.

Tumour suppressor genes have to be inactivated to render the role as tumorigenic factors. The mechanism involved are deletion ,insertion and point mutation. These genes are recessive and to inactivate the gene both alleles need to be inactivated to conform a biological change. Then there is also a different entity called, Dominant negative mutation in which its enough if only one allele is inactivated. In both allele inactivation cases, mutation is of a combination type ,with point mutation of one allele and complete loss of other allele called LOH(loss of heterozygosity).Epigenetic mechanism also play a major role[123].Tumour suppressor genes which have a high frequency mutation in pancreatic cancer are CDKN2A,TP53 and SMAD4.

The CDKN2A is the most commonly mutated tumour suppressor gene in 85% of pancreatic cancers. It encodes p16[124].The somatic mutation mechanisms involved are intragenic mutation and homozygous deletion and epigenetic activation [125,126,127] by gene silencing. Germline mutation of this gene occurs in familial atypical multiple mole melanoma syndrome, It predisposes to pancreatic cancers.

The second commonly mutated gene is TP53 which encodes p53 portion and shows somatic mutation in 50-75% of pancreatic cancers [128,129,130,131].The germline mutation of the same gene leads to LiFraumani syndrome predisposing to pancreatic cancers [132,133].The third gene is SMAD4 which is somatically mutated in 55% of pancreatic cancers and undergoes germline mutation to develop Familial carcinoma syndrome, juvenile polyposis predisposing to pancreatic cancers[134].Some genes exhibit low mutation especially genes encoding transforming growth factor-Beta, predisposing to pancreatic cancers[135].Mutation of MAP2K4, another tumour suppressor gene[136] is reported in many cancers including pancreating cancers but at a very low percent of 3-6%

Genome maintenance gene:

Dysfunction in genome maintenance gene leads to increased mutation and leads to tumourigenesis in cells .The mechanism involved are same as tumour suppressor genes .Fanconi Anaemia is due to gene mutation in genome maintenance genome.BRCA2 is one another found in 7-10% of pancreatic cancers.

Ras pathway:

This is a growth promoting pathway which is connected to pancreatic cancer through KRAS and BRAT genes. The kras protein is a guanine nucleotide building protein encoded by kras gene in codon 12,13 and 62.It has intrinsic GTP ase activity that hydrolyses GTP to GDP .In its active state it is bound to GTP and in inactive state to GDP. When there is a mutation, GTP hydrolysis is impaired, constitutive activation of kras results in continuous transmission of growth

promoting signals or exaggerated response to growth promoter signals. Through one or other pathways it causes active transcription[137] .

The Rb Pathway:

This pathway is the regulator of G1/S transisition in cell cycle and is linked to pancreatic cancer by mutation of CDKN2A gene encoding p16 protein. Inactivation of it is seen in pancreatic cancers. Binding of p16 to CDK4 inactivates it. Mutation of p16 interferes with p16/CDK4 binding and results in the promotion of G1/S transition.

P53 pathway:

This is linked with pancreatic cancers by mutation of TP53 gene which encodes protein p53 which mainly regulates cell cycle control and programmed cell death producing cellular responses to oncogenic stress.p53 is usually expressed at low levels due to high protein turnover. Nascent p53 can be activated or inactivated by proteolysis. Activated p53 binds to specific DNA sequences[138] which induces large variety of target genes especially those mutated in cell cycle regulation.CDKN1A,SFN and GADD45 and also genes involved in apoptosis. BAX and BBC3 also induce the transcription of MDM2.It establishes the negative feedback of p53 protein levels.

Mutation in the gene causes p53 inactivation by impairing its DNA binding capability**.** This interfers with transcription[139]**.** Since P53 is central in regulation of cell cycle, its mutation results in dysregulation of this cell growth controlling mechanism.

TGF_Beta:

These cell receptors are linked to pancreatic cancers by mutations on SMAD4,TGFBR1,TGFBR2,ACVR1B and ACVR2 genes. Ligands bound to these receptors bring about changes through smad proteins. smads operates through different mechanisms. Mutation in SMAD4 interferes in signaling pathways by many mechanisms. Mutation in SMAD4[140] is implicated in the negative of cell growth via regulation of cell cycle ,any mutation ton it is oncogenic[141].Less frequently TGFB1,TGFB2, ACVR1B and ACVR mutation can cause disruption in the signaling pathways [142,143,144].

FANCONI ANAEMIA/BRCA2:

This is implicated in pancreatic cancers through mutation in FANCC,FANCG and FANCD1/BRCA2 genes. In DNA repairs and cell cycle control, these genes are genome maintenance genes too and have tumour suppressor function.DNA interstitial cross linking agents are among the most commonly used chemotherapeutic agents. FANCC and FANCG mutations confer an increased sensitivity to these drugs and effect is observed in any FANCC and FANCG pathway disruption [145,146,147] .

Similar mutations and pathways that acts in ampullary and periampullary carcinoma like Kras,P53,Beta catenin,Wnt signalling pathways, APC gene mutation etc., have been investigated to a lesser extent than those described in the literature for pancreatic carcinoma.

CHAPTER IV

SCOPE AND PLAN OF WORK

SCOPE OF THE STUDY

1. This study was chosen since there aren't any published report on the mutation of Kras and P53 genes in periampullary carcinoma in our regional population.

2. The study will unravel the association of such mutations in patients who develop periampullary carcinoma when there are no other risk factors involved like family history of cancer, smoking, alcohol and other dietary risk factors.

3. If in the study samples, mutation rate is found to be higher, then serious thought has to be given regarding routine genetic screening of specimens to add biological therapy in the adjuvant treatment in our regional population.

PLAN OF WORK

1. Development and finalizing the protocol

2. Wide Literature search will be done in the Internet, Journals, Books etc., for references pertaining to the study.

3. Institutional Ethical committee clearance after designing the study and the methodology.

4. Collection of data regarding periampullary cancers operated in the Institute of Surgical Gastroenterology, Stanley Medical College Hospital.

5. Collection of pathology report of the specimen subjected for Histopathological examination after surgical resection.

6. Collection of samples(n=90) i.e. Formalin Fixed Paraffin Embedded tissue blocks from the department of Pathology.

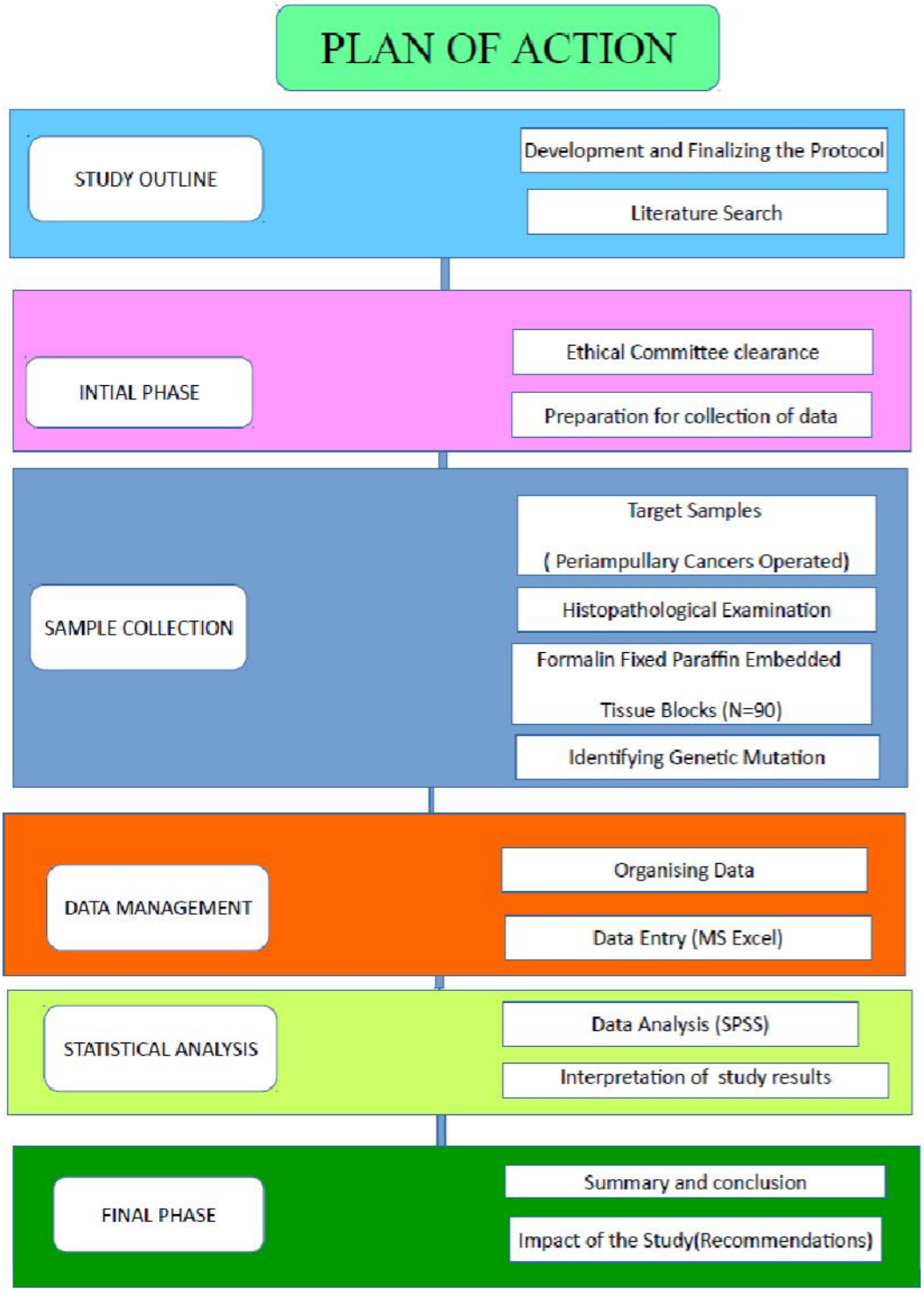
7. Subjecting the paraffin blocks to the Department Of Human Genetics, Bharathiar University, Coimbatore, for PCR-RFLP DNA Analysis of KRAS and P53 Genetic mutation (Kras codon 12,Exon 1 and P53 codon 128-184,Exon 5).

8. After the datas are obtained, the collected data will be organized and data entry will made in the MS Excel sheet. analysis of the stage of the disease at presentation between mutated and non-mutated cases by comparing the pathology report.

9. Statistical analysis of the data to done to look for the association of the selected variables with the mutated samples, with the help of a statistician by SPSS software.

10. Interpretation of study results will be carried out.

11. Finally the Summary and conclusions as well as the recommendations(Impact of the study) on the outcome of the study will be discussed and presented as Thesis.


PLAN OF ACTION
STUDY OUTLINE
Development and Finalizing the Protocol
Literature Search
INTIAL PHASE
Ethical Committee clearance
Preparation for collection of data
SAMPLE COLLECTION
Target Samples
(Periampullary Cancers Operated)
Histopathological Examination
Formalin Fixed Paraffin Embedded
Tissue Blocks (N=90)
Identifying Genetic Mutation
DATA MANAGEMENT
Organising Data
Data Entry (MS Excel)
STATISTICAL ANALYSIS
Data Analysis (SPSS)
Interpretation of study results
FINAL PHASE
Summary and conclusion
Impact of the Study(Recommendations)

CHAPTER V

MATERIALS AND METHODS

STUDY DESIGN:

This is a prospective, cross sectional, single institution study of the data collected from the database of the Department of Surgical gastroenterology, Department of General surgery and Department of pathology at Stanley Medical college Hospital, Chennai from 2009 to 2013.

SAMPLE COLLECTION:

90 samples of Formalin fixed paraffin blocks, prepared from Whipple's Pancreatico Duodenectomy specimens, operated for periampullary carcinoma were collected from the department of Pathology(Fig 6).The Paraffin blocks, representative of tumor tissue, were chosen after studying the H&E stained slides of the tumor tissue based on the pathology report collected from the department database which was again cross-checked with the specimen report registry maintained at the Department of Pathology.

INCLUSION CRITERIA:

All samples which are adenocarcinoma of periampullary region, as mentioned in the final histopathology report were included in the study.

EXCLUSION CRITERIA:

Samples which was mentioned in the pathology report as carcinoma of head of the pancreas and lesions other than adenocarcinoma arising in the periampullary region like neuroendocrine tumors,GIST(Gastrointestinal stromal tumors),lymphomas, tuberculosis and benign strictures were excluded from the study.

SAMPLING TECHNIQUE:

Purposive sampling

INSTITUTIONAL ETHICAL COMMITTEE CLEARANCE

After DNA analysis and estimation of the presence or absence of Kras and P53 mutation status, association between mutation and pathological staging was compared using the detailed pathology report collected from the department database. The study protocol was presented to the Stanley Medical college Hospital Ethical Committee and Institutional Ethical Committee clearance was obtained before the start of the study.

STATISTICAL ANALYSIS:

1. Data were analysed using SPSS 17
2. Qualitative variables were given in frequencies and percentages
3. Quantitative variables were given in mean and Standard deviation

4. Independent t Test were used to compare the mean size between mutant and non-mutant.

5. Associated factors of mutation were identified using chi-square test.

6. Backward Multiple logistic regression were used to identify the predominant factors associated with mutation status.

7. $P < 0.05$ were considered as significant.

DNA EXTRACTION

Extraction of core

1. Before Isolation of DNA from the tumor tissue, H&E stain is prepared from the top slide of the tissue in the block. This is read by an experienced pathologist. Then the normal and tumor regions marked. This slide used to identify the corresponding regions in the block.

2. Then a 14G needle is used as a cutting tool to pierce the block in the region of interest. 1 to 3 mm core is cut out by turning the needle in the block. The depth must be sufficient to fully pierce the tissue. Part of the tissue is cut in to 5um sections on the microtome for DNA extraction(Fig.7). The stage is wiped with alcohol and the blade is replaced after each block there by avoiding cross contamination.

3. The newly cut core is transferred to a 2 ml polypropylene microcentrifuge tube. The tube is labelled appropriately.

4. The coring or cutting process may be repeated as necessary. Each core is placed in a separate tube.

5. It is always recommended that the seal is blocked with melted paraffin later.

Removal of Paraffin

(It is to be noted that, Xylene is a hazardous chemical hence step 6 must be done in a fume hood, The resulting waste must be handled accordingly).

6. The core is treated in the following steps in 2.0 ml polypropylene microcentrifuge tubes (if 15 ml polypropylene centrifuge tube is used, the centrifugation is adjusted accordingly):

    a. The core is immersed in 1ml of xylene for 10minutes and pellet at 14000 rotations per minute for about 10minutes.The hypernatant is aspirated.

    b. Repeat the step and immerse in 1 ml of 100% Ethanol for about 10 minutes and pellet at 14,000 rpm for another 10 minutes.The supernatant is aspirated.

    c. The step is repeated and immerse in 1 ml of 80 % Ethanol for about 10 minutes and pellet at 14,000 rpm for another 10 minutes.The supernatant is then aspirated.

d. Again repeat the step and immerse in 1 ml of 50 % Ethanol for a period of 10 minutes and pellet at 14,000 rpm for another 10 min.The supernatant is now aspirated.

e. The step should be repeated again.

7. Now 1ml of H_2O is added and incubated overnight at 4°C.

Digestion of Protein

8. Next Pellet sample at 14,000 rpm for about 10 minutes and the supernatant is aspirated.

9. 700 ul of Nucleic Acid Lysis buffer (NALB) is then added.

10. 50 ul of Proteinase K (30 mg/ml) is also added.

11. Incubation is done for 24 hours at 65°C.

12. 50 ul of Proteinase K (30 mg/ml) is then added.

13. Incubation for a period of 24 hours is done at 65°C.

Precipitation & Isolation of DNA

14. For the elimination of endogenous RNA in the sample, 10 ul of RNAse A (10mg/ml) is added and incubation is done at 37°C for 30 minutes.

15. 250 ul of 6 M NaCl (saturated) is then added to it

16. This is left to stand at room temperature for about 10 minutes.

17. Next Pellet sample at 14,000 rpm fo aboutr 10 minutes.

18. The supernatant is transferred to a clean microcentrifuge tube.

19. 1 ml of ice-cold (or –20°C) 100% Ethanol is then added.

20. It is Carefully mixed and placed at –20°C for 20 minutes.

21. Then Pellet sample at 14,000 rpm for a period of 10 minutes.

22. The supernatant is then discarded carefully.

23. The pellet is washed with 1.5 ml of 70% Ethanol.

24. Again Pellet sample at 14,000 rpm for about 10 minutes.

25. The supernatant is carefully discarded.

26. The pellet is allowed to air dry on the benchtop for about 10 to 15 minutes.

27. Then 30 to 80 ul of TE Buffer is added

    Nucleic Acid Lysis Buffer which containts,

    10 mM Tris Base (1.21 g/L)

    400 mM NaCl (32.4 g/L)

    2 mM Na2EDTA (0.75 g/L) and

    0.7% SDS (7.0 g/L)

Agarose Gel Electrophoresis

Principle

Agarose gel electrophoresis procedure is used to separate DNA fragments based on their molecular weight. It is an intrinsic part of all routine experiments carried out in the field of molecular biology.

Preparation of 1% Agarose Gel

1. 1X TAE was prepared by dilution of 50 X TAE buffer stock solutions. (0.4 mL of 50X TAE was made up to 200 mL with distilled or dematerialized water).
2. 0.5 g of agarose is added to 50 mL of 1X TAE. This yields 1% agarose gel.
3. The solution is boiled till agarose gets dissolved completely.
4. In the meanwhile, the combs were placed in electrophoresis set in such a manner that it is approximately 2 cm away from the cathode.
5. Ethidium bromide (EtBr) is then added to the molten agarose at a concentration of 0.5 µg/mL (from a stock of 10mg/mL in water) and it is mixed gently.

The agarose solution is then poured in the central part of the tank when the temperature reaches approximately 60º C. Air bubbles should be avoided. The thickness of the gel should be around 0.5 to 0.9 cm. The gel is kept undisturbed at the room temperature for the agarose to get solidified.

Electrophoresis

1. 1X TAE buffer is then poured into the gel tank till the buffer level stood at 0.5 to 0.8 cm above the gel surface.
2. The combs is lifted gently after ensuring that wells remain intact.
3. The power cord is connected to the electrophoretic power supply according to the convention i.e. red for anode and black for cathode.
4. DNA samples is then loaded in the wells, in the desired order and the order recorded with a tracking dye (6X Tri-track loading dye, Bangalore genei).
5. The voltage is set at 50 V after which the power supply is switched on.
6. The power is switched off, when the tracking dye from the well reaches 3/4th of the gel.
7. After the electrophoresis, DNA samples are visualized under UV transilluminator, (they will appear fluorescent and no destaining is required in this case).

Quantification of DNA

Standard DNA is prepared by using salmons sperm DNA at various concentrations (10, 25, 50, 75 g/mL). The control DNA was serially diluted in distilled water.50 □L of the isolated genomic DNA was diluted in 1 mL distilled

water and OD was measured at 260nm using spectrophotometer. An OD of 1 corresponds to 50 □g/mL for double stranded DNA. Using standard curve obtained from the standard DNAs OD, the concentration of the template DNA was quantified. Similarly, OD was measured at 280nm. The ratio of reading at OD 260 and 280nm provides an estimate of purity of DNA.

Polymerase Chain Reaction (PCR) amplification(Fig.8,Fig.9):

KRas and P53 gene primers was designed by NCBI database and synthesised from Bangalore Genei, India and the primers were chosen as follows:

Table shows the forward and reverse primers used, their restriction enzymes of Kras and p53 genes in Ampulla of Vater patients

Gene	Exon Region	Codon	Forward and reverse primers	Bp	Restriction enzymes
P53	5	128-184	FP:5'-TTCCTCTTCCTGCA-GTACTCC-3' RP:5'-GCCCCAGCTGCTCACCATCG-3'	215	RsaI
Ki-ras	1	12	FP:5'-TAGGCAAGAGTGCCTTGACG-3' RP:5'-CCCTCCCCAGTCCTCATGTA-3'	190	RsaI

The reaction volume used was

Template DNA (200 ng) ---------- 4.0 μL

Forward and Reverse primers (1μM) ---------- 1.0 μL each

PCR Master Mix (2X) ----------12.5 µL

MilliQ water----------8.5 µL

Total volume ----------25.0 µL

The PCR conditions for amplification were as follows

An initial denaturing step at 95°C for about 10 minutes should be followed by 30 cycles of

95 °C (30 s) - Denaturation step

60 °C (30 s) - Annealing step

72 °C (1 min) - Extension step

and a final elongation step of 72 °C (10 min).

The PCR products are electrophoresed on 1% agarose gel containing EtBr.This is then viewed under ultraviolet light.

Restriction digestion (Fig.8,Fig.9)

For the assay of KRas gene polymorphism, the PCR conditions were as follows:

The allelic variants were identified by the use of restriction enzymes that differentiate between alleles.

For digestion of PCR product with bstNI restriction enzyme (Fermentas), the following protocol was used directly after amplification:

PCR reaction product----------10 µL

Nuclease free water----------18 µL

10X buffer R ---------- 2 µL

bstNI enzyme---------- 2 µL

The components were mixed gently and spun for a few seconds. Then the reaction mix was incubated at 37°C for 8 to 12 hrs. The digestion products were visualized on 4% Metaphor agarose gel containing EtBr.

The primers (Bangalore Genei, India) used for amplifying genomic DNA.

The reaction volume used was

Template DNA (200 ng) ---------- 4.0 µL

Forward and Reverse primers (1µM) ---------- 1.0 µL each

PCR Master Mix (2X) ----------12.5 µL

MilliQ water----------8.5 µL

Total volume ---------- 25.0 µL

The PCR conditions for amplification were as follows

An initial denaturing step at 95°C for about 10 minutes followed by 30 cycles of

95 °C (30 s) - Denaturation step

60 °C (30 s) - Annealing step

72 °C (1 min) - Extension step

and a final elongation step of 72 °C (10 min).

The PCR products were electrophoresed on 1% agarose gels containing EtBr.This is viewed under ultraviolet light.

For the assay of P53 gene polymorphism, the PCR conditions were as follows:

The allelic variants were identified by the use of restriction enzymes that differentiate between alleles.

For digestion of PCR product with bstUI restriction enzyme (Fermentas), the following protocol was used directly after amplification:

PCR reaction product----------10 μL

Nuclease free water----------18 μL

10X buffer R ---------- 2 μL

bstUI enzyme---------- 2 μL

The components were mixed gently and spun for a few seconds. Then the reaction mix is incubated at 37°C for about 8 to 12 hrs. The digestion products are then visualized on 4% Metaphor agarose gel which contains EtBr.

The reaction volume used was

Template DNA (200 ng) ---------- 4.0 μL

Forward and Reverse primers (1μM) ---------- 1.0 μL each

PCR Master Mix (2X) ----------12.5 μL

MilliQ water----------8.5 μL

Total volume ---------- 25.0 μL

The PCR conditions for amplification were as follows

An initial denaturing step at 95°C (10 min) followed by 30 cycles of

95 °C (30 s) - Denaturation step

60 °C (30 s) - Annealing step

72 °C (1 min) - Extension step

and a final elongation step of 72 °C (10 min).

The PCR products are then electrophoresed on 1% agarose gels containing EtBr. This is now viewed under ultraviolet light.

Figure 6. Paraffin Blocks Collected from Department of Pathology

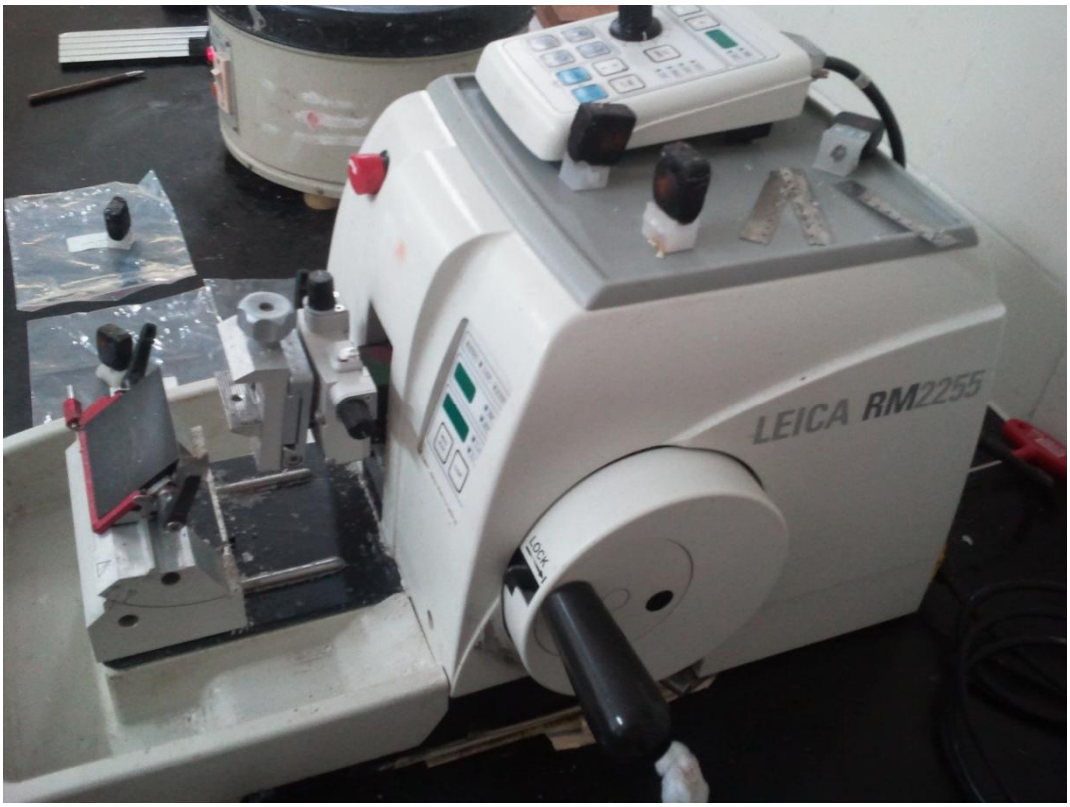
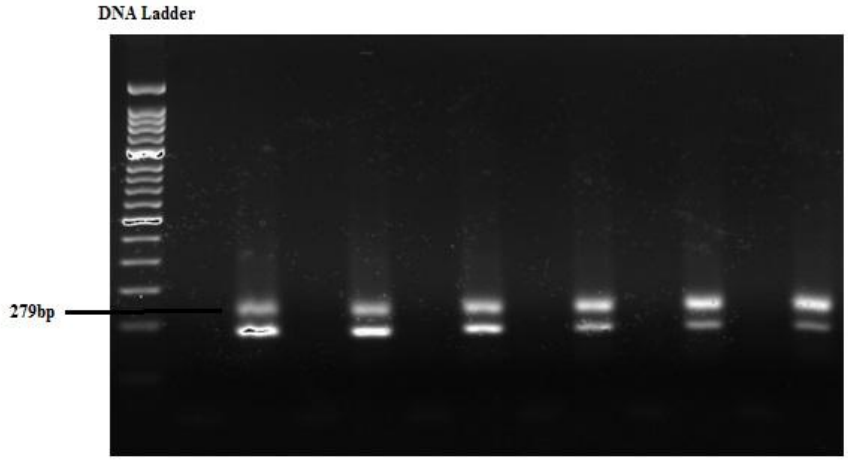
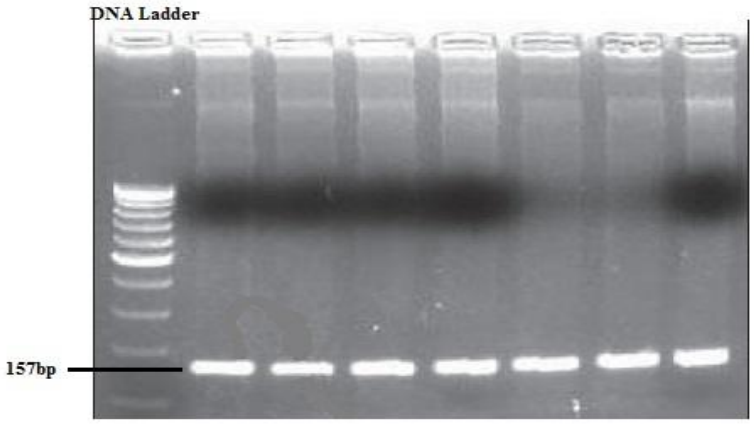


Figure 7. Paraffin Blocks Microdissected to 5µ size for DNA Extraction

Figure 8: Representing p53 and Kras genes PCR amplified DNA sequences

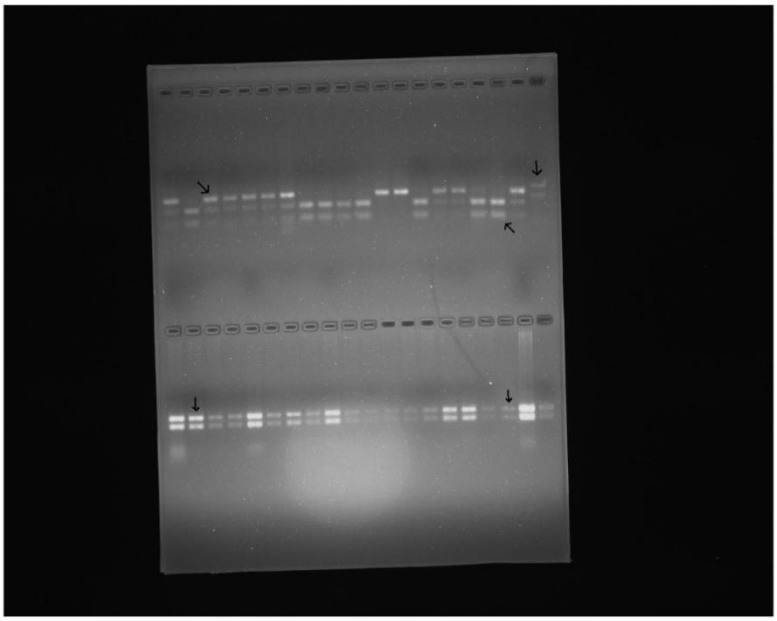


p53 gene



Kras gene

Figure 9: Representing p53 (279bp) and Kras (157bp) restriction digested DNA fragments



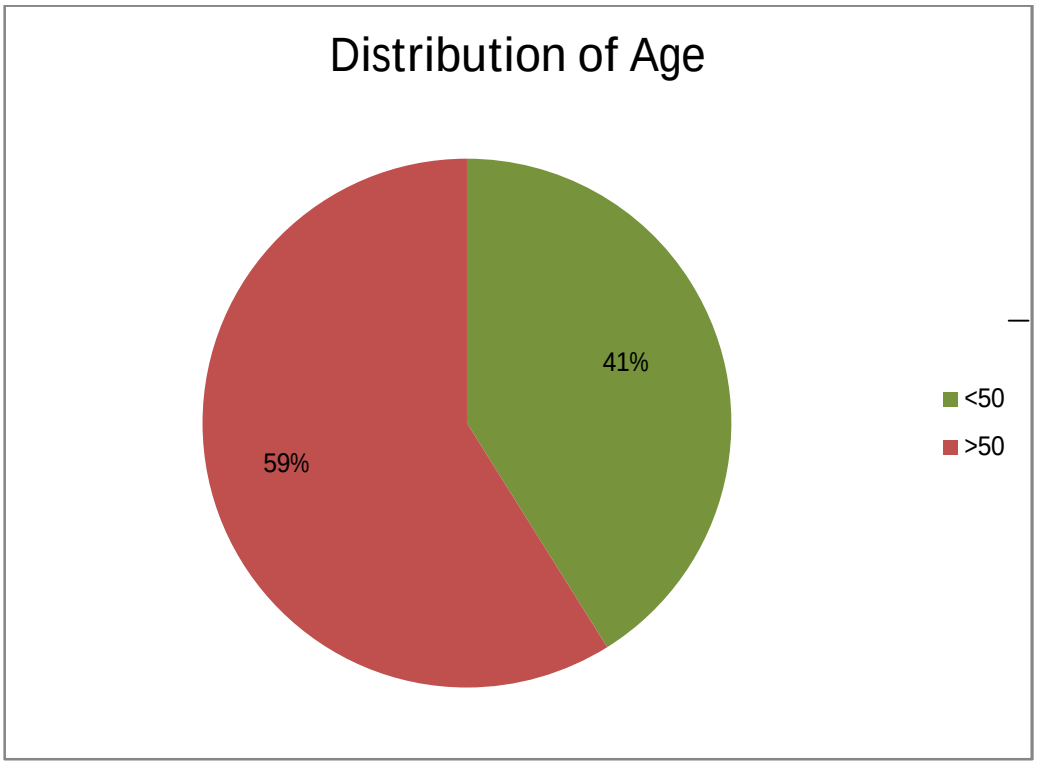
CHAPTER VI

STATISTICAL ANALYSIS AND RESULTS

Totally 90 samples were selected as per inclusion criteria and those samples considered for identifying the kras and P53 Gene mutations.

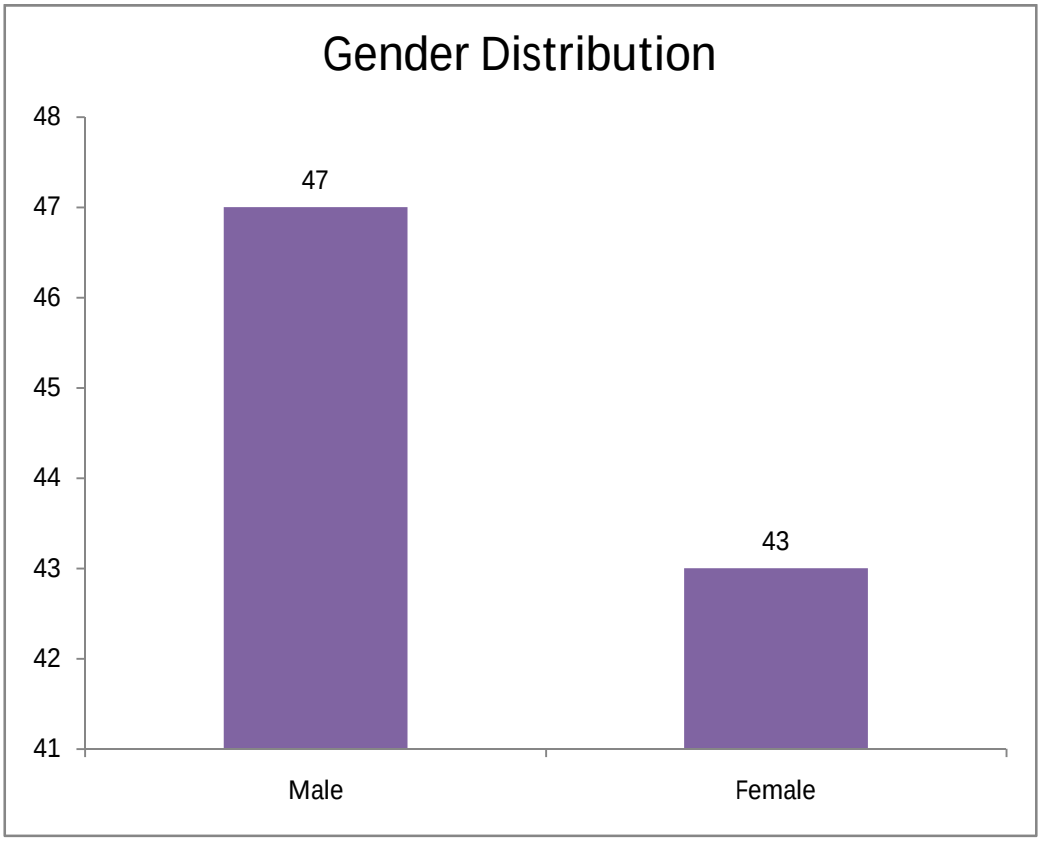
Age	Frequency	Percent
≤50	37	41.1
>50	53	58.9
Total	90	100.0

Majority of the samples belong to greater than 50 years of age with mean(Sd) 53.04(7.44) ranging from 30 to 70 years.



Gender distribution among the samples were almost equally distributed

GENDER	Frequency	Percent
Male	**47**	**52.2**
Female	**43**	**47.8**
Total	**90**	**100.0**



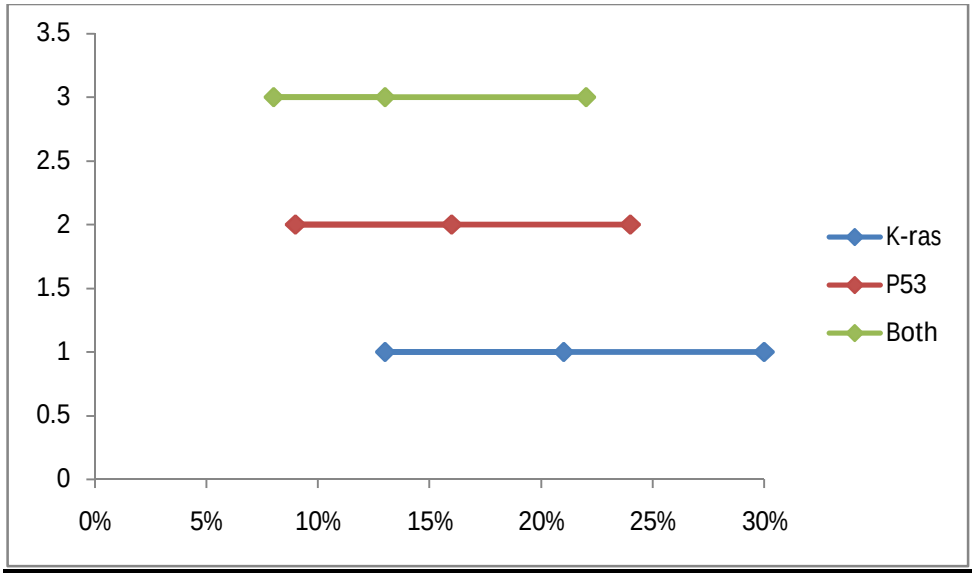
A) PREVALANCE

Table I Prevalence of Type of Mutation

Mutation Type	Prevalence	95% CI for Prevalence	
		Lower Limit	Upper Limit
K-ras	21%	13%	30%
P53	16%	9%	24%
Both	13%	8%	22%

Table 1 depicts that the prevalence of K-ras were 21% which is high with 95% confidence interval 13% to 30% as compared with P53 and both(K-ras and P-53)

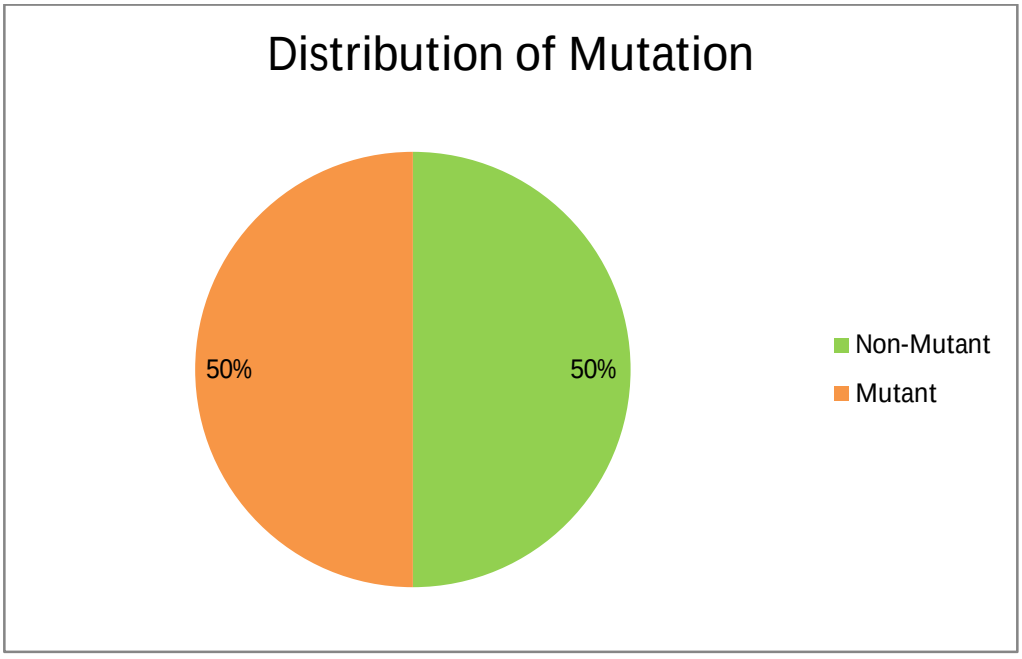
Fig1- Forest Plot for Prevalence Gene Mutations



B) FREQUENCY DISTRIBUTION OF THE VARIABLES

Mutation Status	Frequency	Percent
Non-Mutant	45	50
Mutant	45	50
Total	90	100

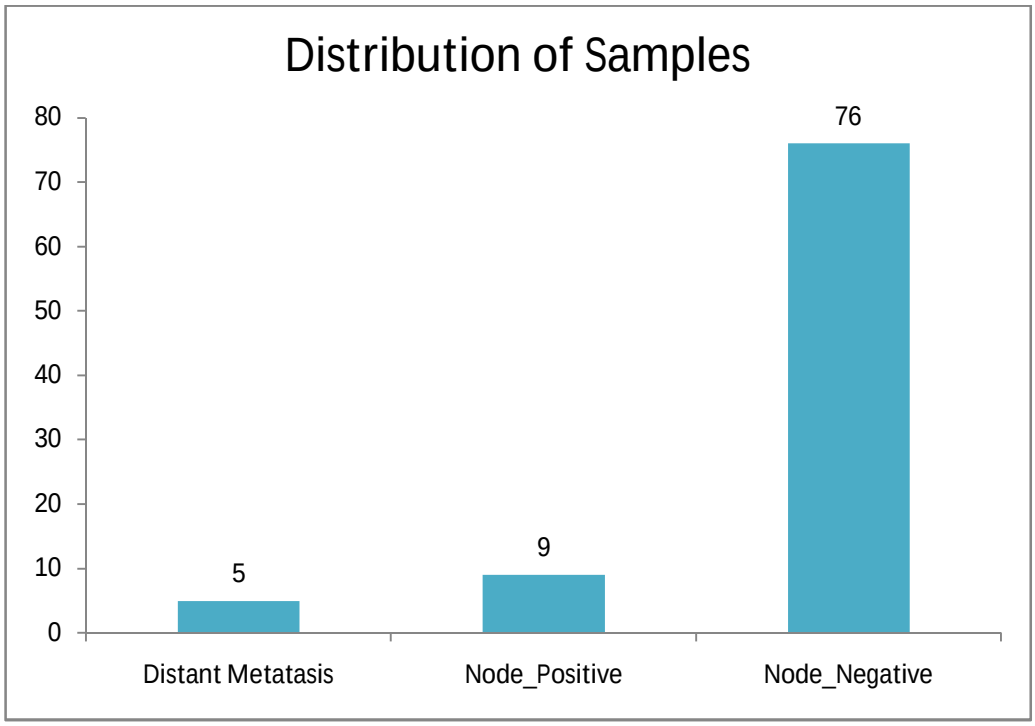
Mutation and Non- Mutation are equally distributed among samples taken.



Distribution of Nodes

NODE	Frequency	Percent
Positive	9	10.58
Negative	76	89.42
Total	85	100

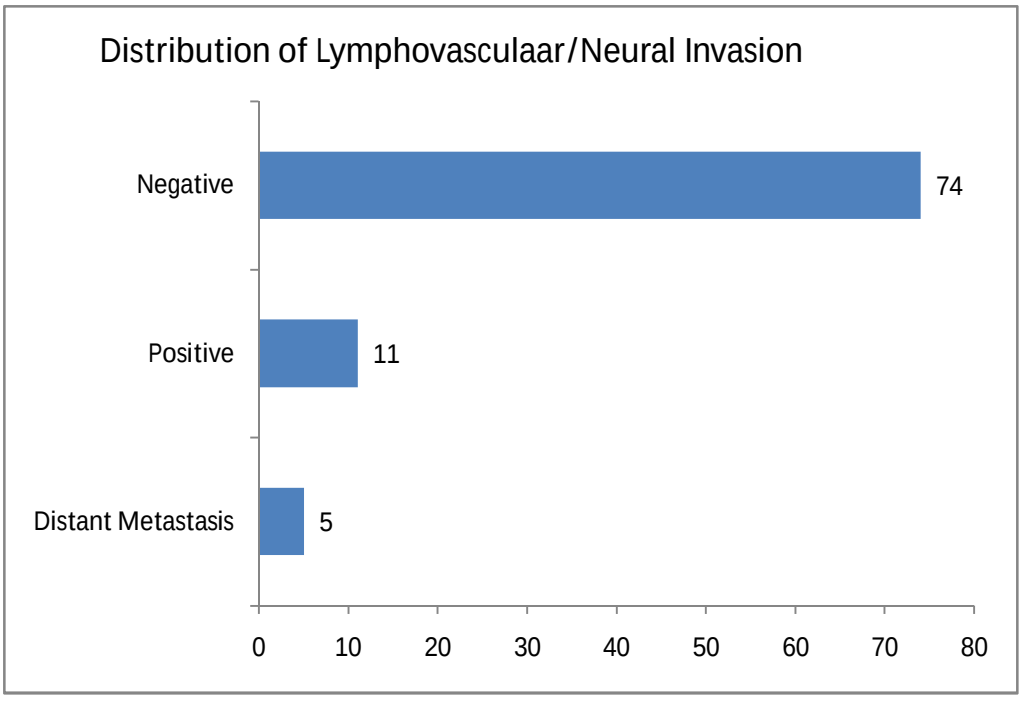
Among the 90 samples 5 were distant metastasis. Out of the remaining 85 samples 9 (10.58%) of the samples had positive nodes.



Distribution of Lymph vascular/Neural Invasion

LV/NEU -INVA	Frequency	Percent
Positve	11	12.9
Negative	74	87.1
Total	85	100

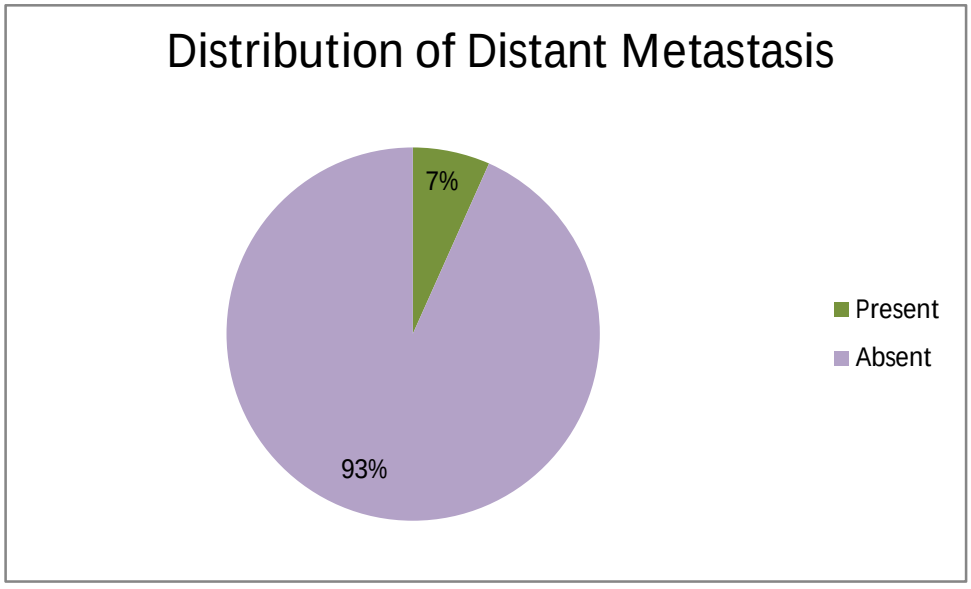
Out of 85 samples 11(12.9%) was found to be positive for Lymphovascular/ Neural Invasion as depicted in the graph



Distribution of Distant Metastasis

Distant Metatasis	Frequency	Percent
Present	6	6.7
Absent	84	93.3
Total	90	100

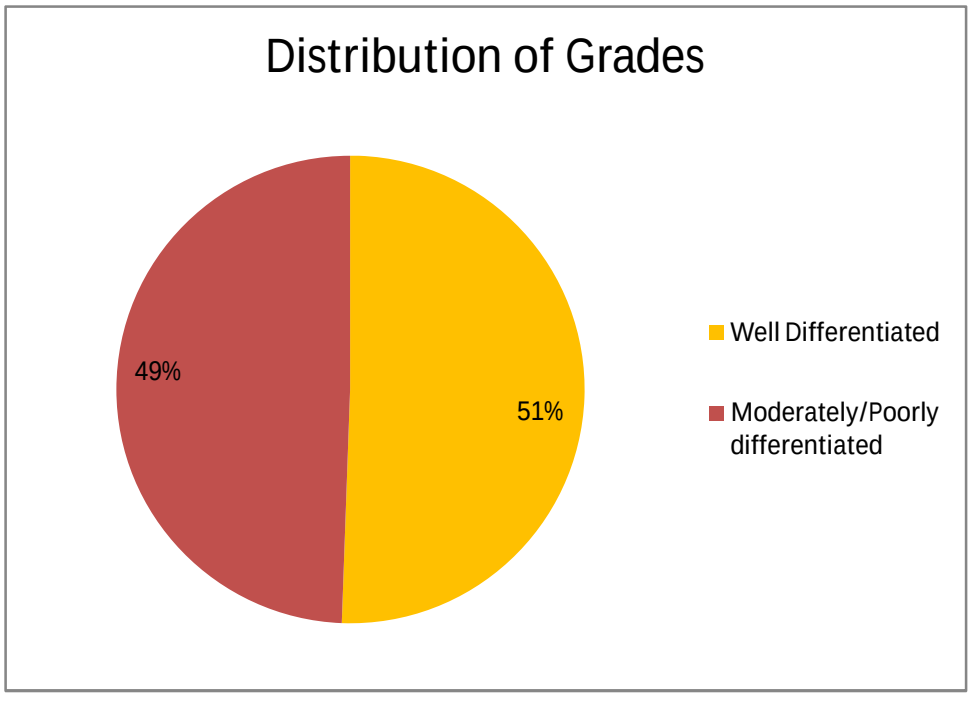
The below pie chart shows that among the 90 samples , 6(6.7%) were positive for Distant Metastasis . Among the 6 samples positive for distant metastasis, five were from liver metastasis and one sample was included in the distant metastasis category, since it was positive for non-regional nodal metastasis (Aorto-caval node).



DISTRIBUTION OF GRADES

GRADE	Frequency	Percent
Well Differentiated	43	50.5
Moderately/Poorly Differentiated	42	49.5
Total	85	100

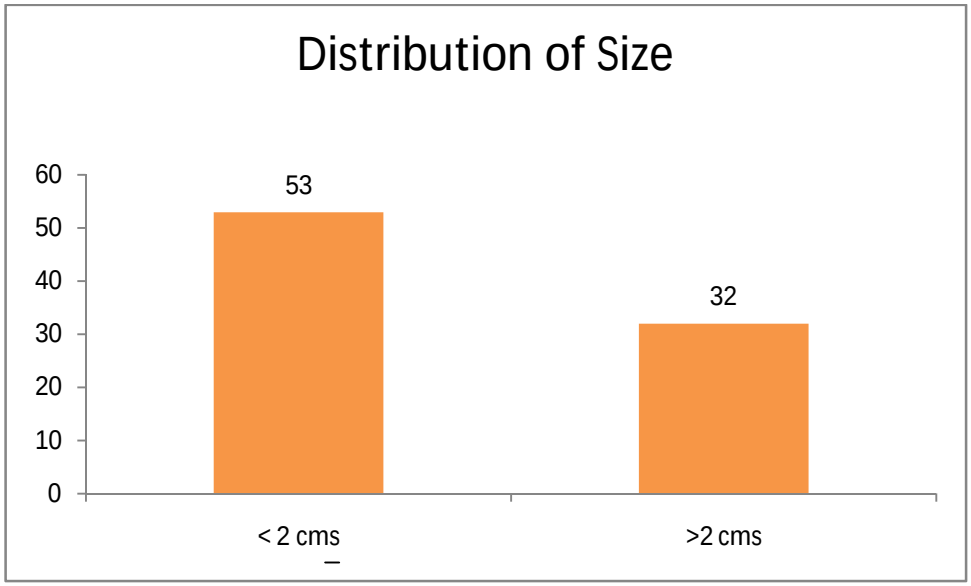
Among the samples well differentiated and moderately/poorly differentiated samples were equally distributed. Five samples do not belong to any specific grades because they belonged to distant metastasis.



DISTRIBUTION OF SIZE

Size(in cms)	Frequency	Percent
≤2	53	58.9
>2	32	35.6
Total	85	94.4

Among the samples the size was found to be greater than two centimeters in 35.6%.

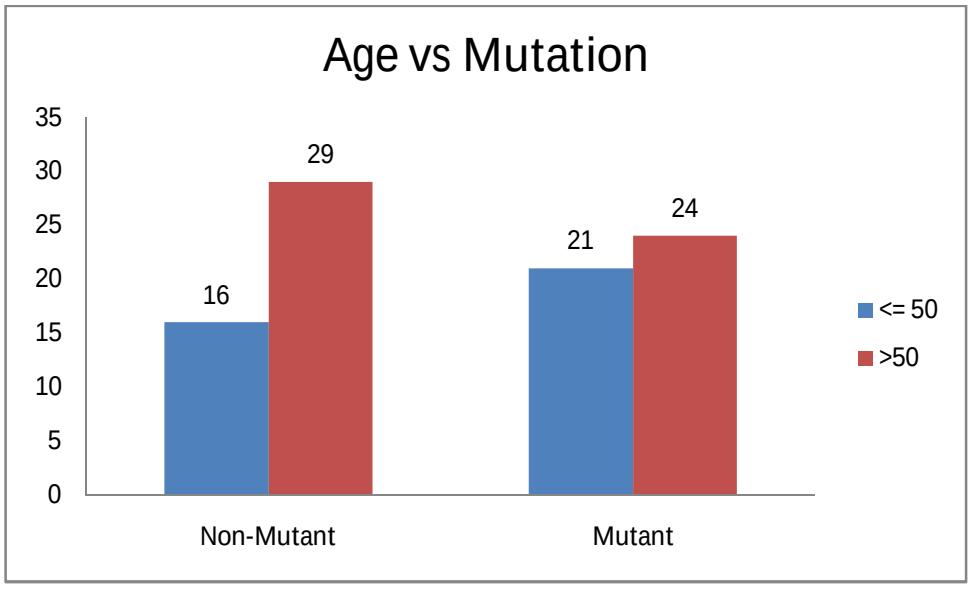


c) TEST FOR ASSOCIATION

Chi-Square tests were used to find the association of Mutation status with all the variables.

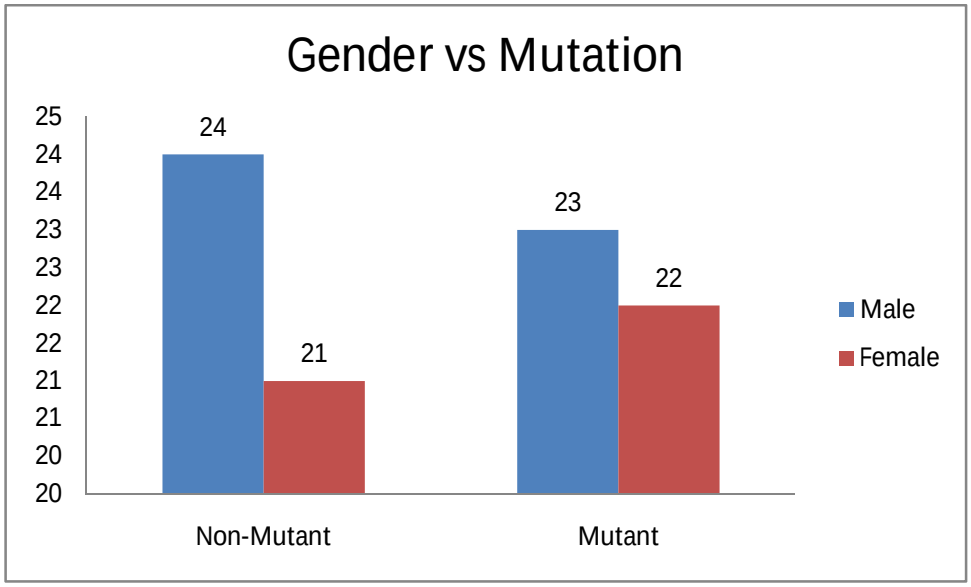
Age	Mutation		Chi-Square value	P-value
	Non-Mutant	Mutant		
≤50	16(35.6%)	21(46.7%)	1.047	2.84
>50	29(64.4%)	24(53.3%)		

Age greater than 50 years were found to be high among the mutant cases (53.3% vs 46.7%). There was no statistical significance (P>0.05) between age and mutation.



Gender	Mutation		Chi-Square value	P-value
	Non-Mutant	Mutant		
Male	24(53.3%)	23(51.1%)	0.045	0.833
Female	21(46.7%)	22(48.9%)		

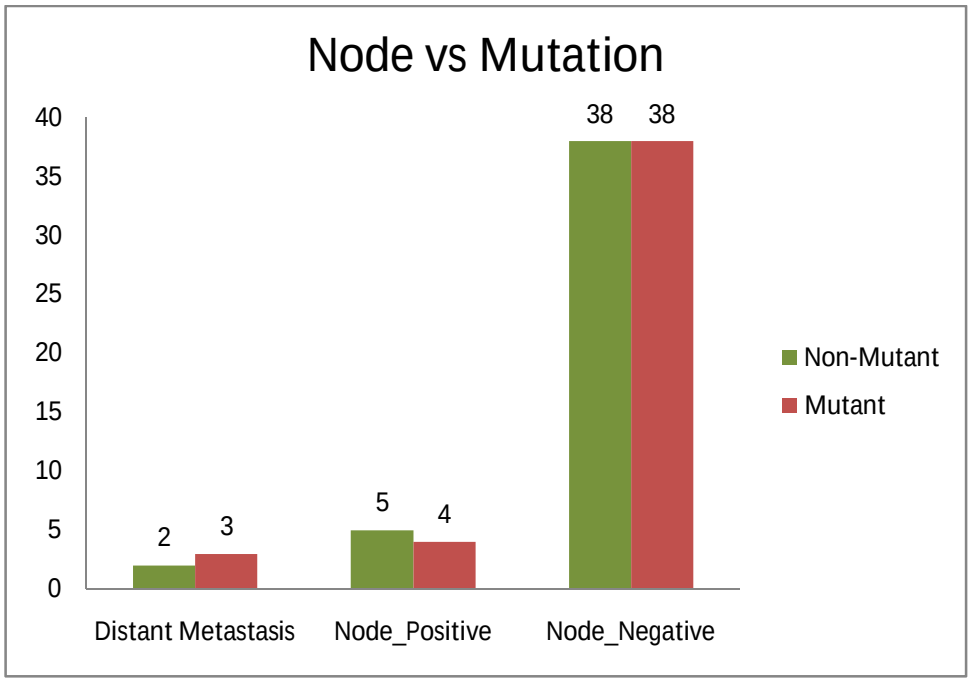
Association was not significant (p>0.05) between Gender and mutation.



NODE VS MUTATION

Node	MUTATION STATUS		Chisquare	p value
	Non-Mutant	Mutant		
Distant Metastasis	2(4.4%)	3(6.7%)	0.311	0.856
Positive	5(11.1%)	4(8.9%)		
Negative	38(84.4%)	38(84.4%)		

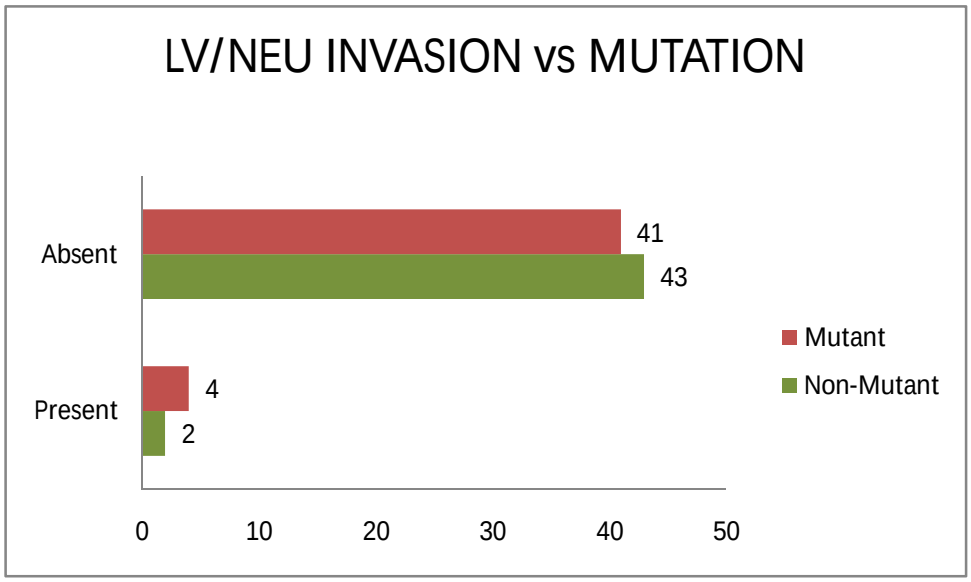
There was no significant($p>0.05$) association between the node and mutation status (χ^2 = 0.311 and p=0.856)



LYMPHOVASCULAR/NEURAL INVASION VS MUTATION

	MUTATION STATUS			
Lv/neu invasion	Non-Mutant	Mutant	Chisquare	p value
Distant Metastasis	2(4.4%)	3(6.7%)	0.345	0.842
LV/Neu Positive	5(11.1)	6(13.3)		
LV/Neu Negative	38(84.4%)	36(80%)		

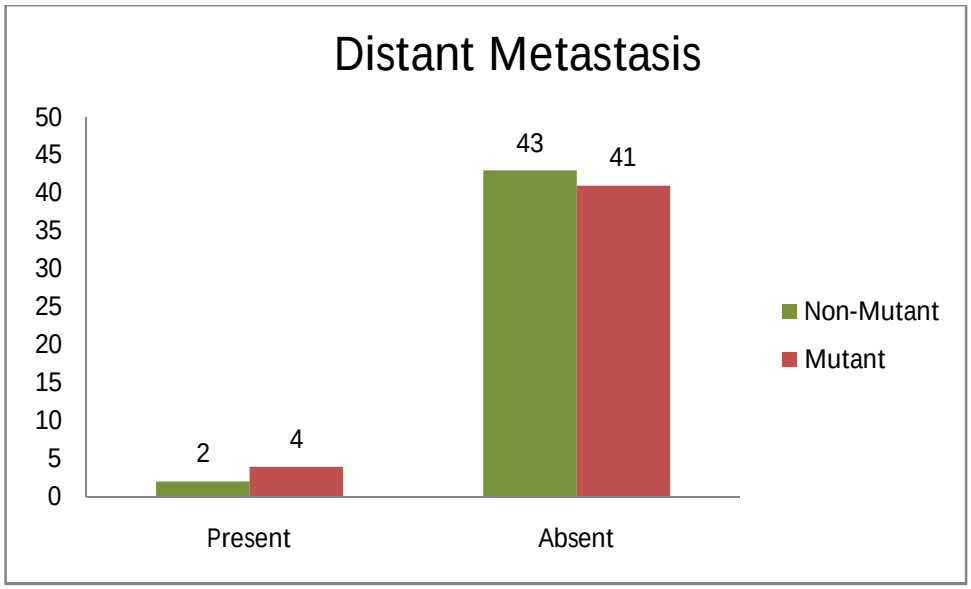
There was no significant ($p>0.05$) association between the Lymphovascular / neural invasion and mutation status (χ^2 = 0.345 and p=0.842)



DISTANT METASTASIS VS MUTATION

Distant metastasis	MUTATION STATUS		Chisquare	p value
	Non-Mutant	Mutant		
Positive	2(4.4%)	4(8.9%)	0.714	0.398
Negative	43(95.6%)	41(91.1%)		

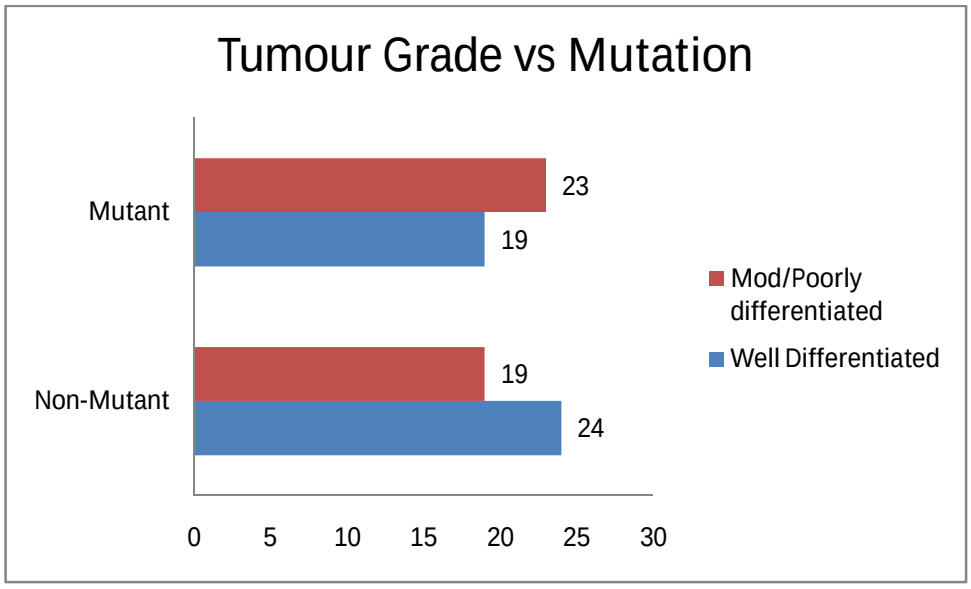
On comparing Distant Metastasis and Mutation status, it resulted χ^2 = 0.714 and p=0.398 which implies that there is no significant association between them.



TUMOUR GRADE VS MUTATION

Grade	MUTATION STATUS		Chisquare	p value
	Non-Mutant	Mutant		
Distant Metastasis	2(4.4%)	3(6.7%)	1.162	0.559
Well Differentiated	24(53.3%)	19(42.2%)		
Moderately/Poorly Differentiated	19(42.2%)	23(51.1%)		

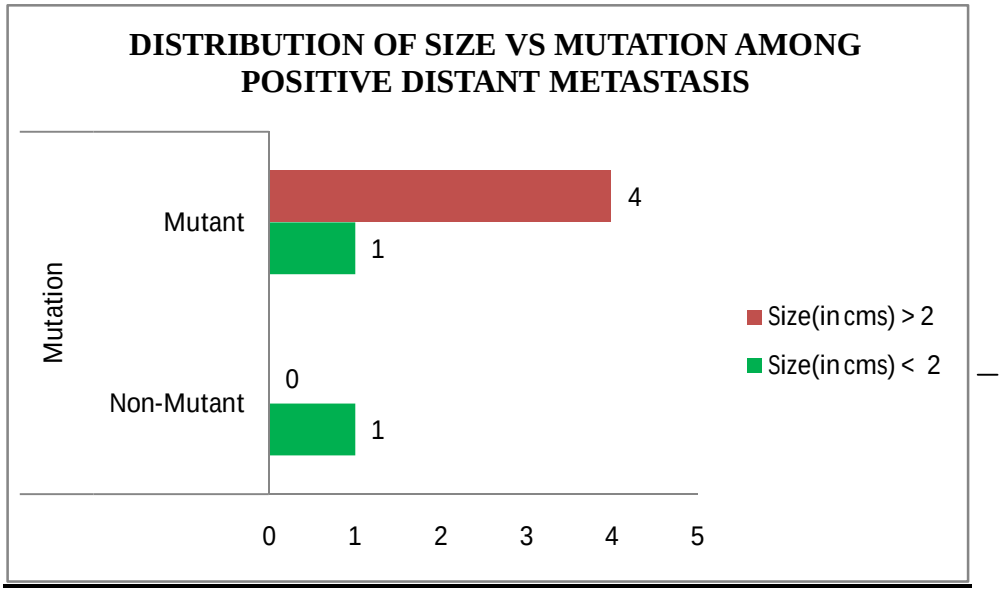
There was no significant ($p>0.05$) association between the Grade and mutation status (χ^2 = 1.162 and p=0.559)



COMPARISON OF DISTANT METASTASIS VS SIZE AND MUTATION STATUS

Distant Metastasis			Mutation		Total
			Non-Mutant	Mutant	
Positive	Size(in cms)	≤ 2	1	1	2
		> 2	0	4	4
	Total		**1**	**5**	**6**

Out of six cases of distant metastasis, 4 cases were more than 2cms(MD CT scan measurement of size) which were all positive for mutation. In the two cases which were less than 2 cms one is mutant

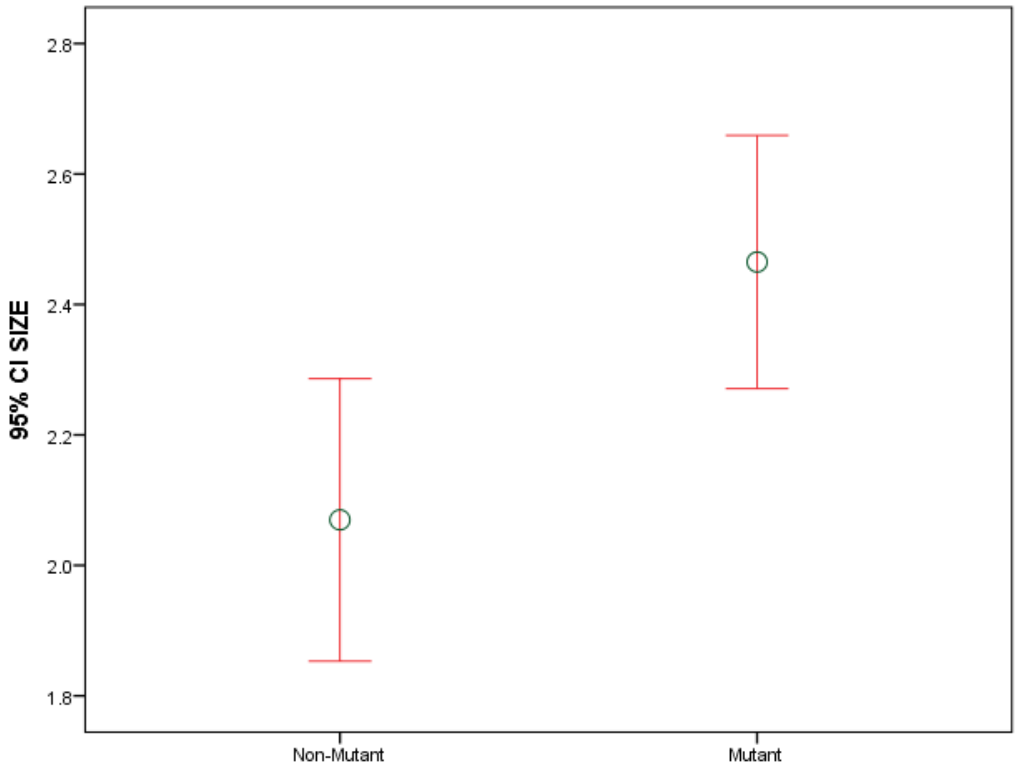


C) TEST FOR DIFFERENCE OF MEAN SIZE

Independent t test were used to test the mean size difference among the parameters under study

MUTATION STATUS	Mean size	Standard Deviation size	t	P value
Mutant	2.47	.631	2.744	0.007
Non-Mutant	2.07	.704		

Error plot for 95% Confidence Interval Size among Mutation Status



There is a significant mean difference $p<0.05$ between the size and mutation status which is depicted in figure and table above

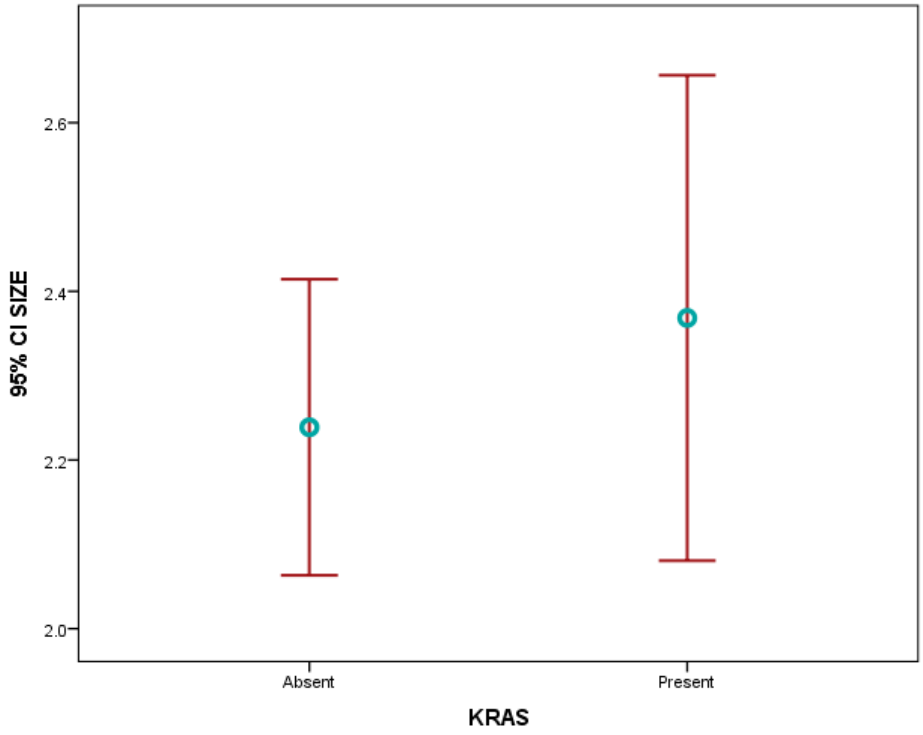
MEAN SIZE(incms) COMPARISON OF KRAS MUTANT

Group Statistics

KRAS		N	Mean	Std. Deviation	t	p value
SIZE(incms)	Present	19	2.37	.597	0.717	0.475
	Absent	67	2.24	.720		

There is no significant mean difference p>0.05 between the size and KRAS which is depicted in table above. The figure reveals that there is a mean difference in size but it is not statistically significant.

Error plot for 95% Confidence Interval Size among Mutation Status



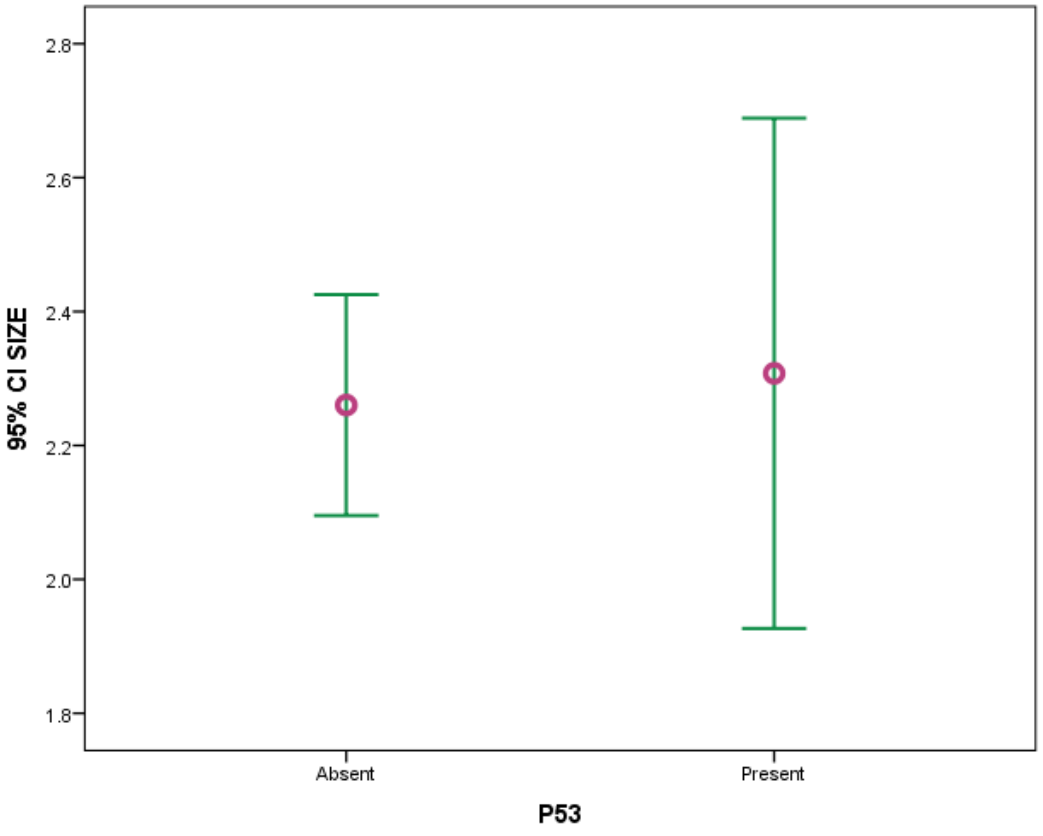
MEAN SIZE(incms) COMPARISON OF P53 MUTANT

Group Statistics

P53		N	Mean	Std. Deviation	t	p value
SIZE	Present	13	2.31	.630	0.226	0.822
	Absent	73	2.26	.708		

There is no significant mean difference p>0.05 between the size and P53 which is depicted in table above. The figure given below depicts that there is no mean difference in size which is also not statistically significant.

Error plot for 95% Confidence Interval Size among Mutation Status



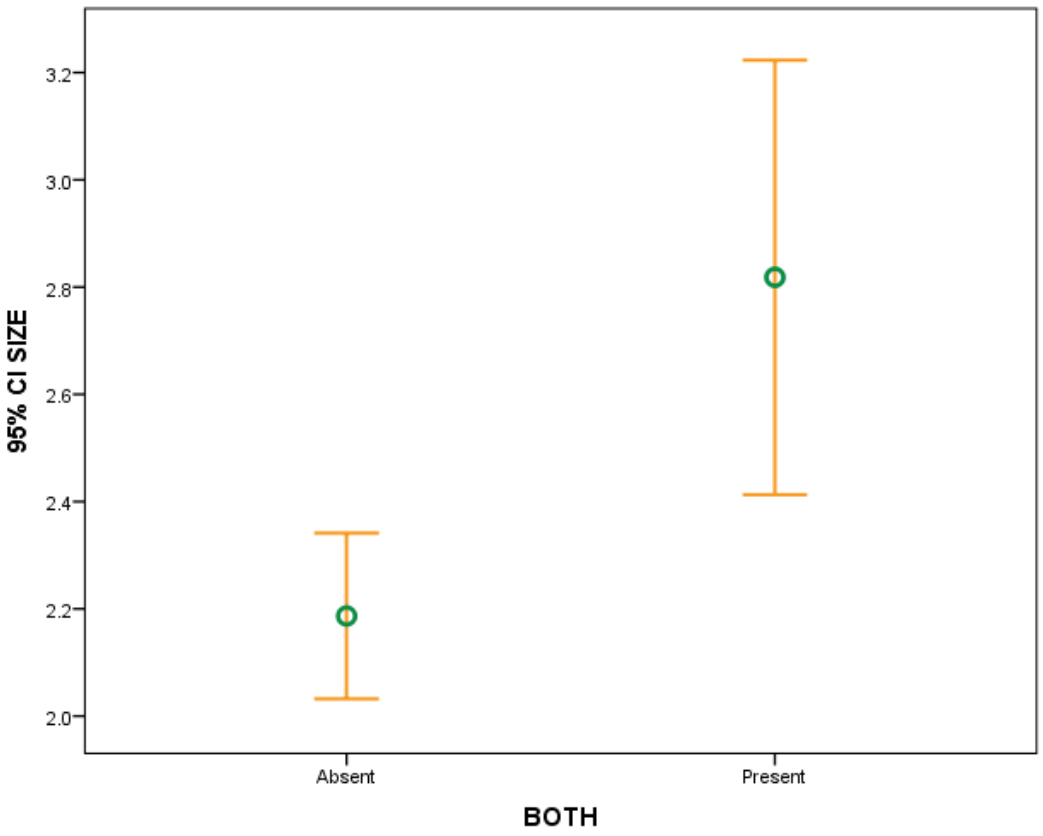
MEAN SIZE(incms) COMPARISON OF KRAS AND P53 MUTANT

Group Statistics

KRAS AND P53		N	Mean	Std. Deviation	**t**	**p value**
SIZE	Present	11	2.82	.603	2.946	0.004
	Absent	75	2.19	.672		

There is significant mean difference $p<0.05$ between the size and both KRAS and P53 which is depicted in table above. The figures also shows the same.

Error plot for 95% Confidence Interval Size among Mutation Status



D) MULTIPLE LOGISTIC REGRESSION

Variables found to be clinically meaningful were included in the model of multiple logistic regression. Hosmer and Lemeshow Goodness of fit are used for assessing how well the model fits the data. It resulted a non significant value, which is an indication of a model that really predicts the population fairly well**.**

Hosmer and Lemeshow Test

Step	Chi-square	df	Sig.
1	3.138	6	.791
2	3.347	5	.647
3	2.867	3	.413
4	.422	1	.516

Assuming Mutation status as a dependent variable and other factors as independent variables the backward logistic regression method was selected which carried out four iterative steps. The results were tabulated below.

Backward Multiple Logistic Regression

		odds ratio	95% C.I.for odds ratio		Wald statistic P value
			Lower	Upper	
Step 1	SIZE	11.69	3.40	139.98	.012
	NODE(1)	1.92	1.15	22.78	.593
	DISTMETS	1.47	1.17	2.51	.032
	GRADE(1)	2.15	1.36	6.78	.570
	LVNEUINVA(1)	3.07	1.31	99.90	.873
Step 2	SIZE	11.82	3.43	141.95	.011
	NODE(1)	1.97	1.17	20.10	.608
	DISTMETS	1.47	1.17	2.52	.032
	GRADE(1)	2.14	1.36	6.59	.555
Step 3	SIZE	11.46	3.40	128.75	.011
	DISTMETS	1.46	1.17	2.48	.029
	GRADE(1)	2.18	1.37	6.81	.587
Step 4	SIZE	12.13	3.53	140.25	.009
	DISTMETS	1.41	1.17	2.19	.011

In the final step it showed that the odds of occurrence of mutation is 12.13 times higher due to Size and 1.41 times due to Distant Metastasis. It is evident from the tabulated results it was statistically significant(Wald statistics p value<0.05) and it does not have one in the 95% confidence interval.

RESULTS

Totally 90 samples were selected as per inclusion criteria and those samples considered for identifying the presence of Kras and P53 Gene mutations. Further, in the present study, main consideration such as Sex, Gender, Tumor size, node, Lymphovascular/Neural Invasion, Distant Metastasis were taken and analyzed for the association of K ras and P53 gene mutations.

Majority of the samples belong to greater than 50 years of age with mean(Sd) 53.04(7.44) ranging from 30 to 70 years. The prevalence of K-ras is 21%, which is high with 95% confidence interval of 13% to 30%,when compared with P53(15.56%) and both(K-ras and P-53)(13.33%) mutation. Among the 90 samples 5 were from liver metastasis (distant metastasis). Out of the remaining 85 samples 9 (10.58%) of the samples were from node positive cases.

Out of 85 samples, 11(12.9%) was found to be positive for Lymphovascular/Neural Invasion, 6(6.7%) were positive for Distant Metastasis . Among the 6 samples positive for distant metastasis, five were from liver metastasis and one sample was included in the distant metastasis category, since it was positive for non-regional nodal metastasis (Aorto-caval node). Out of six cases of distant metastasis, 4 cases were greater than 2cms size (Pre-operative MDCT scan measurement of size) which were all positive for mutation. In the other two cases, with size less than 2 cms, one was positive for mutation.

Among the samples well differentiated and moderately/poorly differentiated samples were equally distributed. Five samples did not shown any

specific grades because they belonged to distant metastasis. Among the samples, the size was found to be greater than two centimeters in 35.6%. There was no statistical significant ($p>0.05$) mutation status and association to the Gender, Lymphovascular/neural invasion, Distant metastases and Tumor grade.

In the present study, There is significant mean difference ($p<0.05$) between the size and both KRAS and P53 mutation. The odds of occurrence of mutation is 12.13 times higher due to Size and 1.41 times due to Distant Metastasis.

CHAPTER VII

DISCUSSION

The Latin term cancer was coined by celsus. It was Galen who used the term “oncos” to describe tumors. This is the birth of the word “oncology”[148] . Cancer involves a fundamental dysregulation of tissue growth transforming normal cells into cancer cells. In this process genes play a major role[149].As described earlier, the genetic alteration may involve an entire chromosome or a mutation involoving a single DNA nucleotide. This study was designed to looked for such a mutation in periampullary carcinoma.

There is a lot of geographical variation across countries in the prevalence of Kras and P53 mutations in Periampullary carcinoma especially in carcinoma of Ampulla of Vater, ranging from 0-60%(Taiwan 0%,Thailand 8%,USA 32%,Japan 60%)[150]. Based on previous studies in the literature this study was conducted to look for kras mutation in Exon 1 at codon 12.P53 mutation was looked for in Exon 5 of codon 128-184.Because of paucity of studies in the literature on this mutation in Indian patients, especially in our regional population, this study was conducted to look for the prevalence of similar mutation in periampullary carcinomas.

This study result has shown mutation in 50%(Kras 21.11%,P53 15.56% and both Kras and P53 mutation in 13.33%) of the total of 90 samples selected for the study as per the inclusion criteria. This indicates that our regional population has

higher mutation than Taiwan, Thailand and USA population but lower than what has been reported in Japan.

The earlier presentation of periampullary carcinoma and high resectability rate of upto 80% and 5 year survival rate of 6 to 61%[151],assigns this cancer into the variety of good prognosis tumors. This study though has not studied the prognosis, after analyzing the prevalence of the mutation, the second part of this study analyzed the if these mutated cases presented with higher stage of the tumors and if there is any differences in mutation pertaining to age and gender. Majority of the samples(58.9%) were greater than 50 years of age. Though not statistically significant , this study showed a slightly higher mutation status in those aged > 50 years of age[<50yrs(46.7%) vs >50yrs(53.3%)].This may be a normal occurrence or it indicates a step wise progression of adenoma to carcinoma sequence occurring at a later age[152] **.** Similarly our study results did not show any major gender differences between mutated and non mutated cases[M:F=51.1% : 48.9%].

As in previous studies[153] **(Howe et al)**, this study also concurred that there was no significant correlation with Lympho vascular/neural invasion, and tumor grade and nodal metastasis. In fact contrary to the set hypothesis, the study results has shown a gross difference in association between nodal status and mutation status. Almost 84,4% of mutation positoive samples did not have nodal matastasis. Studies have demonstrated poor survival in ampulla of cancer patients with certain genetic alterations(ref for survival).Though this study was not intended to look for the prognosis and survival, this opens new areas to study the survival status in mutated cases in our regional population. In Howe et al study, the only

statistical significant association with regard to kras mutation and clinicopathological features was the tumor size. Tumors < 2cms size had 27.5% mutation and >2cms size tumors had 48.8% incidence of mutation.In this study also by logistic regression analysis, correlation was found to be significant for tumors >2cms and for tumors with distant metastasis(Odds of occurrence was 12.3 times higher for mutated cases).

Westgaard et al in 2013 has studied on the intestinal type and pancreatico-biliary type of ampullary adenocarcinoma and its difference with other periampullary malignancies. They have reported that ampullary carcinomas have a better prognosis than adenocarcinoma arising from lower end of bile duct and head of the pancreas.The lack of availability of percise molecular tests to differentiate between the intestinal type and PB type has left with dependence on the pathological findings to differentiate these tumors[154].Overall Carcinoma of ampulla of vater represents 21% of patients undergoing a Whipple pancreaticoduodenectomy surgery[155], with a better chance of survival after resection than pancreatic head carcinoma and is described as "the most curable of all GI tract cancers[156].

Kim et al retrospectively reviewed forty three patients who underwent Pancreaticoduodenectomy and found that the stage of the disease at presentation and degree of differentiation were predictors of failure by univariate analysis.But stage of the disease alone was the predictor of failure in multivariate analysis.It was recommended that higher stage disease will require adjuvant therapy[157].In a study by **Brown et al** it was shown that Pancreaticoduodenectomy for node negative ampullary carcinoma was curative in 80% of patients[158]. The tumor recurrence rate, as reported in the literature, varies from 28 to 44%. Recurrence in the form of

metastasis can occur in the liver, aortocaval lymph node metastases or as locoregional tumor recurrence [159,160]. Postoperative adjuvant chemotherapy [161] and radiochemotherapy [162,163] have been shown to improve survival outcomes in patients with periampullary carcinomas. In current studies, however, patients considered for postoperative adjuvant therapy often had adverse prognostic factors, such as positive lymph node involvement, higher tumor stage, or poor tumor differentiation, compared with patients who were treated with surgery alone [164]. Whether genetic mutation also influenced the prognosis were not investigated in these studies. **Achilles et al** studied APC mutation in sporadic cases and in FAP patients[165].They reported that ampulla tumors in sporadic cases differ from FAP patients both in the frequency of occurrence(17% vs 64%) and the in the site of mutation. This suggested a different molecular pathology in both these conditions.FAP(Familial adenomatous polyposis) is a condition were ampullary and duodenal malignancy occur at a higher frequency [166,167].Further in FAP the ratio of adenomas versus carcinoma is of the order of 4:1[168].Hence it was postulated that adenoma to carcinoma sequence occurs in ampullary carcinoma as in colo-rectal malignancies[169].An interesting study by **Ohike et al** has shown that high tumor budding(determined by number of clusters of tumor cells in one microscopic field) was associated with highly invasive and node-positive ampullary adenocarcioma with a poorer 5 year survival when compared to low budding tumors.The authors went on to suggest that pathology reports should include tumor budding in ampullary adenocarcinoma[170].On similar lines of the hypothesis set in this study, correlating mutation status with tumor stage at presentation, **Park et al** investigated on the P53 overexpression in carcinoma of Ampulla of Vater.The study showed that P53 overexpression was found in carcinoma with adenomatous component and had a

better prognosis than in denovo ampulla of vater carcinoma.It was also found that p53 positive cases were higher in advanced carcinoma group and in metastatic group.According to this study p53 overexpressed group had metastatic disease in upto 63.3% [171]. In certain cases of carcinoma of the ampulla of Vater, adenomatous components are located in the vicinity of carcinomas, transition of adenoma to carcinoma is seen with the presence of carcinoma foci. The existence of adenoma was more frequent in the earlier stage[172].**Sitthideatphaiboon et al** identified 63 patients between January 2006 and December 2012, to identify the prevalence of kras mutation alone in Thailand. All these patients were histologically proved to be ampullary adenocarcinoma.PCR analysis and pyrosequencing techniques were used for DNA analysis prepared from Formalin fixed paraffin embedded tissues. Kras mutation was detected in 46% of patients in codon 12 and 13, with 96.6% of mutation in the samples were at codon 12.They correlated this with clinical-pathological features and survival outcomes. The study concluded that wild type Kras patients had better survival, although this was not statistically significant [173]. This study was also designed to look for the mutation status and its association with higher stage of the disease. Kras codon 12 mutation was found in 21.1% of samples, with 13.33% showing both kras and p53 mutation. Isolated p53 mutation was found in 15.56% of samples.

Rashid et al in 2002 has published their study on mutation in in biliary tract cancers.In this population based study, Kras mutation was higher i.e 61% for ampullary cancers,15.2% for biliary tract tumors and only 2.7% for gall bladder carcinoma.Mutated cases in bile duct carcinoma had poor survival than non-mutated cases [174].This study has 62 cases of ampullary carcinoma,22 cases of common origin carcinoma(origin of tumor not ascertained by the pathologist) and 4 cases of distal

bile duct carcinoma. Among the 62 samples from ampullary carcinoma 28 samples (45%) had mutation. In samples which are from common origin,13 had mutation(59%).There were only 4 samples in which the origin of the tumor was fixed at distal CBD. Out of this 3 samples(75%) was positive for mutation. It can be inferred that the high percentage of mutation in samples with common origin was due to the difficulty in fixing the origin of tumor by the pathologist .The possibility that tumors would have originated in in ampulla and involved the sphincter of Oddi and pancreas. The high percentage(75%) of mutation seen in distal CBD cancers may also be due to the small sample size.The results has to be further analysed with a larger sample size. Similar to this study results, Scarpa et al[175] has shown higher kras mutation in 3 out of 4 cases which mainly involved the intraduodenal portion of the bile duct. The same group has also published that P53 mutations are involved in the transformation of adenoma to high grade carcinomas and kras mutations was restricted to cancer arising from bile duct component in the ampulla[176] .

Studies have described somatic mutations in APC gene in FAP patients with occurrence of ampullary and duodenal non ampullary tumors[177] .APC gene mutation has to be investigated further in our regional population. But studies in pancreatic cancer has not shown APC mutations [178,179] except in Japan[180].

Zhoa et al has studied the morphological character of tumor and mutation status i.e. between ulcerative and non ulcerative types of ampullary carcinoma.P53 overexpression was higher in ulcerative than non-ulcerative variety.The same study also revealed, the association of Kras mutation on the intestinal and pancreaticobiliary type of carcinoma of ampulla of Vater. Kras codon 12 mutations were frequent in intestinal type than PB type[181] .**Hetchman et al**

published his results on sequencing 279 cancer genes in ampullary carcinomas in relation to the histological subtypes.Kras mutation was was frequent in Pancreatico biliary type and APC gene mutation was frequent in intestinal type of ampullary carcinoma[182] .This indicates that such studies will need to be carried out in our regional population to look for its association with longterm survival and planning adjuvant treatment.

Thinking a step further,**Franko et al** hypothesized that genetic analysis of pancreatic adenocarcinoma and ampullary carcinoma may correlate with post operative survival.Loss of heterozygosity and Kras mutation correlated well with survival after resection[183] .In continuance of the present study, further research on survival status in mutated cases needs to be analyzed in our regional population.

Analysis of this study and other studies in the literature has shown limited data on the topic in our regional population. There is a gross variation in occurrence of this mutation and its association with the morphological and clinico-pathological charecteristics across the globe. This clearly shows ethnic, racial and geographical differences do exist in mutations involving periampullary carcinoma. It may also be attributed to the different techniques adopted in DNA isolation and sequencing methodology performed in various countries.This also underlines the importance of further molecular studies to be conducted in future, to detect other genetic alterations involved(apart from kras and P53 genes) in the pathogenesis and progression of periampullary cancers. Analysis of this study has clearly shown that as of now genetic mutation should be carried out in all cases of periampullary carcinoma presenting with primary tumour size >2cms as well as in cases with distant metastasis for further follow up and adjuvant therapy.

CHAPTER VIII

SUMMARY AND CONCLUSION

This present study has observed that

1. A high prevalence of K-ras and P53 mutation in periampullary carcinoma in the regional population. More samples were positive for K-ras mutation than P53 mutation(Many studies has shown increased p53 mutation than K-ras mutation ,especially in pancreas carcinoma).

2. The mutation status has not affected the variables such as Age, Gender, nodal metastasis, lympho-vascular/neural invasion and grade of the tumor.

3. By logistic regression analysis, the Odds of occurrence of mutation was 12.3 times due to size and 1.4 times due to distant metastasis.

4. Nearly half of the mutated cases were less than T2 lesions indicating that K-ras and p-53 mutations occur early in

   carcinogenesis process of periampullary carcinoma.

5. Whether this mutation status in periampullary carcinoma affects the longterm survival(as in various other studies) has to be seen in the follow-up studies in future.

6. A study with a larger sample size will give a clear understanding about these facts in our regional population.

7. Though K-ras mutation commonly occurs in codon 12 and codon 13,future genetic studies may concentrate on mutation in other codons and also different types of mutation like DPC4,APC,Beta Catenine pathway etc.,

8. Future studies involving the gene expression pathways may open avenues in different genes that gets mutated in periampullary carcinoma.

9. Better understanding of the molecular biology of periampullary carcinoma is of interest, not only in basic research but will have clinical implications in assessing risk of developing periampullary cancers,its early diagnosis, treatment and for prognosis.

CHAPTER IX

RECOMMENDATIONS
(IMPACT OF THE STUDY)

1. This study has unravelled the important fact that K-ras and P53 mutation is highly prevalent in our regional population.
2. It has opened avenues for further research on the importance of genetic mutation on the pathological upstaging of periampullary carcinoma with a larger sample size.
3. Future research on the topic may be carried as a cohort study on the survival status in the mutated cases.
4. Due to the high prevalence of K-ras and P53 mutation in the regional population, further studies can be carried out on the genetic signalling pathways that are affected in the carcinogenesis.
5. In future such genetic studies may become mandatory in patients operated for periampullary carcinoma to prognosticate the disease.
6. Follow-up studies on the mutated cases will assist the medical oncology research for considering the addition of Gene targeted therapy along with chemotherapy and radiotherapy to decrease the the local recurrence and distant metastatic potential of periampullary carcinoma.
7. This study has underlined the importance of the need for further studies on targeted therapies to improve the overall survival benefit in patients operated for periampullary carcinoma.

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APPENDIX
MASTER CHART

S.No	Specimen No	Age	Sex	Pathological Staging	Grade	Origin	Mutation	
							K-Ras	P53
							K-Ras	P53
1	110/09 A2	51	F	T2 N0 MX	MDCA	Ampulla	+	-
2	285/09 A2	55	F	T2 N0 MX	WDAC	Common	-	+
3	675/09 A1	47	M	T3 N0 MX	WDAC	Ampulla	-	-
4	1857/09 A1	34	M	T3 N0 MX	WDAC	Ampulla	-	+
5	2112/09 A1	38	M	T2 N0 MX	WDAC	Ampulla	+	-
6	2636/09 A1	58	F	T2 N0 MX	WDAC	Ampulla	-	+
7	2357/09 A	62	M	T2 N0 MX	WDAC	Common	-	-
8	2371/09(Liv METS) /PERI AMP.	41	M	T2 N0 M1 (IMAGING)	ADCA(Deposit)	Not ascertained	+	+
9	2830/09 A2	60	M	T2 N0 MX	WDAC	Ampulla	-	-
10	3070/09 A1	59	M	T2 N1 MX	WDAC	Ampulla	-	-
11	3182/09 A1	65	M	T2 N0 MX	WDAC	Ampulla	+	-
12	3240/09 A1	48	M	T2 N0 MX	WDAC	Ampulla	+	+
13	3980/09 A1	40	F	T2 N0 MX	WDAC	Ampulla	-	-
14	5754/09 A2	61	F	T3 N0 MX	WDAC	Ampulla	-	-
15	5831/09 A	55	M	T2 N0 MX	MDAC	Common	-	+
16	5959/09 A2	34	M	T3 N0 MX	MDAC	Common	-	-
17	220/10 A1	50	F	T2 N0 MX	MDAC	Common	-	+
18	253/10 A2	58	M	T2 N0 MX	MDAC	Common	-	-
19	329/10 A2	53	F	T2 N0 MX	MDAC	Common	+	-
20	376/10	60	M	T3 N0 MX	MDAC	Common	-	-
21	759/10 A1	48	M	T3 N0 MX	MDAC	Common	+	+

22	1204/10 A	50	F	T2 N0 MX	PDAC	Common	+	-
23	1744/10 B2	70	F	T3 N0 MX	WDAC	Common	-	-
24	2156/10 A2	52	F	T3 N0 MX	MDAC	Common	+	-
25	2480/10 A2	55	F	T3 N0 MX	WDAC	Common	-	+
26	2698/10 A2	58	M	T1N1MX	WDAC	Ampulla	-	-
27	2734/10 A2	40	M	T2 N0 MX	WDAC	Common	+	-
28	3505/10 A2	38	F	T2 N0 MX	MDAC	Common	+	+
29	4456/10 A2	55	F	T4 N0 MX	MDAC	Common	-	-
30	4790/10 A2	62	M	T2 N0 MX	WDAC	Common	-	-
31	5109/10 A1	40	M	T2 N0 MX	WDAC	Common	+	-
32	5242/10 B1(LIV METS)/Peri.amp	55	F	T2 N0 MX (IMAGING)	ADCA	Common	-	-
33	5423/10 A3	65	F	T4N0MX	MDAC	Common	-	+
34	483/11 A,RB(A)	52	M	T2N1MX	MDAC	Ampulla	-	-
35	737/11 A1	60	M	T3 N0 MX	MDAC	Ampulla	+	-
36	1067/11 A2,RB(A)	40	F	T2 N0 MX	MDAC	Ampulla	-	-
37	1166/11 A2	50	F	T2 N2 M1 (Aortocaval node +ve)	MDAC	Ampulla (mets)	+	-
38	1284/11 A2	42	M	T2 N0 MX	MDAC	Ampulla	-	-
39	1431/11 A3	65	F	T2 N0 MX	MDAC	Common	-	+
40	1909/11 A2	62	F	T3 N0 MX	MDAC	Ampulla	-	-
41	2982/11 A3	60	F	T3 N0 MX	WDAC	Ampulla	-	+
42	3171/11 A2	55	M	T3 N1 M0	MDAC	Ampulla	+	+
43	3372/11 A1	49	F	T1N0MX	MDAC	Ampulla	-	-
44	3562/11 A2,RB11	55	F	T3 N0 MX	MDAC	Ampulla	+	-
45	3637/11 A2	62	M	T2 N1 M0	MDAC	Ampulla	-	-

46	3766/11 A1	65	F	T2 N0 MX	WDAC	Ampulla	-	-
47	4264/11 A3	64	M	T3 N0 MX	MDAC	Ampulla	+	+
48	4536/11 A1	48	F	T2N0M0	WDAC	Ampulla	-	-
49	4783/11 A2	60	M	T2N0MX	WDAC	Ampulla	-	+
50	4889/11 A2	55	M	T3 N0 M0	MDAC	Ampulla	-	-
51	43/12 A1.A2,A3	55	M	T1 N1 MX	WDAC	Ampulla	-	-
52	101/12 A1,A2,A3	46	F	T2 N0 M0	WDAC	Ampulla	+	-
53	160/12 B,LIV Mets	42	F	T3(Imaging)	ADCA (Deposits)	Ampulla	-	+
54	416/12 B(LIV MET)	65	M	T3(Imaging)	ADCA (Deposits)	Ampulla	-	-
55	419/12 A1,A2	42	F	T2 N0 MX	MDAC	Ampulla	+	+
56	644/12 A1,A2	40	F	T1 N0 MX	MDAC	Ampulla	+	-
57	1011/12 A2,RB	58	F	T2 N0 MX	MDAC	Ampulla	-	-
58	1424/12 A1	45	F	T4 N0 MX	WDCA	Ampulla	+	+
59	1824/12 A1,A2	50	M	T1 N0 MX	MDAC	Ampulla	-	-
60	1869/12 A2,A3	30	F	T2 N0 MX	MDAC	Distal CBD	-	+
61	2263/12 A,B(LIVER METS)	52	F	T3(Imaging)	ADCA(Deposits)	Not ascertained	+	+
62	2264/12 A2,A3	49	F	T2 N0 MX	WDCA	Ampulla	-	-
63	2529/12 A5,A6	60	M	T3 N0 MX	WDCA	Distal CBD	+	-
64	2749/12 A4	48	F	T1 N0 MX	MDAC	Distal CBD	-	-
65	3170/12 A1,A2	50	F	T2 N1 MX	MDAC	Ampulla	-	-
66	3943/12 A1,A2	40	M	T3 NO MX	MDAC	Ampulla	+	-
67	4030/12 A2,A3,A4	59	M	T3 NO MX	MDAC	Ampulla	-	-
68	4153/12 A1,A2,A3,A4	65	F	T2 N0 MX	WDCA	Distal CBD	+	+
69	4935/12 A1,A3,A4	62	F	T1 N0 MX	MDAC	Ampulla	-	-
70	356/12B	49	M	T1 N0 MX	MDAC	Common	-	-

71	3237/12 A1,A2	45	F	T2 N0 MX	WDCA	Ampulla	-	-
72	992/13 T1	67	M	T2 N0 MX	MDAC	Ampulla	+	-
73	1588/13 T1	57	M	T2 N0 MX	WDCA	Ampulla	-	-
74	1718/13 RB(A1)	45	M	T1 N0 MX	MDAC	Ampulla	+	+
75	2341/13RB(AA1)	70	M	T2 N0 M0	MDAC	Ampulla	-	+
76	3050/13 RB1	70	F	T1 N0 MX	WDAC	Ampulla	-	-
77	3281/13 T1, RB2	57	M	T2 N0 MX	WDAC	Ampulla	-	-
78	4544/13 T2	65	M	T2 N0 MX	MDAC	Ampulla	+	-
79	4881/13 T3	65	F	T1 N1 MX	MDAC	Ampulla	-	-
80	5018/13 T2	52	M	T1 N0 MX	WDAC	Ampulla	-	+
81	5019/13 T3	47	M	T1 N0 MX	WDAC	Ampulla	-	-
82	5047/13 T1	38	M	T3 N0 MX	WDAC	Ampulla	+	+
83	5199/13 T1	60	M	T3 N0 MX	WDAC	Ampulla	+	-
84	5282/13 A1	63	M	T3 N0 MX	WDAC	Ampulla	-	-
85	5617/13 T2	40	M	T1 N0 MX	MDAC	Ampulla	-	-
86	5922/13 T1	55	M	T2 N0 MX	WDAC	Ampulla	+	-
87	6265/13 T1	33	M	T2 N0 MX	WDAC	Ampulla	-	-
88	6354/13 T1	55	F	T2 N0 MX	MDAC	Ampulla	-	-
89	SS 813/13	60	F	T2 N0 MX	WDAC	Ampulla	-	-
90	SS 826/13	59	F	T3 N0 MX	WDAC	Ampulla	-	-

M-Male,F-Female,T-Tumor size,N-Nodal status,M-Metastasis,WDAC-Well-Differentiated adenocarcinoma,MDAC-Moderately Differentiated adenocarcinoma,PDAC-Poorly differentiated adenocarcinoma.

Results of Tissue Specimen using *K-ras* and *P53* gene

2009 specimens to Bharathiar University - Results			
S.No.	**Specimen No.**	***K-ras***	***P53***
1.	110/09 A2	+	-
2.	285/09 A2	-	+
3.	675/09 A1	-	-
4.	1857/09 A1	-	+
5.	2112/09 A1	+	-
6.	2636/09 A1	-	+
7.	2357/09 A	-	-
8.	2371/09(LIV METS)/PERI AMP.	+	+
9.	2830/09 A2	-	-
10.	3070/09 A1	-	-
11.	3182/09 A2	+	-
12.	3240/09 A1	+	+
13.	3980/09 A1	-	-
14.	5754/09 A2	-	-
15.	5831/09 A	-	+
16.	5959/09 A2	-	-
2010 specimens to Bharathiar University - Results			
1.	220/10 A1	-	+
2.	253/10 A2	-	-
3.	329/10 A2	+	-
4.	376/10(Pre –op)	-	-
5.	759/10 A1	+	+
6.	1204/10 A	+	-
7.	1744/10 B2	-	-
8.	2156/10 A2	+	-
9.	2480/10 A2	-	+
10.	2698/10 A	-	-
11.	2734/10 A2	+	-
12.	3505/10 A2	+	+
13.	4456/10 A2	-	-
14.	4790/10 A2	-	-
15.	5109/10 A1	+	-
16.	5242/10 B1(LIV METS)/Peri.amp	-	-
17.	5423/10 A3	-	+
2011 specimens to Bharathiar University - Results			
1.	483/11 A,RB(A)	-	-
2.	737/11 A1	+	-
3.	1067/11 A2,RB(A)	-	-
4.	1166/11 A2	+	-

5.	1284/11 A2	-	-
6.	1431/11 A3	-	+
7.	1909/11 A2	-	-
8.	2982/11 A3	-	+
9.	3171/11 A2	+	+
10.	3372/11 A1	-	-
11.	3562/11 A2,RB11	+	-
12.	3637/11 A2	-	-
13.	3766/11 A1	-	-
14.	4264/11 A3	+	+
15.	4536/11 A1	-	-
16.	4783/11 A2	-	+
17.	4889/11 A2	-	-
2012 specimens to Bharathiar University - Results			
1.	43/12 A1,A2,A3	-	-
2.	101/12 A1,A2,A3	+	-
3.	160/12 B,LIV Mets	-	+
4.	416/12 B(LIV Met)	-	-
5.	419/12 A1,A2	+	+
6.	644/12 A1,A2	+	-
7.	1011/12 A2,RB	-	-
8.	1424/12 A1	+	+
9.	1824/12 A1,A2	-	-
10.	1869/12 A2,A3	-	+
11.	2263/12 A,B	+	+
12.	2264/12 A2,A3	-	-
13.	2529/12 A5,A6	+	-
14.	2749/12 A4	-	-
15.	3170/12 A1,A2	-	-
16.	3943/12 A1,A2	+	-
17.	4030/12 A2,A3,A4	-	-
18.	4153/12 A1,A2,A3,A4	+	+
19.	4935/12 A1,A3,A4	-	-
20.	356/12 B	-	-
21.	3237/12 A1,A2	-	-
2013 specimens to Bharathiar University - Results			
1.	992/13 T1	+	-
2.	1588/13 T1	-	-
3.	1718/13 RB(A1)	+	+
4.	2341/13 RB(A,A1)	-	+
5.	3050/13 RB1	-	-
6.	3281/13 T1,RB2	-	-
7.	4544/13 T2	+	-
8.	4881/13 T3	-	-

9.	5018/13 T2	-	+
10.	5019/13 T3	-	-
11.	5047/13 T1	+	+
12.	5199/13 T1	+	-
13.	5282/13 A1	-	-
14.	5617/13 T2	-	-
15.	5922/13 T1	+	-
16.	6265/13 T1	-	-
17.	6354/13 T1	-	-
18.	SS 813/13	-	-
19.	SS 826/13	-	-

Table: Percentage of *K Ras* and *P53* gene mutation

S.No	Gene	Total No of Samples	Percentage
1	**Null Mutation**	**45**	**50.0%**
2	***K-ras/P53***	**12**	**13.33%**
3	***K-ras***	**19**	**21.11%**
4	***P53***	**14**	**15.56%**

Analysis by

Verified

Dr.V.BALACHANDAR
Young Scientist-DST Fast Track
Human Molecular Genetics Lab
Department of Zoology
School of Life Science
Bharathiar University
Coimbatore -641 046

supervisor

Dr.K.Sasikala M.Sc., M.A.Z., Z.S.I., M.A.F.R.C
UGC - Emeritus Professor
Human Molecular Genetics Laboratory,
Department of Zoology,
Bharathiar University,
Coimbatore - 641 046. Tamil Nadu, India.

INSTITUTIONAL ETHICAL COMMITTEE,
STANLEY MEDICAL COLLEGE, CHENNAI-1

Title of the Work	: Prevalence of K-Ras mutation and P-53 over expression in carcinoma of Ampulla of Vater and its impact on the Pathological staging
Principal Investigator	: Dr.L.Anand
Designation	: Asst.Prof.of SGE
Department	: Department of Surgical Gastroenterology Government Stanley Medical College, Chennai-1

The request for an approval from the Institutional Ethical Committee (IEC) was considered on the IEC meeting held on 11.06.2012 at the Modernized Seminar Hall, Stanley Medical College, Chennai-1 at 2PM

The members of the Committee, the secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the principal investigator.

The Principal investigator and their team are directed to adhere to the guidelines given below:

1. You should inform the IEC in case of changes in study procedure, site investigator investigation or guide or any other changes.
2. You should not deviate form the area of the work for which you applied for ethical clearance.
3. You should inform the IEC immediately, in case of any adverse events or serious adverse reaction.
4. You should abide to the rules and regulation of the institution(s).
5. You should complete the work within the specified period and if any extension of time is required, you should apply for permission again and do the work.
6. You should submit the summary of the work to the ethical committee on completion of the work.

13/6/12

MEMBER SECRETARY,
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Case Report

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Bleeding Complication after Pancreatic Surgery: Role of Harmonic Scalpel

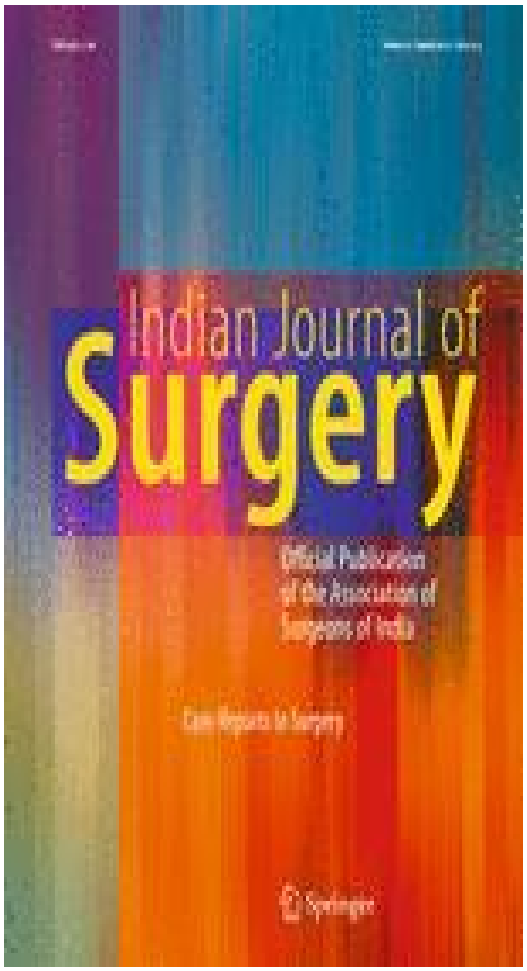
- **Anand Lakshmanan**
- **, P. Ravichandran**
- **, S. Jeswanth**
- **, R. Sukumar**

Abstract

In this modern era of technological advancements, though many centers are contemplating complex surgical procedures on the pancreas, morbidity is still high and around 30–35 %. Post-operative bleeding complications are the most worrisome of all, which need vigilance by the operating team. Early recognition and prompt management using endoscopy, intervention radiology or urgent surgery, with a low threshold for relaparotomy is needed to avoid mortality. After successfully completing more than 500 Whipple's operations and over 300 Frey's procedures in the last 10 years, our bleeding complication, which is around 2 %, has substantially increased. This increase over the last couple of years is seen with usage of harmonic scalpel in pancreatic surgery. Here we report our recent encounter with bleeding in the post-operative period after Whipple's pancreaticoduodenectomy and Frey's procedure, where harmonic scalpel was used. We have recommended our suggestion to avoid this complication, by adopting a simple technique. We have achieved optimal results by applying this technique in our subsequent cases.

Keywords

Bleeding complication Frey's procedure Harmonic scalpel Whipple's pancreaticoduodenectomy



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CASE REPORT

Bleeding Complication after Pancreatic Surgery: Role of Harmonic Scalpel

Anand Lakshmanan · P. Ravichandran · S. Jeswanth · R. Sukumar

Received: 4 July 2012 / Accepted: 20 September 2012

Abstract In this modern era of technological advancements, though many centers are contemplating complex surgical procedures on the pancreas, morbidity is still high and around 30–35 %. Post-operative bleeding complications are the most worrisome of all, which need vigilance by the operating team. Early recognition and prompt management using endoscopy, intervention radiology or urgent surgery, with a low threshold for relaparotomy is needed to avoid mortality. After successfully completing more than 500 Whipple's operations and over 300 Frey's procedures in the last 10 years, our bleeding complication, which is around 2 %, has substantially increased. This increase over the last couple of years is seen with usage of harmonic scalpel in pancreatic surgery. Here we report our recent encounter with bleeding in the post-operative period after Whipple's pancreaticoduodenectomy and Frey's procedure, where harmonic scalpel was used. We have recommended our suggestion to avoid this complication, by adopting a simple technique. We have achieved optimal results by applying this technique in our subsequent cases.

Keywords Bleeding complication · Frey's procedure · Harmonic scalpel · Whipple's pancreaticoduodenectomy

Introduction

Bleeding in the post-operative period is a dreaded complication after pancreatic surgery. Though the incidence is 5–16 % [1],managing the complication requires earlier detection and timely intervention by an experienced team comprising of gastroenterologists, intervention radiologists and general surgeons. We share our recent encounter of early bleed after Whipple's surgery and Frey's procedure, its timely management and the lessons learned from the experience.

A. Lakshmanan (✉) · P. Ravichandran · S. Jeswanth · R. Sukumar
WARD 601, Institute Of Surgical Gastroenterology,
Stanley Medical College Hospital,
Chennai 600 001 Tamilnadu, India
e-mail: dr_anand_l@yahoo.com

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Case 1

A 39-year-old male presented to our department with painless progressive jaundice and pruritus of 1 month duration. Investigations revealed obstructive jaundice due to periampullary carcinoma. Whipple's operation was done after transecting the neck of the pancreas using harmonic scalpel. Pancreaticogastrostomy was done by the dunking technique. On post-operative day 1, the patient brought out 1 l of coffee ground blood through the ryles tube with hemoglobin level falling >3 g/dl, suggestive of severe early bleed as per the ISGPS classification [2]. The patient was resuscitated with blood transfusion. After stabilization, upper gastrointestinal endoscopy was done to look for the site of bleed. At endoscopy, the stomach was found to be filled with blood clots. As attempts to flush and suck it with saline were futile and angiogram could not be done, he was taken for emergency laparotomy. At laparotomy, anterior gastrostomy revealed an arterial spurter (transverse pancreatic artery) (Fig. 1) from the pancreatic stump. It was suture ligated using 3–0 prolene suture. Anterior gastrostomy was closed in two layers. Post-operative recovery was uneventful.

Case 2

A 17-year-old girl presented with upper abdominal pain for 2 years duration. Investigations revealed tropical pancreatitis with inflammatory head mass. Head coring with

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De novo germ-line mutation of *APC* gene in periampullary carcinoma with familial adenomatous polyps. A novel familial case report in South India

L. Anand [a,b*], K. Padmavathi [d], V. Dhivya [d], I. Mahalaxmi[d], V. Balachandar[c,d]*

[a] *Department of Surgical Gastroenterology, Government Mohan Kumaramangalam Medical College Hospital, Salem, India*

[b] *Institute of Surgical Gastroenterology, Government Stanley Medical College Hospital, Chennai, India*

[c] *Human Molecular Genetics Laboratory, Department of Zoology, Bharathiar University, Coimbatore – 641046, Tamil Nadu, India*

[d] *Human Molecular Genetics and stem cells Laboratory, Department of Human Genetics and Molecular Biology, Bharathiar University, Coimbatore – 641046, Tamil Nadu, India*

***Corresponding Authors**

Email Addresses

dr_anand_l@yahoo.com (L. Anand)

geneticbala@yahoo.co.in (V. Balachandar)

geneticbala@gmail.com (V. Balachandar)

Short Title: ***APC* gene mutation in periampullary carcinoma**

Abstract

Periampullary carcinoma is a malignant tumour arising from the ampulla of vater. Adenomatous polyposis coli *(APC)* gene has a key role in stabilizing β-catenin pathway, in which hypermethylation in *APC* gene could lead to proteasome degradation of β-catenin. The aim of this case report is to identify the *APC* gene mutation and its influence on β-catenin pathway in patient with periampullary carcinoma. A 51-year-old woman was diagnosed with yellow discolouration of sclera, passing deep yellow coloured urine and pruritus. A family history of ovarian cancer had been reported in her mother. Her radiological, pathological and laboratory examination were suspected for periampullary carcinoma. She underwent whipple's pancreaticoduodenectomy, and the histopathology of the resected specimen showed a well differentiated adenocarcinoma involving the ampulla of vater. Further, the tumour region was subjected to genetic screening by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP), cytogenetic analyses such as karyotyping and immunohistochemical techniques. These results showed non-sense mutation in *APC* gene at codon 1309, chromosomal alterations at 5q21 and irregular accumulation of β-catenin in nuclear membrane. The family history revealed a strong association of ovarian cancer (maternal) with a similar *APC* gene mutation. We conclude that periampullary carcinoma patient exhibit FAP due to *de novo* germ-line mutation of *APC* gene that engenders an inactivation of β-catenine /TCF mediated transcription function, with a strong family history.

Keywords: Periampullary carcinoma; Adenomatous polyposis coli; β-catenin; Pedigree; Offspring

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INTRODUCTION

Periampullary cancers are relatively uncommon malignancy. They arise from the region which is less than 2 centimeters from the Ampulla of Vater. The common bile duct merges with the pancreatic duct of Wirsung to form a common channel which however through the ampulla into the duodenum. The distal most portion of the common bile duct appears dilated (i.e., from the ampulla of Vater) and is also surrounded by the sphincter of Oddi,the sphincter muscle described by Boyden, which spirals upward around the terminal part of the duct.

Periampullary Carcinoma tends to manifest early due to biliary outflow obstruction.This is in contrast to to pancreatic neoplasms which are often advanced at the time of diagnosis. Periampullary carcinoma includes cancers arising from Ampulla of Vater, Intrapancreatic distal end of common bile duct, Mucosa of 2nd part Duodenum close to Ampulla of vater and tumors of pancreatic head involving Ampulla[1-2]. It is a relatively uncommon malignancy accounting for 6% of all gastrointestinal malignancies[3].When compared to pancreatic cancer periampullary cancers present early, since it blocks the flow of bile into the intestine early in histogenesis.

Patients afflicted with periampullary cancers present with symptoms of painless progressive jaundice, pruritus (itching of skin),passing high colored urine and clay colored stools.Diagnosis is by clinical examination of hepatomegaly and palpable gallbladder. The diagnosis is confirmed by ultrasonogram of abdomen and Multidetector CT scan of abdomen. CT scan will reveal either a tumor mass in the periampullary region or dilated intrahepatic and extrahepatic biliary tree with abrupt

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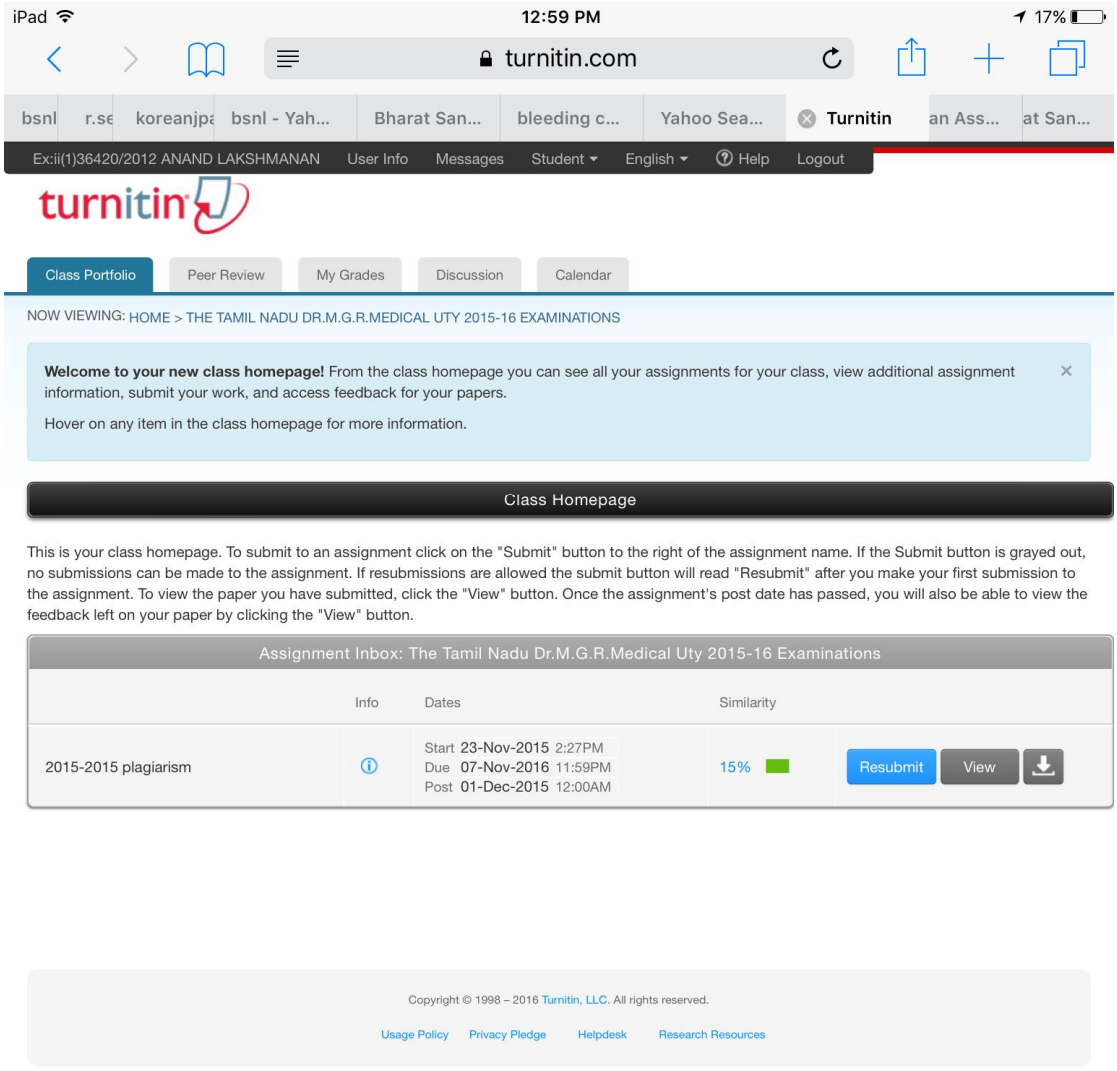
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