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THE PREVALENCE OF K-RAS MUTATION IN PANCREATIC ADENOCARCINOMA AT THE JOHANNESBURG ACADEMIC HOSPITALS

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Medicine (Anatomical Pathology)

Johannesburg, 2019

DECLARATION

I declare that this research is my own, unaided work. It is being submitted for the Degree of Master of Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

13 day of December 2019 at 07h00

ABSTRACT

Introduction:

Pancreatic adenocarcinoma (PDAC) is a disease with a high mortality and morbidity worldwide and current treatment options have proven largely ineffective. The tumour has certain well recognised risk factors of which genetic risk factors are critical in the development thereof. *KRAS* gene is the most commonly mutated gene and is an early event in the carcinogenesis.

Materials and Methods:

A retrospective study was performed of cases received at Johannesburg Academic Hospitals, to determine the frequency of *KRAS* mutations in pancreatic adenocarcinoma. SNOMED search from January 2006 and December 2010 revealed 132 cases of PDAC of which 40 randomly selected cases underwent *KRAS* testing that proved successful in 28 cases. Five 3 μm thick tissue sections of tumour were cut, and subjected to deoxyribonucleic acid (DNA) extraction. The DNA product was amplified for *KRAS* exon 2 using polymerase chain reaction (PCR) followed by DNA sequencing of the purified PCR amplicons.

Results:

Of the 28 patients, 11 were female (39.3%) and 17 males (60.7%). The age of the patients ranged from 38 to 78 years with mean of 60.6 years (standard deviation of 9.6). *KRAS* mutational analysis was performed on forty cases, successful in 28, 19 of which showed *KRAS* mutations (67.9%).

Conclusion:

A high frequency of *KRAS* mutations in PDAC of 67.9% was noted in this study. The key to managing this devastating neoplasm lies in targeted therapies against the *KRAS* pathway and mutational testing for *KRAS* in pancreatic adenocarcinoma may become routine in the future.

PUBLICATIONS/PRESENTATIONS ARISING FROM THIS STUDY

CONFERENCE PRESENTATION:

Poster presentation entitled “Prevalence of K-ras mutations in a cohort of 30 cases of pancreatic adenocarcinoma at the Johannesburg Academic Hospitals”, at the XXIXth Congress of the International Academy of Pathology held September 29 to October 5, 2012 in Cape Town, South Africa.

CITATION DECLARATION

The citations, referencing and bibliography in this research report have been performed using Mendeley referencing software, Version 1. 19. 4 via the Mendeley Cite-O-Matic citation MSWord Plug-in. In the case of multiple citations, the references are listed in ascending year of publication.

DEDICATION

To my sister Mphumzile Thelma Ngwenya for all your help and support.

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TABLE OF CONTENTS

THE PREVALENCE OF K-RAS MUTATION IN PANCREATIC ADENOCARCINOMA AT THE JOHANNESBURG ACADEMIC HOSPITALS .....

DECLARATION ..... *i*

ABSTRACT ..... *ii*

PUBLICATIONS/PRESENTATIONS ARISING FROM THIS STUDY ..... *iii*

CITATION DECLARATION ..... *iv*

DEDICATION ..... *v*

ACKNOWLEDGEMENTS ..... *vi*

TABLE OF CONTENTS ..... *vii*

LIST OF FIGURES ..... *ix*

LIST OF TABLES ..... *x*

ABBREVIATIONS ..... *xi*

CHAPTER 1 ..... *1*

1 INTRODUCTION *1*

1.1 Aetiology **3**

1.2 Prognosis **5**

1.3 Literature review **6**

1.3.1 KRAS Oncogene 6

1.3.2 KRAS in carcinogenesis 9

1.3.3 Clinical implications of KRAS mutation 11

1.3.4 KRAS mutational analysis 12

1.4 Aims and Objectives **13**

CHAPTER 2 ..... *14*

2 MATERIALS AND METHODS *14*

2.1 Patients: **14**

2.2 Pathology: **14**

2.3 KRAS Mutational Assessment **15**

2.3.1 DNA extraction from tumour tissue 15

2.3.2 PCR amplification of KRAS exon 2 16

2.3.3 Purification of PCR Amplicons using BioSpin PCR purification kit according to the departmental protocol 17

2.3.4 Cycle sequencing of purified amplicons using the Qiagen Bioflux Purification kit. 17

2.3.5 Analysis of generated sequences 18

2.4 Inclusion and exclusion criteria: **19**

2.5 Data Analysis **20**

CHAPTER 3 ..... *21*

3 RESULTS *21*

3.1 Patient Details **21**

3.2 Histopathological assessment **24**

3.3 KRAS mutational analysis **33**

3.4 Clinicopathological correlation of KRAS mutation status **42**

CHAPTER 4 ..... *47*

4 DISCUSSION *47*

CHAPTER 5 ..... *54*

5 CONCLUSION: *54*

6 REFERENCES: *55*

7 APPENDICES: *61*

ETHIC CLEARANCE CERTIFICATE **61**

KRAS REFERENCE SEQUENCE **62**

TURNITIN RECEIPT **63**

LETTER REGARDING TURNITIN **64**

LIST OF FIGURES

Figure 1.1 KRAS signalling pathway in a cell. 8

Figure 2.1 Flow diagram of PCR and DNA Sanger sequencing 15

Figure 3.1 Box plot of the age of the population. 21

Figure 3.2 Histogram of age. 22

Figure 3.3 Photomicrograph demonstrating normal pancreatic parenchyma. 24

Figure 3.4 Photomicrograph of PDAC (thick arrow) with adjacent normal pancreatic parenchyma (thin arrow). 25

Figure 3.5 Photomicrograph of cytomorphology of PDAC. 26

Figure 3.6 Photomicrograph illustrating an invasive pancreatic adenocarcinoma with extension into peri-pancreatic adipose tissue. 27

Figure 3.7 Pie chart of proportions of WHO tumour stage. 28

Figure 3.8 Photomicrograph of PanIN II. 29

Figure 3.9 Photomicrograph of PanIN III. 30

Figure 3.10 Photomicrograph illustrating lymphovascular invasion. 31

Figure 3.11 Photomicrograph of PDAC showing perineural and intraneural invasion. 32

Figure 3.12 Bar graph of 260/280 Nanodrop 1000 DNA quantification. 33

Figure 3.13 Photomicrograph of PCR gel electrophoresis of cases 1 to 9. 35

Figure 3.14 Electropherogram of the wild-type *KRAS* forward and reverse sequence. 37

Figure 3.15 Electropherogram of GAT *KRAS* mutation seen in the majority of cases. 38

Figure 3.16 Electropherogram of GCT *KRAS* mutation. 39

Figure 3.17 Electropherogram of CGT *KRAS* mutation. 40

Figure 3.18 Electropherogram of GTT *KRAS* mutation. 41

Figure 3.19 Boxplot of age by *KRAS* mutation. 42

LIST OF TABLES

Table 1.1 Syndromes and genes associated with an increased risk of pancreatic adenocarcinoma 4

Table 1.2 Genetic mutations in pancreatic adenocarcinoma 5

Table 2.1 PCR master mix reagents calculation..... 16

Table 2.2 Thermal cycler conditions for PCR..... 17

Table 2.3 Cycle sequencing master mix reagents using the Big Dye Terminator V3.1 Cycle Sequencing kit..... 18

Table 2.4 Cycling parameters for Cycle Sequencing 19

Table 3.1 Skewness/Kurtosis tests for Normality 22

Table 3.2 Characteristics of the study population (n=28) 23

Table 3.3 DNA Extraction and Quantification log Assay:..... 34

Table 3.4 KRAS mutation results of the study population..... 36

Table 3.5 Age by *KRAS* mutational status 43

Table 3.6 Gender by *KRAS* mutation 43

Table 3.7 Grade of tumour by *KRAS* mutation 44

Table 3.8 Pancreatic intraepithelial neoplasia by *KRAS* mutation 45

Table 3.9 Frequency of *KRAS* mutation presented by clinicopathological features 46

Table 4.1 Frequency of KRAS mutations in PDAC in various studies..... 50

ABBREVIATIONS

A	Adenine
ala	Alanine
arg	Arginine
asp	Aspartic acid
bp	Base pairs
C	Cytosine
CDKN2A	Cyclin dependent kinase inhibitor 2A
cys	Cysteine
ddNTP	Dideoxynucleotides triphosphates
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DPC4	Deleted in pancreatic cancer, locus 4
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated protein kinases
FFPE	Formalin fixed paraffin embedded tissues
G	Guanine
GAP	Guanosine triphosphatase-activating protein
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factors
GTP	Guanosine triphosphate
GTPases	Guanosine triphosphatases
H&E	Haematoxylin and eosin

Her2/neu	Human epidermal growth factor receptor 2
HOP	Head of pancreas
HRAS	Harvey rat sarcoma viral oncogene homolog
IPMNs	Intraductal papillary mucinous neoplasms
KRAS	Kirsten rat sarcoma viral oncogene homolog
Leu	Leucine
LVI	Lymphovascular space invasion
MAPK	Mitogen activated protein kinases originally called ERK
MLH1	MutL homolog 1
MSH2	Human mutS homolog 2
MSH6	Human mutS homolog 6
n	Number
NRAS	Neuroblastoma Ras viral oncogene homolog
PanIN	Pancreatic intraepithelial neoplasia
PCR	Polymerase chain reaction
PDAC	Pancreatic Adenocarcinoma
phe	Phenyalanine
PMS2	Posmeiotic segregation increased 2
PNA	Peptide nucleic acid
PNI	Perineural invasion
PRSS1	Serine protease 1
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma
ser	Serine

SNOMED	Systemised Nomenclature of Medicine
std. dev	Standard deviation
STK11	Serine/threonine kinase 11 also known as liver kinase B1 (LKB1)
T	Thymine
TP53	Tumour protein p53
try	Trypsin
val	Valine
WHO	World Health Organisation

CHAPTER 1

1 INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a malignant epithelial neoplasm composed of infiltrating glands deposited within a desmoplastic stroma. The stroma may be abundant and outnumber the neoplastic cells (Hruban & Klimstra, 2014). PDAC is the most common neoplasm in the pancreas and accounts for up to 90% of all pancreatic tumours (Bosman F. T., Carneiro F., Hruban R. H., 2010).

Despite their highly aggressive behaviour, pancreatic adenocarcinomas are usually well differentiated and may resemble the normal pancreatic ductal parenchyma. Hruban & Klimstra, (2014) listed eight histopathological features which are helpful in the diagnosis of pancreatic adenocarcinoma. These included haphazard arrangement of glands, perineural invasion, vascular invasion, glands adjacent to large blood vessels, luminal necrosis, incomplete lumina, 'naked' glands within fat and an increase in nuclear size (four times the normal ductal epithelial nuclear size).

Studies have shown that pancreatic adenocarcinoma develops in a stepwise fashion from normal duct epithelium to dysplasia and subsequent invasive carcinoma (Oda et al., 2014; Koorstra et al., 2008; Hruban et al., 2000). The World Health Organisation Classification of Tumours of the Digestive system, 2010 recognises three precursor lesions: pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm and mucinous cystic neoplasm.

PanINs are the most common precursors of pancreatic adenocarcinoma and the term was coined by the researchers Hruban et al., (2001). PanINs were described as small (<5 mm in diameter), flat or papillary intraepithelial neoplasms located within the pancreatic ducts. They were classified into three main grades according to architectural and cytological features. PanIN1 shows no cytologic atypia and is further subdivided into PanIN 1A and PanIN 1B.

PanIN 1A is a flat lesion composed of tall columnar cells with basally located nuclei displaying no cytologic atypia. PanIN 1B are similar to PanIN 1A except that these have papillary architecture. PanIN 2 lesions may be flat or papillary and have cytologic abnormalities including nuclear irregularities, hyperchromasia and loss of polarity. PanIN 3 lesions are papillary or micropapillary, seldom flat, and display marked cytological abnormalities, similar to that of carcinoma. However, in contrast to carcinoma, no invasion is present in PanIN 3 lesions.

Mucinous cystic neoplasms of the pancreas are epithelial neoplasms that form cysts which do not communicate with the main duct system (Bosman F. T., Carneiro F., Hruban R. H., 2010). The cysts are encapsulated and lined by columnar mucinous epithelium with underlying ovarian-type stroma. They are mainly located in the distal pancreas and occur most commonly in women between the ages 40-50 years. In the World Health Organisation Classification of Tumours of the Digestive system 2010, mucinous cystic neoplasms are divided into low grade dysplasia, intermediate grade, high grade dysplasia and invasive mucinous adenocarcinoma.

Intraductal papillary mucinous neoplasms (IPMNs) are macroscopic epithelial neoplasms larger 10mm, involving the main pancreatic duct or branches. The epithelium is papillary and composed of mucinous columnar cells (Hruban et al., 2004). IPMNs are graded as follows; low grade dysplasia, intermediate grade dysplasia and high grade dysplasia.

It is important for histopathologists to recognise and report the presence of precursor lesions in PDAC. Some studies have ascertained that high grade PanIN (grades 2 and 3) is associated with an overall better prognosis (Oda, Aishima, Morimatsu et al., 2014) in contrast to cases of PDAC in which low grade PanIN was evident and those in which no in-situ lesions were identified.

1.1 Aetiology

The causes of pancreatic adenocarcinoma remain largely unknown. However, certain environmental agents and genetic syndromes are known to be associated with an increased risk for the development of PDAC. Several genetic and epidemiologic studies have established a number of risk factors which are inclusive of cigarette smoking, older age, alcohol consumption, family history, diabetes mellitus, coffee consumption, obesity, high fat diet and low intake of fruits and vegetables (Bosman, Carneiro, & Hruban, 2010). The most well established risk factor is cigarette smoking. People with a history of smoking have a 2-to-3 fold increased risk of developing PDAC (Yeo, et al., 2002). Mizuno, et al., (2014) noted that patients who had a history of cigarette smoking presented at a younger age than patients who had no history of smoking.

According the WHO Classification of tumours of the Digestive systems (2010), pancreatic cancer is more common in males than in females with the highest rates noted in African Americans (Bosman F. T., Carneiro F., Hruban R. H., 2010). In a study by Mizuno, et al., (2014) 59.9% of their patients were male. Although coffee has been suggested as a risk factor, a prospective study in a large cohort of participants with the highest consumption of coffee in the world found no increased risk of pancreatic cancer (Bidel, Jousilahti, Pukkala, Hakulinen & Tuomilehto, 2013).

Only 10% of patients with pancreatic adenocarcinoma have a family history of cancer and these patients present at a younger age (Bardeesy & DePinho, 2002). Mizuno et al., (2014) noted that patients with a family history of PDAC were diagnosed at an early age compared with other patients; however, this was not a significant finding. Syndromes and genes associated with an increased risk of PDAC are illustrated in

Table 1.**1**. Peutz-Jeghers syndrome is caused by germline mutations involving the *STK11* gene which encodes for a protein that regulates protein kinases and as a result, regulates cell growth (Zhan et al., 2018). Patients with Peutz-Jeghers syndrome are at high risk of developing internal malignancies including PDAC.

Hereditary Breast-Ovarian cancer syndrome is caused by germline mutations in the DNA mismatch repair genes *BRCA1 and BRCA2*. *BRCA1/2* germline mutations have been identified in 11% of families with familial PDAC(Kim et al., 2009). Familial atypical mole-malignant melanoma(FAMMM) is due to germline mutations in *CDKN2A* gene which codes for p16(INK4A) and p14(ARF), which are responsible for cell cycle regulation (Zhan et al., 2018). Patients with FAMMM have a 13-20 increased risk of PDAC (Zhan et al., 2018). Lynch syndrome (hereditary nonpolyposis colorectal carcinoma) is due to germline mutations in DNA mismatch repair genes *MLH1*, *PMS2*, *MSH2* and *MSH6*. Patients with Lynch syndrome are at high risk of developing colorectal carcinoma, endometrial carcinoma and PDAC (Zhan et al., 2018). It is important to identify patients who have a strong family history of PDAC because they should be encouraged to undergo screening for possible early detection of resectable tumours.

Table 1.1 Syndromes and genes associated with an increased risk of pancreatic adenocarcinoma

Syndrome	Gene	Main features
Peutz-Jeghers	*STK11/LKB1*	Gastrointestinal hamartomatous polyps, buccal hyperpigmentation and increased risk of multiple cancers
Hereditary Breast-Ovarian cancer	*BRCA1* and *BRCA2*	Breast cancer, ovarian cancer
Familial atypical mole-malignant melanoma(FAMMM)	*CDKN2A/p16*	Melanoma
Lynch syndrome	*MLH1, PMS6, MSH2 & MSH6*	Carcinoma of the colon, endometrium and stomach
Hereditary pancreatitis	*PRSS1*	Recurrent episodes of pancreatitis

Adapted from Flanders & Foulkes (1996).

The genetic basis of pancreatic adenocarcinoma involves an increase in cytologic atypia in the ducts and an associated increase in the number of genetic mutations. These are summarised in Table 1.2 (Bardeesy & DePinho, 2002). The main genes involved in pancreatic carcinoma pathogenesis include tumour suppressor genes; *TP53*, *p16/CDKN2A* and *DPC4/SMAD4* and the Kirsten rat sarcoma viral oncogene homolog (*KRAS).* *TP53* mutations have been observed in most human cancers and PDAC is no exception. Some studies have reported *TP53* mutations in up to 61% of PDACs (Felsenstein et al., 2018). *DPC4/SMAD4* mutations have been observed in approximately 50% of PDACs and *p16/CDKN2A* alterations in almost all cases (Biankin et al., 2002).

Table 1.2 Genetic mutations in pancreatic adenocarcinoma

Stage of progression	**Genetic alteration**	**Chromosome**
1. Early stage	*K-RAS* activation	12p
	HER-2/neu expression	17q
2. Intermediate	*p16* activation	9p
3. Late	*p53*	17p
	DPC4	18q
	BRAC2	13q

Adapted from (Bardeesy & DePinho, 2002)

1.2 Prognosis

PDACs are amongst the most lethal forms of cancer as the 5 year survival is less than 5% (Siegel et al., 2014) and the median survival rate is only 6 months after diagnosis (Flanders & Foulkes, 1996). Most patients present with metastatic disease at diagnosis and only 10-20% of tumours are resectable at the time of diagnosis (Flanders & Foulkes, 1996).

In 2018, GLOBOCAN reported 458 918 new cases of PDAC worldwide, with an almost equal number of deaths (433, 242 cases) (Bray et al., 2018). Previously, in 2014 an estimated 46 420 new cases of pancreatic cancer were reported in the United States with an astounding estimated death rate of approximately 39 590 cases (Siegel et al., 2014). Moreover, 4 600 cases of pancreatic adenocarcinoma were reported in Canada during 2012 in association with an alarming number of 4 300 deaths (Hurton et al., 2014). PDAC was found to be the fourth leading cause of cancer death in both men and women in the United States (Siegel et al., 2014) and in Canada (Hurton et al., 2014).

Unfortunately, traditional surgical and non-surgical methods have failed to improve the prognostic outlook of patients afflicted with PDAC. Consequently, there has been a plethora of research aimed at understanding the pathogenesis of pancreatic adenocarcinoma in order to develop targeted therapies and to find markers for screening and early diagnosis.

1.3 Literature review

1.3.1 KRAS Oncogene

Oncogenes promote cell growth in cancer cells. They are derived from normal cellular counterparts called proto-oncogenes. Proto-oncogenes function by regulating cell proliferation and differentiation.

Research on RAS began in the 1960s when a paper was published by Jennifer Harvey on a murine leukaemia virus that induced sarcomas in rodents (Harvey, 1964). The Kirsten murine sarcoma virus was discovered in 1967 by Werner Kirsten (Malumbres & Barbacid, 2003). Ed Scolnick and colleagues postulated that the aforementioned viruses were recombinant viruses and called them the Harvey sarcoma virus and Kirsten sarcoma virus (Malumbres & Barbacid, 2003). Consequently, in 1983 the Ras oncogenes were identified in animal tumours accompanied by the KRAS oncogene being identified in lung adenocarcinoma and not in native lung tissue (Malumbres & Barbacid, 2003). Additionally, in 1984 the *NRAS* oncogene was identified in human neuroblastoma neoplastic cells (Malumbes et al., 2003).

KRAS is a member of the RAS family of proto-oncogenes, which includes *NRAS* and *HRAS* (Hingorani & Tuveson, 2003). These encode small guanosine triphosphatases (GTPases) that regulate cell proliferation, survival, differentiation and apoptosis (Hingorani & Tuveson, 2003). The *KRAS* oncogene is located on chromosome 12p. It encodes for the RAS protein, which in normal cells is tethered to the cytoplasmic aspect of the cell membrane and is bound to guanosine diphosphate (GDP). As a result, RAS exists in an inactive state.

When stimulated by growth factors, RAS is activated by exchanging GDP for guanosine triphosphate (GTP) (Hingorani & Tuveson, 2003). Activation of RAS protein stimulates downstream signalling pathways, such as RAS/RAF/mitogen-activated protein kinase pathway (MAPK) and phosphatidylinositol 3-kinase/AKT pathways, to transmit growth promoting signals to the nucleus (Bos, 1989). In normal cells, this is transient, with GTPase converting GTP to GDP (an inactive state). Although the RAS protein has intrinsic GTPase activity, this is too slow and requires GTPase activating proteins (GAP) to speed up the hydrolysis of GTP to GDP resulting in the resting state (Hingorani & Tuveson, 2003).

Guanine nucleotide exchange factors (GEF) switch on the RAS protein by facilitating the exchange of GDP for GTP (Eser et al., 2014) which leads to signalling transduction, whilst guanosine triphosphatase-activating protein (GAP) switches off the RAS protein. This *KRAS* signalling pathway is illustrated in Figure 1.1.

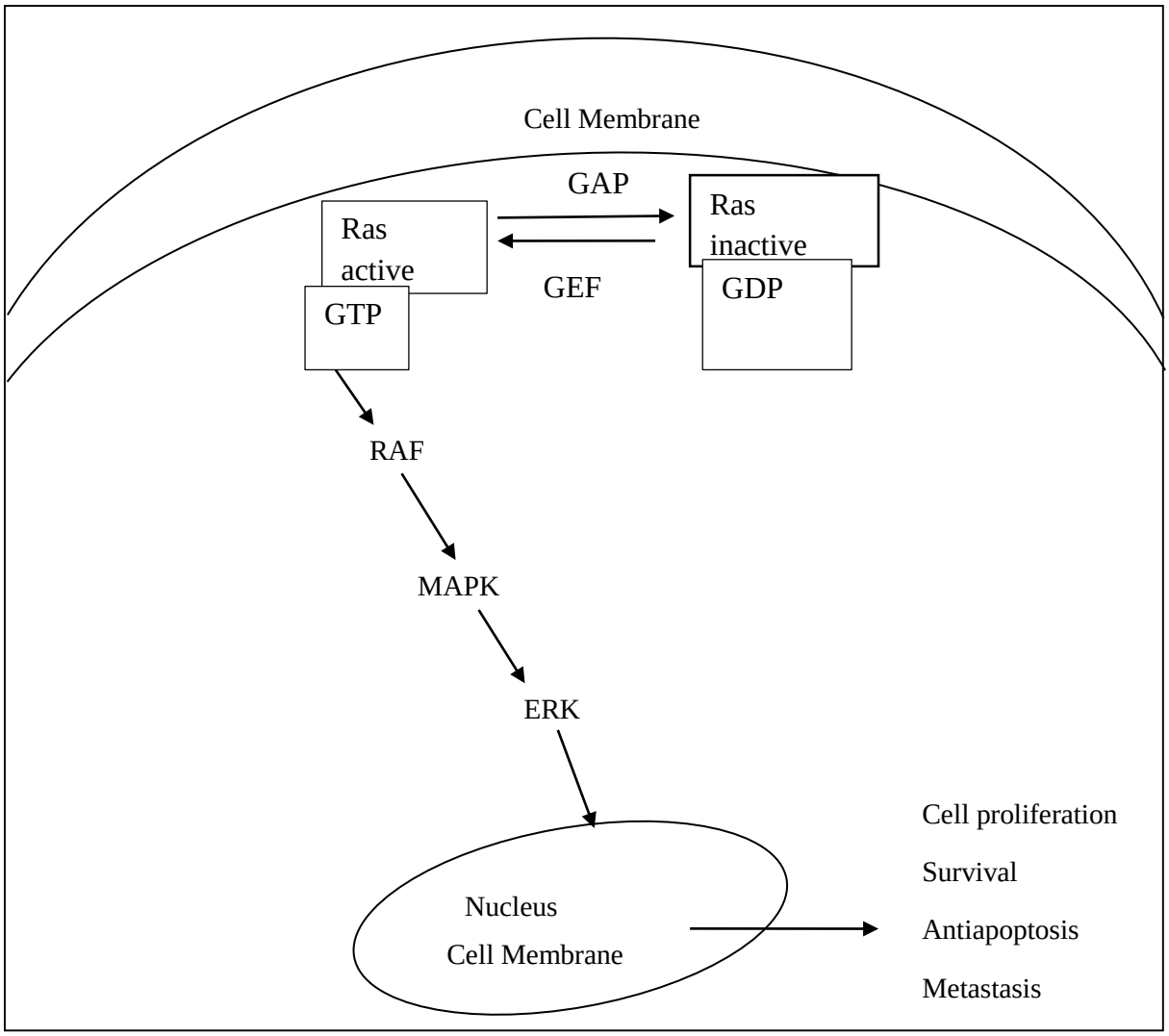


Figure 1.1 KRAS signalling pathway in a cell.

The Ras protein exists in the inactive state and is attached to GDP. When stimulated by external signals, guanine nucleotide exchange factors (GEP) stimulates the hydrolysis of GDP to GTP resulting the Ras active state. This in turn stimulates downstream pathways such as RAF (Rapidly Accelerated Fibrosarcoma), MEK and ERK (Mitogen activated protein kinases originally called ERK) and these results in cell proliferation, survival, differentiation, adhesion, and migration. In neoplastic cells, antiapoptosis, and metastasis are stimulated.

KRAS mutation interrupts the ability of GTPase to hydrolyse GTP and also blocks the GAPs (Bardeesy & DePinho, 2002). As a result, a gain of function of a hyperactive state ensues. Consequently, an increased downstream stimulation occurs. This ultimately results in tumour initiation, growth, invasion and metastasis (Hingorani & Tuveson, 2003).

Studies have shown that *KRAS* mutations are important in metabolic alterations in PDAC, whereby tumour cells are converted into scavengers and hence able to survive extreme conditions such as lack of nutrients and oxygen (Sousa & Kimmelman, 2014). In addition, *KRAS* mutation results in altered metabolism in cancer cells including increased glucose uptake by neoplastic cells resulting in increased glycolysis (Sousa & Kimmelman, 2014). Similarly, there is an increase in lipid intake which are used for production of fatty acids that are important nutrients for the neoplastic cells (Sousa & Kimmelman, 2014).

KRAS mutation also causes use of glutamine for the tricarboxylic acid cycle in which energy in the form of adenosine triphosphate is produced by oxidative phosphorylation within the mitochondria (Sousa & Kimmelman, 2014). Glutamine metabolism has not been identified in normal cells (Eser et al., 2014). These findings are important in explaining the role of *KRAS* mutation in carcinogenesis and may guide the design of treatments that target these cellular processes instead of the KRAS protein itself.

1.3.2 KRAS in carcinogenesis

KRAS mutations have been detected in a number of human cancers, including those that occur in the colon, lung, biliary tract, ovary and gastrointestinal tract. The highest frequency of *KRAS* mutations has been documented in pancreatic cancer (Tada et al., 1990; Wang et al., 2010). In colorectal carcinoma, *KRAS* mutations have been detected in approximately 30 to 50% of cases (Benvenuti et al., 2007; Amado et al., 2008; Hershkovitz et al., 2014; Russo et al., 2014). These studies observed *KRAS* mutations on codon 12 in 80% of cases; the most common amino acids substituted were glycine for aspartic acid.

KRAS mutations have been detected in 20-25% of lung adenocarcinomas (Doebele et al., 2015). Previously, Nadal et al., (2014) reported *KRAS* mutations in 47.5% of patients who had lung

adenocarcinoma. Up to 90% of these mutations were noted on codon 12. The most common type of *KRAS* mutation was glycine (GGT) to cysteine (TGT) substitution in codon 12, which differs from that which occurs in pancreatic ductal and colorectal adenocarcinomas.

The most commonly mutated oncogene in PDAC is the *KRAS* oncogene and mutations have been detected in > 90% of these tumours (Smit et al., 1988; Hruban et al., 1993; Caldas et al., 1994; Bosman F. T., Carneiro F., Hruban R. H., 2010). Most *KRAS* mutations have been identified on codon 12 of the *KRAS* gene. Smit, et al., (1988) reported mutations on codon 12 of the *KRAS* in 28 of the 30 patients in their study. The mutations were guanine-thymidine (purine for pyrimidine) transversions and guanine-adenine (purine for purine) transitions at the second base of codon 12.

Hruban, et al., (1993) noted the presence of point mutations on codon 12 in 83% of tumours in their study. Less than 1% of mutations were identified on codon 13 (Schultz et al., 2012; Shin et al., 2013). The high frequency of *K-RAS* point mutations on codon 12 was also demonstrated in a subsequent study by Wei et al., (2005). In this latter study, K-RAS mutations were found in 25 of 30 patients and 24 mutations were identified on codon 12.

KRAS mutations have not only been detected in the invasive PDAC but also in precursor lesions (Löhr et al., 2005; Schönleben et al., 2007; Kuboki, Shimizu & Hatori, 2015). Kuboki, Shimizu & Hatori, (2015) and Schönleben et al., (2007) demonstrated *KRAS* mutations in 56% and 47% of IPMNs respectively. Most of the mutations where in exon 1, codon 12 of the *KRAS* gene. Previously, Löhr et al., (2005) showed that the frequency of *KRAS* mutation increased with the grade of PanIN. *KRAS* mutations were found in 36% of PanIN 1A lesions, 44% of PanIN 1B and 87% of PanIN 2 and PanIN 3. These findings provide supportive evidence that *KRAS* mutations are an early event in carcinogenesis.

Although most *KRAS* mutations have been detected on exons 2 (codons 12 and 13), studies have also detected exon 3 (codon 61) mutations (Biase et al., 2014). Biase et al. (2014) detected exon 3 (codon 61) mutations in 3.3% and 16.7% of cases in their study using Sanger sequencing and next generation sequencing respectively.

1.3.3 Clinical implications of KRAS mutation

Studies have detected *KRAS* mutations in stool-samples from patients who have pancreatic adenocarcinoma (Caldas et al., 1994). The abovementioned finding is promising in light of establishing screening tests for the early detection of this devastating malignancy. In addition, studies have reported *KRAS* mutations in cell free DNA in patients with PDAC, colorectal carcinoma and non-small cell lung carcinoma (Zhuang et al., 2017). Cell-free DNA are fragments of DNA present in blood and can be identified in plasma (Zhuang et al., 2017). *KRAS* mutations identified in PDAC, have been associated with a poor prognosis [Hazard ratio of 2.81 (95% CI 1.83 – 4.30, $p<0.01$)] (Zhuang et al., 2017). Therefore, cell-free DNA *KRAS* mutational testing is a non-invasive method of detecting patients with pancreatic adenocarcinoma.

Emerging evidence suggests that *KRAS* mutations are associated with poor prognosis in human cancers, including colorectal carcinoma (Amado et al., 2008; Benvenuti et al., 2007; Harbison et al., 2013; Lièvre et al., 2008), lung adenocarcinoma (Nadal et al., 2014) and PDAC (Shin et al., 2013; Sinn et al., 2014).

Currently, *KRAS* mutational analysis is performed in lung and colorectal adenocarcinoma to assess whether tumours will respond to epidermal growth factor receptor (EGFR) inhibitors, such as cetuximab and panitumab (Wang et al., 2010). Several studies have shown that *KRAS* mutations in colorectal adenocarcinoma are associated with poor prognosis and these *KRAS*-mutation containing tumours do not respond to EGFR inhibitors (Benvenuti et al., 2007; Amado et al., 2008; Lièvre et al., 2008; Harbison et al., 2013). Therefore, *KRAS* mutational analysis is mandatory in patients with lung and colorectal adenocarcinomas.

At present there are no established clinical guidelines for testing for *K-RAS* mutations in adenocarcinoma of the pancreas. There are several methods used for *K-RAS* mutational analysis, including polymerase chain reaction (PCR) followed by direct sequencing, real time PCR, probe hybridisation and allele-specific oligonucleotide hybridisation.

1.3.4 KRAS mutational analysis

Several methods have been developed for *KRAS* mutational analysis, and most methods are polymerase chain reaction (PCR)-based. However, no particular method of testing is preferred over the other methods. The most commonly used methods of testing are deoxyribonucleic acid (DNA) sequencing and real time PCR (Wang et al., 2010). Real time-PCR is a method that uses oligonucleotide primers which bind to sequences flanking the mutation sites in codon 12 and 13 (Wang et al., 2010). This test only identifies the targeted mutations only and is more sensitive than DNA Sequencing.

DNA sequencing was developed by Sanger and colleagues in 1970s (Sanger, Goulson & Road, 1975). The Sanger dideoxy chain-termination method used an enzyme called DNA polymerase to synthesise DNA in the presence of a template DNA, four deoxynucleotides triphosphates (dNTP) and a primer (Carrilho, Kist & Franc, 2002). The four nucleotides were adenine (A), cytosine (C), guanine (G) and thymine (T) and hence the deoxynucleotides are dATP, dCTP, dGTP and dTTP. The primer binds to a specific region in the DNA template where synthesis always starts (Carrilho, Kist & Franc, 2002). In addition, low concentrations of chain terminators also called dideoxynucleotides triphosphates (ddNTP) are added into the reaction. Synthesis will continue as long as a deoxynucleotide trisphosphate is added to the template and will stop when a ddNTP is added. When this method was developed, it was performed in four reaction tubes containing one of the ddNTP, DNA template, DNA polymerase, primer and all four deoxynucleotide triphosphates (Carrilho, Kist & Franc, 2002). After the four reactions, the mixture was then separated according to size of the daughter DNA strands using polyacrylamide gel electrophoresis and visualised using autoradiography.

In 1986, Smith et al. modified the Sanger sequencing method by attaching flourophores (fluorescent labelled dyes) onto the primers instead of radioisotopes (Smith et al., 1986). Four flourophores, each with a different colour were developed for each of the bases adenine (A), thymine (T), cytosine (C) and guanine (G). The method was performed in one reaction, instead of four and electrophoresed. The fragments of DNA were identified by colour and the information was stored by a computer. All possible mutations could be identified by using DNA sequencing.

Kwon et al. (2015) compared direct sequencing to peptide nucleic acid (PNA) clamp real-time PCR and found that PNA clamp real-time sequencing was 20 times more sensitive than direct sequencing (Kwon et al., 2015). Most importantly, direct sequencing failed in 25% of cases; the failure attributed to reduced efficiency of PCR amplification.

1.4 Aims and Objectives

The aim of this study was to perform *K-RAS* mutational analysis in pancreatic adenocarcinoma.

The objectives were:

- To determine the age and gender distribution of patients diagnosed with pancreatic adenocarcinoma at the Johannesburg Academic hospitals during the period 2006 to 2010.
- To document the site of tumour grade, perineural invasion, lymphovascular invasion, stage and intraepithelial neoplasia.
- To perform *KRAS* mutational analysis on these cases and assess the frequency of *KRAS* mutation in these patients.
- To ascertain the types of *KRAS* mutations and frequencies.
- To determine whether *KRAS* mutational status correlates with clinicopathological characteristics.

CHAPTER 2

2 MATERIALS AND METHODS

The study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand (clearance certificate M120490, appendix 1).

2.1 Patients:

A retrospective study of patients with histolopathologically proven pancreatic ductal adenocarcinoma was performed from January 2006 to December 2010, at the Charlotte Maxeke Johannesburg Academic Hospital Complex. Systemised Nomenclature of Medicine (SNOMED) search of all patients with a diagnosis of pancreatic adenocarcinoma was performed on the DisaLab system. The pathological reports, slides and paraffin embedded blocks were retrieved and reviewed in order to carry out the *KRAS* mutational analytic tests.

One hundred and thirty-two patients with histolopathologically confirmed PDAC were initially identified, of which 104 patients underwent a pancreaticoduodenectomy (Whipple procedure), four patients underwent distal pancreatectomy. It was likely that the remaining 24 patient had unresectable tumours as no further specimens were received after the initial diagnostic biopsy occurred. Forty (N=40) randomly selected tumours from the Whipple procedure specimens were subjected to DNA sequencing, KRAS gene mutational testing, that was successful in 28 cases. The demographic details of the 28 patients regarding age and gender were retrieved from the pathology reports.

2.2 Pathology:

The slides of the forty cases were retrieved and reviewed by a histopathologist to confirm the diagnosis of PDAC. All tumours were classified using the World Health Organisation Classification of tumours of the Digestive systems (2010). The site of tumour, grade and pathological stage, presence or absence of precursor lesions were documented. The presence or

absence of perineural invasion and lymphovascular space invasion by tumour were also noted. The tissue block containing a predominance of tumour was selected per case for *KRAS* mutational analysis. Macrodissection and removal of non-tumour tissue were performed.

2.3 KRAS Mutational Assessment

Figure 2.1 below shows a flow diagram and summary of the Sanger sequencing method performed on all cases.

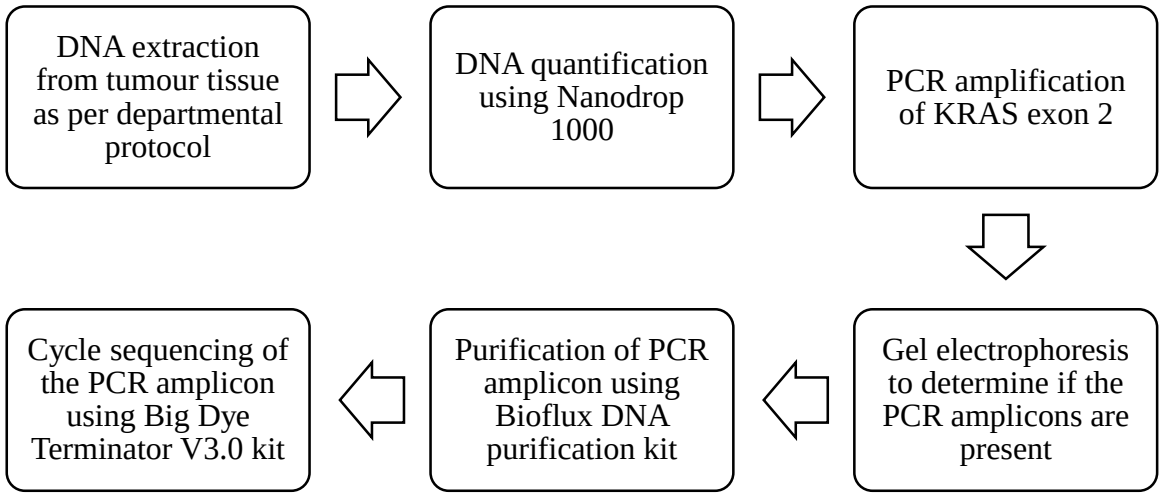


Figure 2.1 Flow diagram of PCR and DNA Sanger sequencing

2.3.1 DNA extraction from tumour tissue

1. Five tissue sections, 3 µm in thickness, of tumour from each case were cut.
2. Deparaffinisation of tissue sections was performed using department protocols.
3. DNA extraction was performed using QIAGEN QIA amp DNA micro kit for extraction of paraffin embedded tissue samples.
4. Quantification of extracted DNA yield undertaken using the Nanodrop1000 spectrophotometer.

2.3.2 PCR amplification of KRAS exon 2

1. An aliquot 1 µl of DNA (50 ng/µl) of each sample was transferred to a labelled tube.
2. The volumes of the reagents to be used in the master mix are shown on Table 2.1. A blank control was set up to ensure that no contamination had occurred.
3. To improve the yield, duplicate samples were set up and combined once the PCR has been checked on a gel.

Table 2.1 PCR master mix reagents calculation

Reagent:	**x1 Sample (µ)**
Taq	0.5
10x buffer	2
dNTPs	2
$MgCl_2$ [1mM]	2
Primer Forward	1
Primer Reverse	1
Distilled water	11

4. The appropriate program was loaded on the thermal cycler as shown in Table 2.2.
5. The PCR amplicons were resolved by running 3 µl of each reaction in a 2% agarose gel. The expected size of the fragment was 173 base pairs.
6. The primers used were:
   Forward Primer: AAGGCCTGCTGAAAATGACTG
   Reverse Primer: CAAAGAATGGTCCTGCACCAG
   The full *KRAS* sequence and mutations are represented in Appendix 2.

Table 2.2 Thermal cycler conditions for PCR

Temperature (°C)	Time (s)	Number of cycles
94.0	60	1
94.0	35	40
60.0	30	
72.0	25	
72.0	120	1
70.5	Decrease 1.5 °C every min	
15.0	Infinity	

2.3.3 Purification of PCR Amplicons using BioSpin PCR purification kit according to the departmental protocol

1. The amplicons were purified using the Qiagen Bioflux Purification kit.
2. Upon completion of the purification, the yield of PCR amplicons were quantified using the NanoDrop.
3. A concentration greater than 10 ng/µl and 260/280 ratio preferably around 1.8 was required to obtain optimum sequencing results.

2.3.4 Cycle sequencing of purified amplicons using the Qiagen Bioflux Purification kit.

1. The volumes of the reagents used in the master mix were calculated according to Table 2.3 and set up on ice.
2. The lids of PCR tubes were removed and 17 µl of the master mix was aliquoted into each tube.
3. The volume of purified DNA added to the master mix was 3 µl.
4. The appropriate program was loaded onto the thermal cycler according to conditions

noted in Table 2.4.

5. The cycle sequencing amplicons were purified using isopropanol.
6. The amplicons were then loaded onto the 3500 DNA analyser.
7. Analysis of sequence for mutations were performed using Sequence Scanner Version 1.0.

Table 2.3 Cycle sequencing master mix reagents using the Big Dye Terminator V3.1 Cycle Sequencing kit

Reagents	1 X Sample (µl)
Terminator ready reaction mix	2
5 x sequencing buffer	4
Primer (forward OR reverse)	6.4
Distilled water	4.6
Total volume of master mix	**17**
Volume of purified DNA to be added to the master mix	3

2.3.5 Analysis of generated sequences

To analyse the sequences, the sequence was aligned to the RefSeqGene?LRG on the NCBI reference sequence on ncbi.nlm.nih.gov website. After logging on the NCBI website, a file was generated to be aligned with the RefSeqGene/LRG. The sequence was then uploaded on to the BLAST page. The results were then reviewed and compared to controls. A report was then printed.

Table 2.4 Cycling parameters for Cycle Sequencing

Temperature (° **C**)	Time	
96	1 minute	
96	10 seconds	25cycles
50	5 seconds	
6	4 minutes	
4	Infinity	

2.4 Inclusion and exclusion criteria:

The following were the inclusion criteria:

1. A histopathological diagnosis of adenocarcinoma of the pancreas.
2. Pathological reports, clinical details, slides and paraffin wax embedded blocks of confirmed cases.
3. Tissue blocks containing sufficient tumour.
4. A readable product after DNA sequencing.

The following were the exclusion criteria:

1. Small biopsies (i.e. fragments) were excluded.
2. Recurrences and repeat specimens were excluded.
3. Cases of neuroendocrine tumours, ampullary adenocarcinomas and cholangiocarcinomas were excluded.
4. Case that did not yield an appropriate result after DNA sequencing were excluded from the study.

2.5 Data Analysis

The data was stored on Microsoft Excel 2016 and before importing the data onto STATA 14.2 software for data analysis, data clean-up was performed to check for errors. Once data clean-up was complete, descriptive statistics was performed. For continuous variables an assessment of normality was performed by checking if the means and medians were almost similar, and by checking Kurtosis and skewness. The mean and standard deviation of the study population was recorded as the data was normally distributed. The categorical variables were represented as frequencies and percentages.

Student t-test was performed to assess P-values to check if age was associated with *KRAS* mutational status. Pearson's chi-squared test was used to check relationships between two categorical variables such as whether KRAS mutation was related to the presence or absence of lymphovascular space invasion. P-values of less than 0.05 were considered statistically significant.

CHAPTER 3

3 RESULTS

3.1 Patient Details

During the course of the study period, 132 cases of PDAC were identified. KRAS mutational analysis was performed on forty, randomly selected cases, and proved successful on 28 cases. Of these 28 patients, 11 were female (39.3%) and 17 males (60.7%). As Figure 3.1 illustrates, the age of the patients ranged from 38 to 78 years.

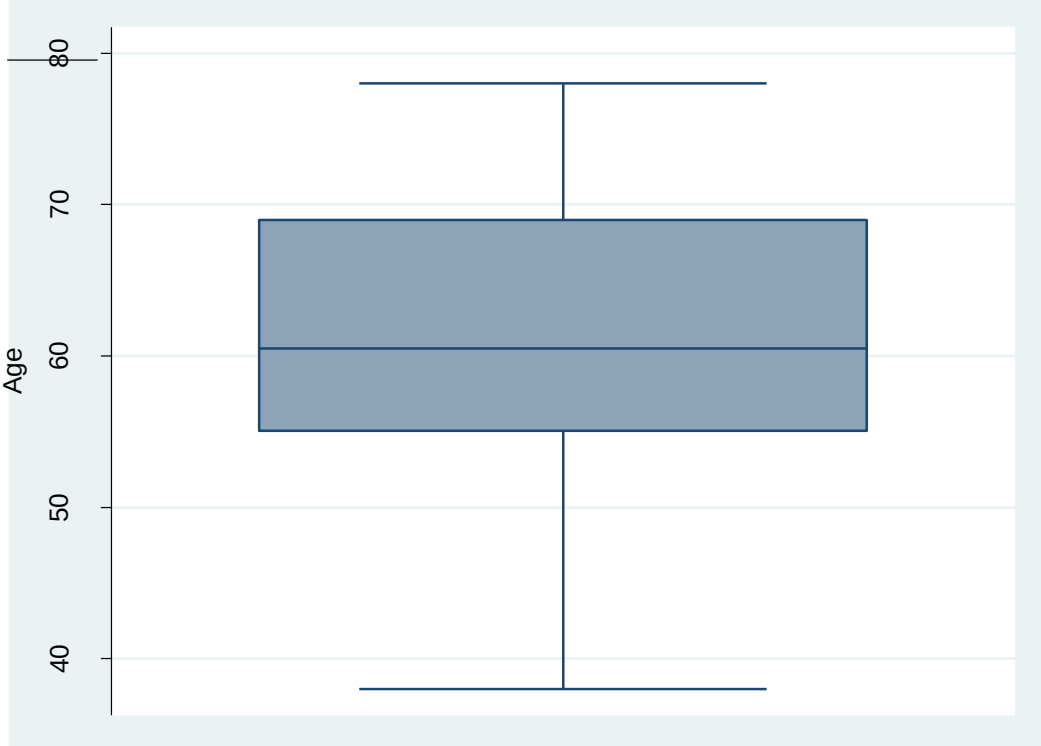


Figure 3.1 Box plot of the age of the population.

Table 3.1 shows the results of Skewness and Kurtosis test used to assess whether the data was normally distributed. The p-value (0.8513) was greater than 0.05 indicating that the data was normally distributed. The histogram of age is represented on Figure 3.2. The mean age of the study population was of 60.6 years (standard deviation of 9.6). A summary of the data is presented as shown in Table 3.2.

Table 3.1 Skewness/Kurtosis tests for Normality

Variable	Number	Skewness	Kurtosis	Chi2(2)	Probability>chi2
Age	28	0.5714	0.9688	0.32	0.8513

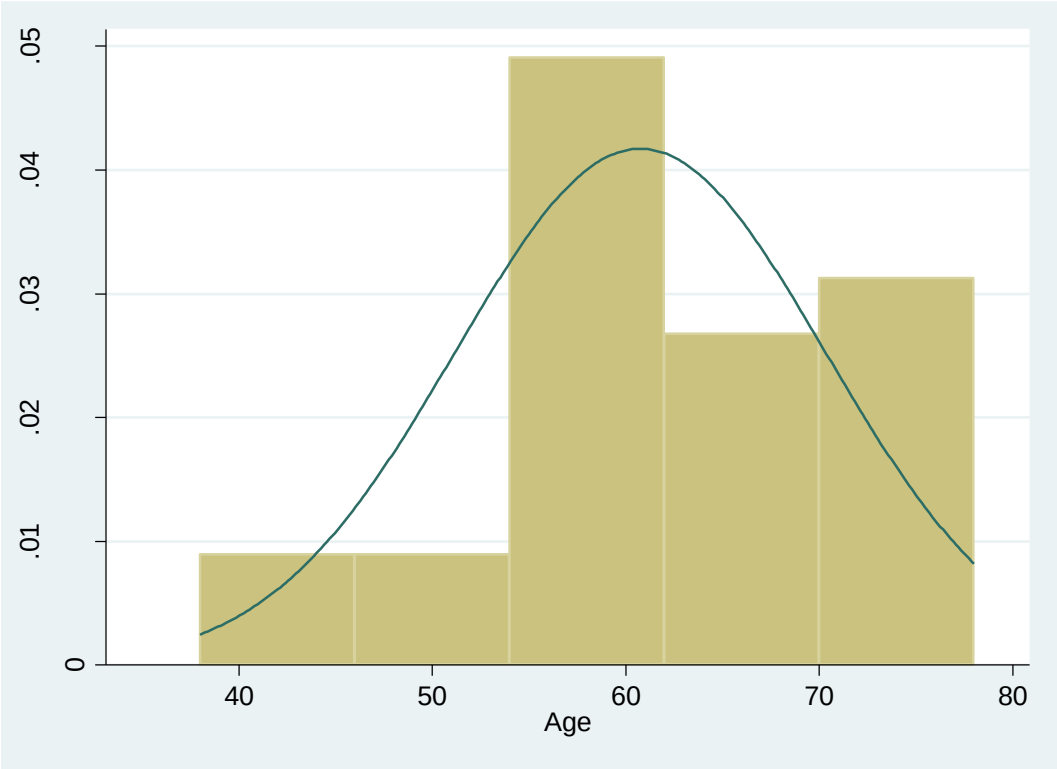


Figure 3.2 Histogram of age.

Table 3.2 Characteristics of the study population (n=28)

Characteristic	Frequency (percentage %)
Gender	
Male	17(60.7)
Female	11(39.3)
Site of tumour	
Head	26(92.9)
Body	2(7.1)
Grade of tumour	
Well differentiated	1(3.6)
Moderately differentiated	17(60.7)
Poorly differentiated	10(35.7)
Stage	
IB	3(10.7)
IIA	5(17.9)
IIB	17(60.7)
III	3(10.7)
PanIN	
Absent	3(10.7)
Present	25(89.3)
PanIN 2	8(28.6)
PanIN 3	17(60.7)
Lymphovascular space invasion	
Present	21(75)
Absent	7(25)
Perineural invasion	
Present	22(78.6)
Absent	6(21.4)
KRAS mutation	
Present	19(67.9)
Absent	9(32.1)

3.2 Histopathological assessment

Histopathological review occurred and the diagnosis of PDAC was confirmed in all cases. An image of normal pancreas is illustrated on Figure 3.3 which shows normal pancreatic ducts embedded within normal acini. PDAC was confirmed by irregularly shaped malignant glands deposited within abundant desmoplastic stroma, as shown in Figure 3.4. The malignant glands demonstrated abnormal architecture in the form of incomplete lumina, close proximity to blood vessels and glands within fibro-adipose tissue. The histopathological features are depicted in Figures 3.5 and 3.6. The malignant cells showed high nuclear to cytoplasmic ratios, hyperchromatic nuclei with four-fold increase in nuclear size compared to normal pancreatic duct nuclei.

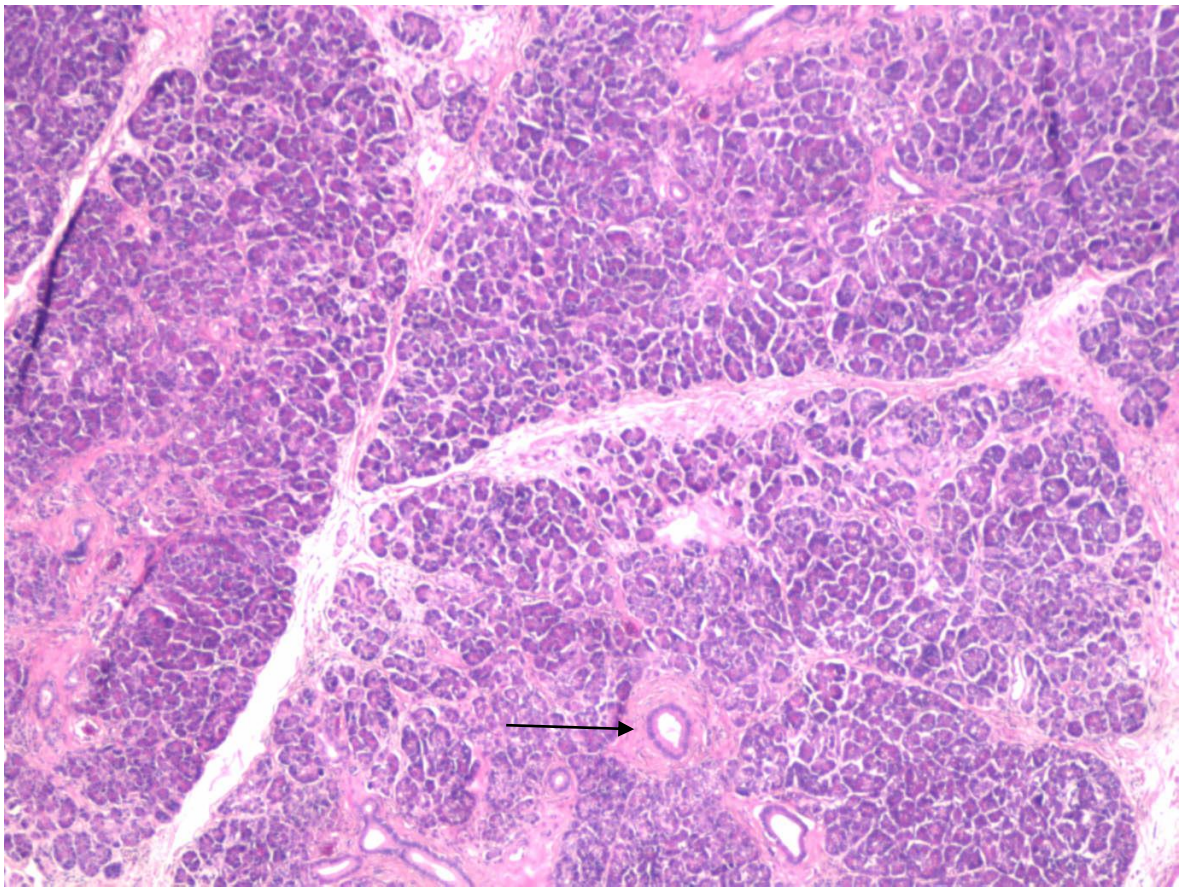


Figure 3.3 Photomicrograph demonstrating normal pancreatic parenchyma.

Normal pancreatic ducts are represented by the arrow, surrounded by acini [Haematoxylin and eosin (H&E) x100 eyepiece x objective].

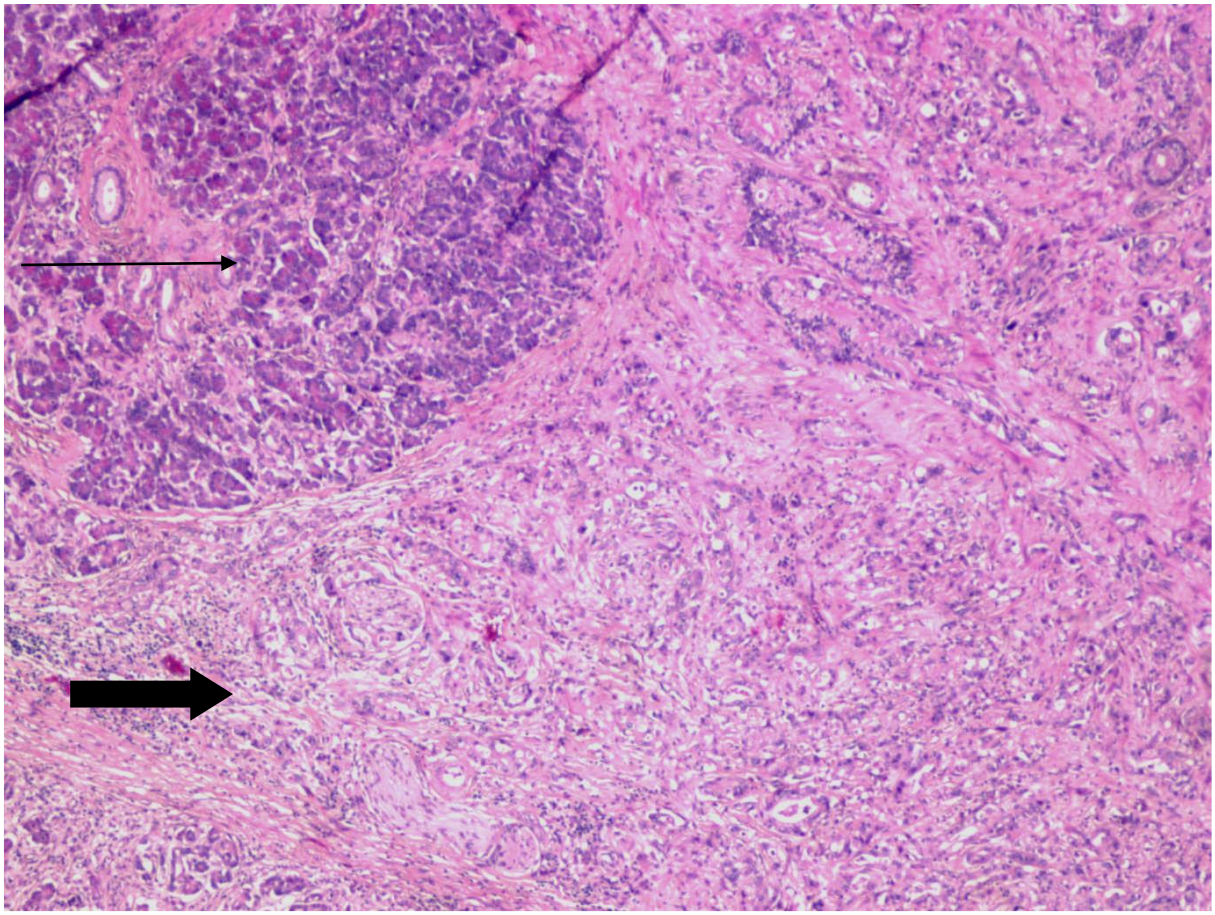


Figure 3.4 Photomicrograph of PDAC (thick arrow) with adjacent normal pancreatic parenchyma (thin arrow).

The tumour is paler compared to the normal parenchyma due to absence of normal acini and abundant desmoplastic stroma (H&E x100).

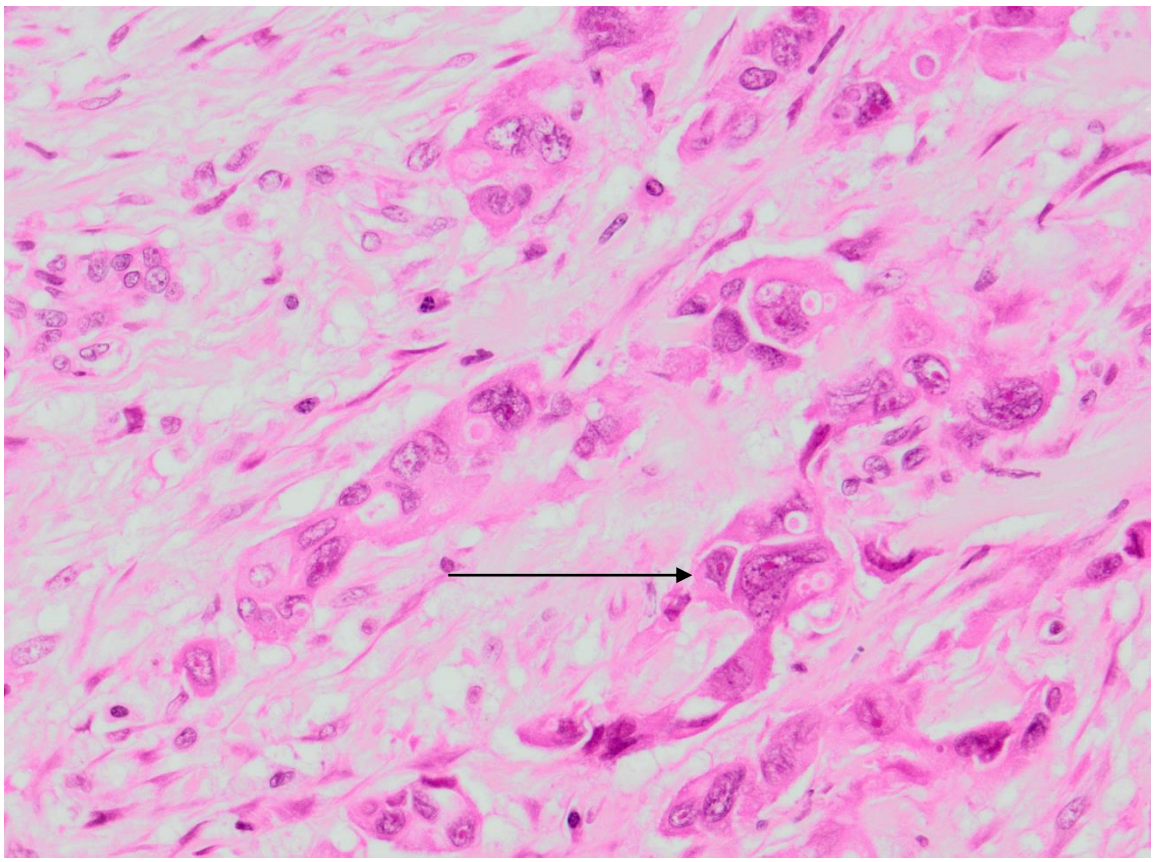


Figure 3.5 Photomicrograph of cytomorphology of PDAC.
Malignant glands are deposited in abundant desmoplastic stroma. The arrow illustrates nuclei with a four-fold variation in nuclear size (H&E x400).

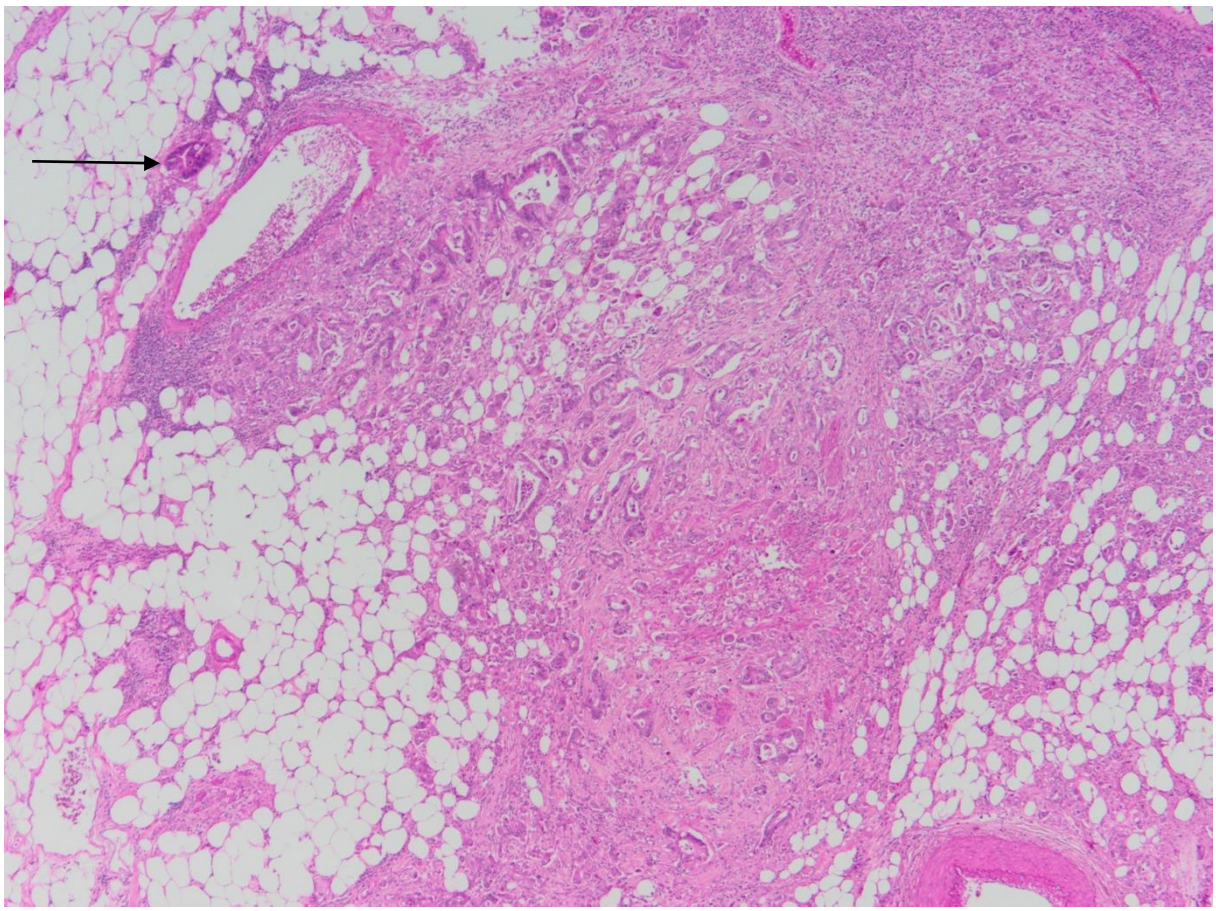


Figure 3.6 Photomicrograph illustrating an invasive pancreatic adenocarcinoma with extension into peri-pancreatic adipose tissue.
The arrow shows a malignant gland surrounded by fat, in close proximity to a vein (H&E x40).

The majority of the tumours (92.9%) were located in the head of pancreas (HOP) and only 7,1% were located on the body of the pancreas. With regard to differentiation, most tumours were moderately differentiated (60.7%), while 35.7% were poorly differentiated and 3.6% were well differentiated. The WHO tumour stages ranged from stage IB to III. As illustrated in Figure 3.7, most patients (60.7%) had stage IIB cancer, followed by 17.9% with stage IIA, and 10.7% with stage IB and III respectively.

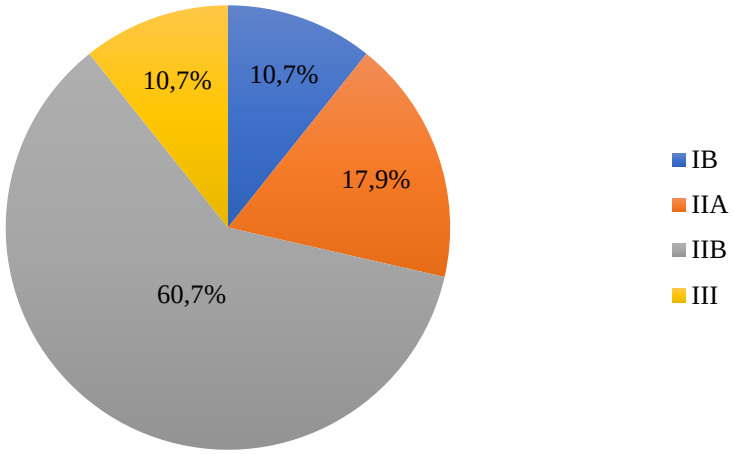


Figure 3.7 Pie chart of proportions of WHO tumour stage.

PanIN was present in 25 patients (89.3%) and absent in 3 patients (10%). Of the patients with PanIN, 8 (28.6%) had PanIN 2 and 17 (60.7%) had PanIN 3. As depicted in Figure 3.7 PanIN 2 was characterised by a pancreatic duct with papillary architecture, loss of polarity, nuclear hyperchromasia and irregularity. PanIN 3 lesions were papillary and display marked cytological abnormalities (Figure 3.9).

Lymphovascular space invasion (LVI) was present in 21 patients (75%) and absent in 7 patients (25%). Figure 3.8 shows a large vein that is completely occluded by malignant glands with residual smooth muscle wall represented at the periphery. Organising tumour thrombus is present. Perineural invasion (PNI) was present in 22 patients (78.6%) and was absent in 6 patients (21.4%). Figure 3.9 demonstrates a nerve in which malignant glands cuffing the nerve and also within the epineurium were noted.

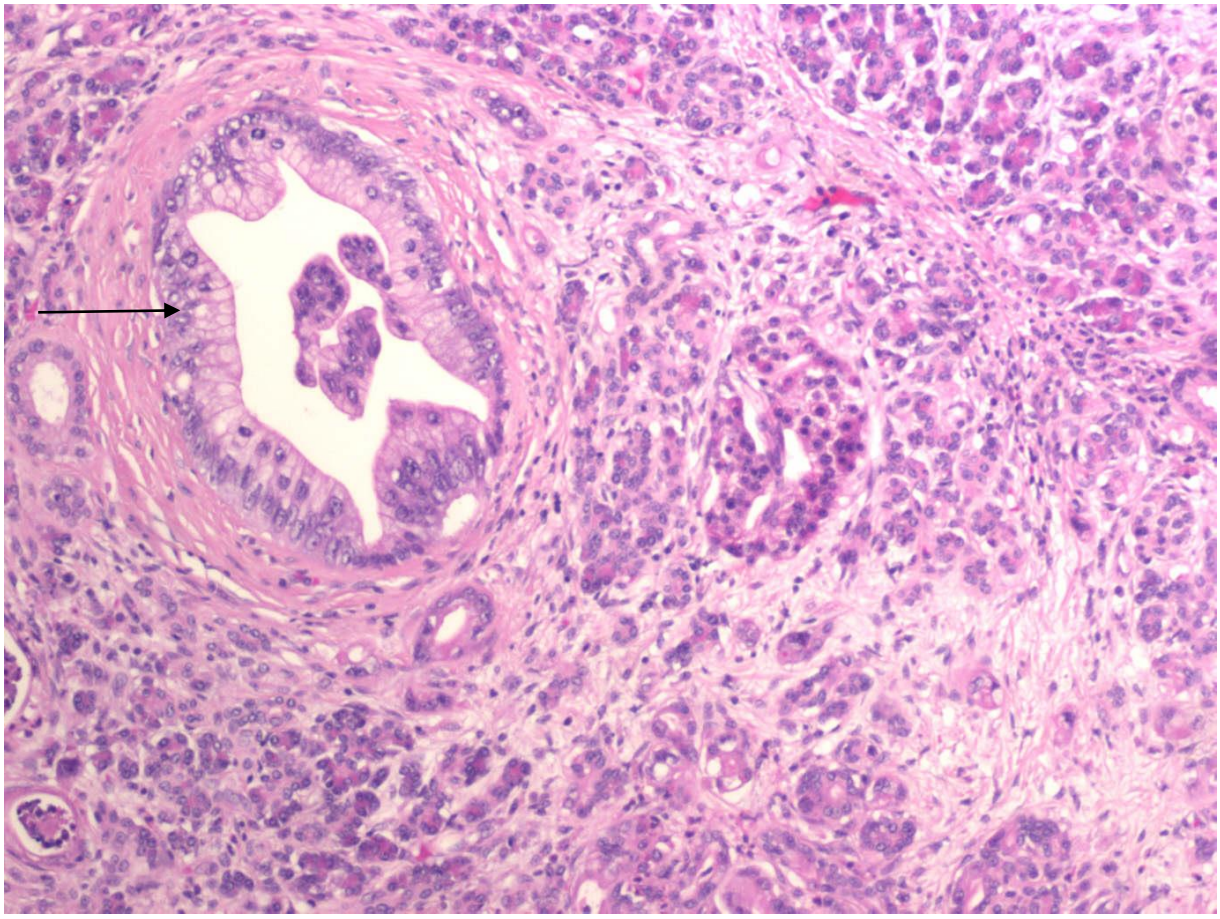


Figure 3.8 Photomicrograph of PanIN II.

The arrow is pointing to a duct with papillary architecture and loss of polarity (H&E x200).



Figure 3.9 Photomicrograph of PanIN III.
The arrow is pointing to a duct with papillary architecture, loss of polarity and nuclear hyperchromasia and irregularity (H&E x100).

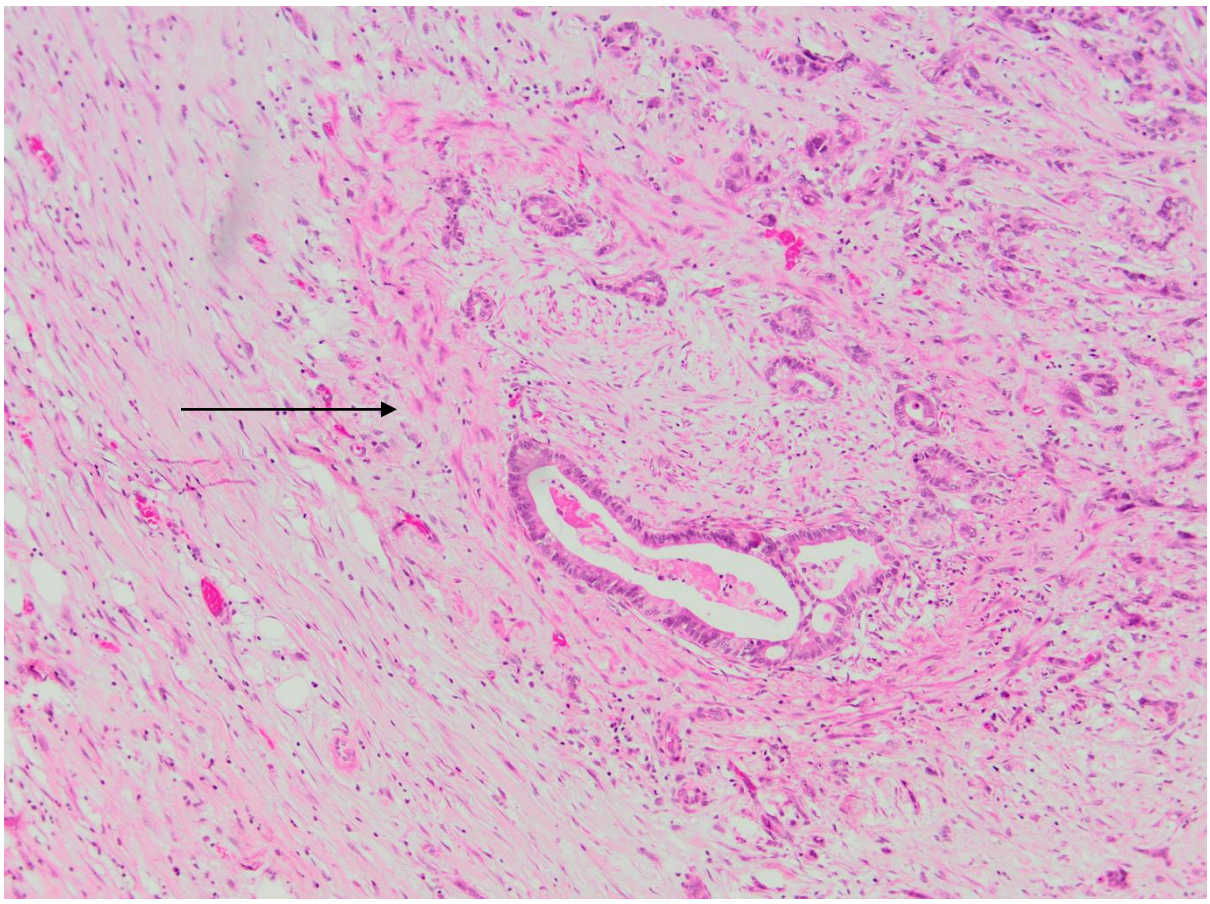


Figure 3.10 Photomicrograph illustrating lymphovascular invasion.
The arrow shows a vein that is completely obliterated by malignant glands. There are features of an organising tumour thrombus (H&E x100).

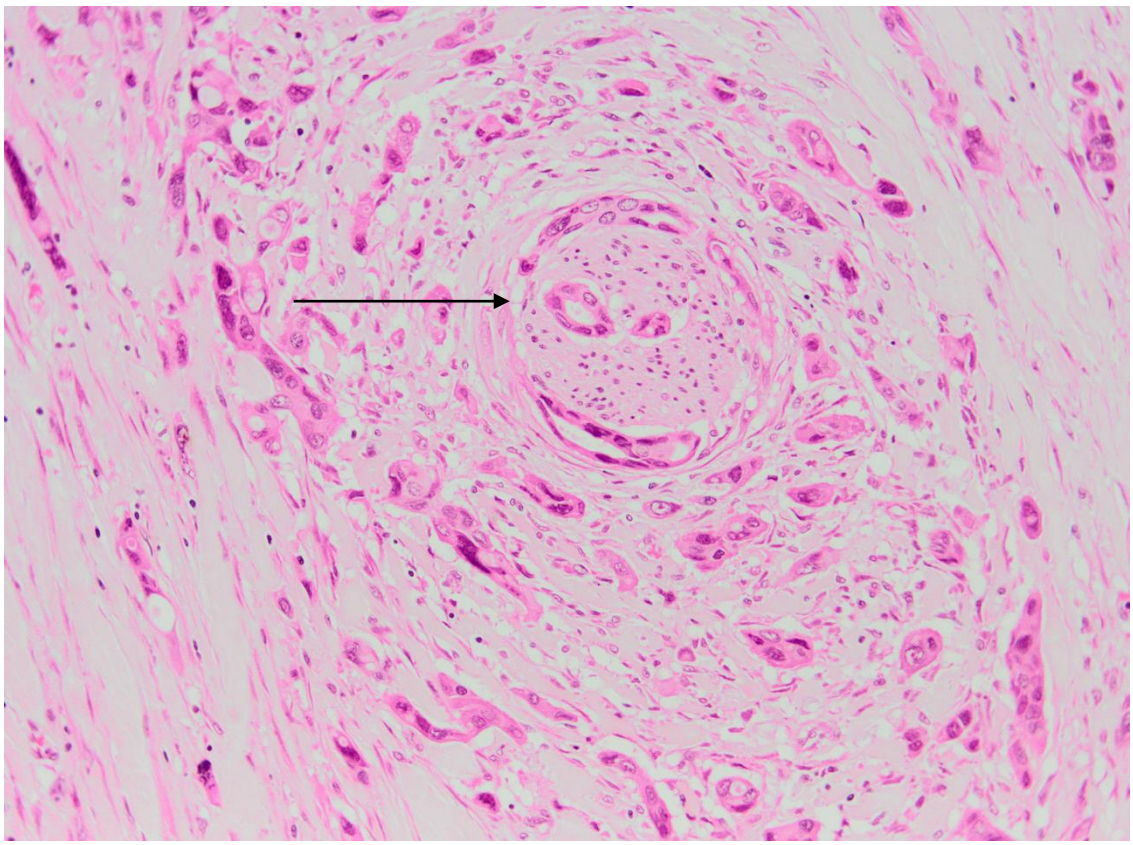


Figure 3.11 Photomicrograph of PDAC showing perineural and intraneural invasion. The arrow shows a nerve that is cuffed by malignant glands (H&E x200).

3.3 KRAS mutational analysis

Upon completion of the purification, the yield of PCR amplicons were quantified using the NanoDrop. As shown in Figure 3.12, the 260/280 ratio ranged from 1.61 to 2.64 with an average of 2.11. Only two cases had ratios below the preferred ratio of 1.8. The DNA concentrations were higher than the preferred concentration of 10 ng/µl. Despite case 7 having a lower concentration of 3.7 ng/µl, optimal sequencing results were obtained.

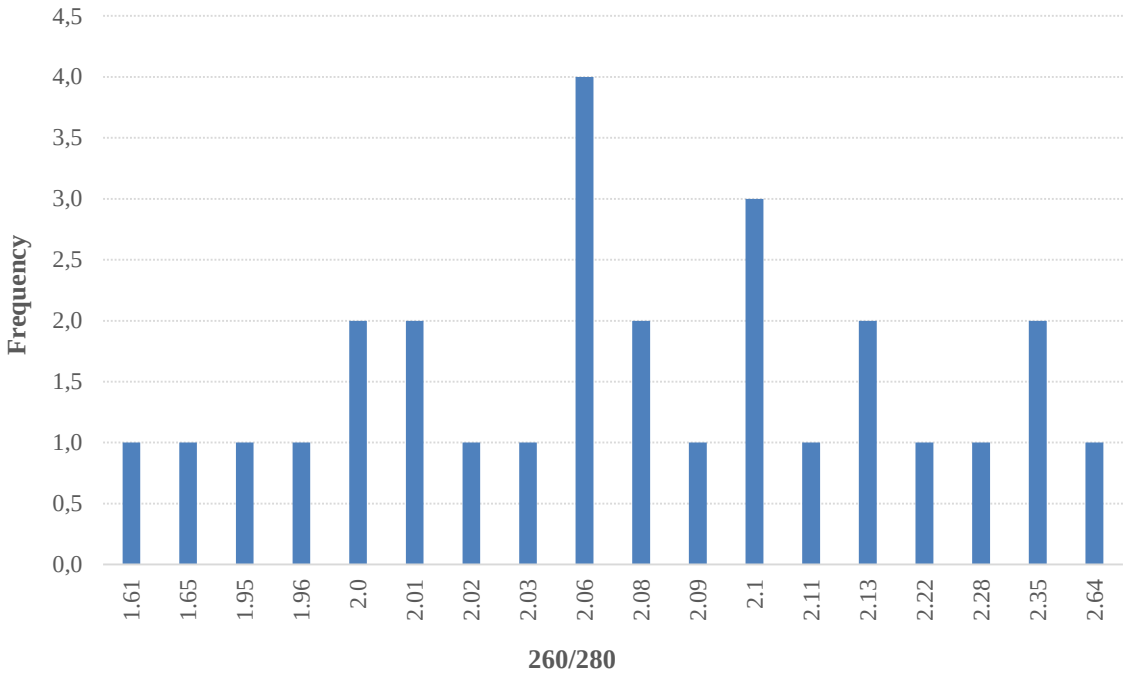


Figure 3.12 Bar graph of 260/280 Nanodrop 1000 DNA quantification

Table 3.3 DNA Extraction and Quantification log Assay:

DNA Extraction and Quantification log using spectrophotometer Nanodrop 1000

Case Number	DNA Quantification	
	260/280	ng/µl
1	2.06	112.8
2	2.22	133
3	2.13	186.9
4	2.06	376
5	2.03	195.3
6	2.1	148.7
7	2.64	3.7
8	2.35	118.5
9	2.01	173.1
10	2.06	170.8
11	2.1	117.2
12	1.61	129.3
13	2	211.6
14	2.28	119.8
15	2.13	94.9
16	2.06	263.3
17	2.11	163.3
18	2.08	150.2
19	2.08	150.7
20	2.35	118.5
21	1.96	27.1
22	2.09	187.5
23	2.01	268.7
24	2	95.2
25	2.02	97.1
26	2.1	121.9
27	1.65	16.4
28	1.95	120.7

On Gel electrophoresis, PCR amplicons were present at 173 base pairs and are illustrated in Figure 3.13. *KRAS* mutation was identified in 19 patients (67.9%) and absent in 9 patients (32.1%). The *KRAS* mutations identified were confirmed on the reverse sequence and were all on codon 12.

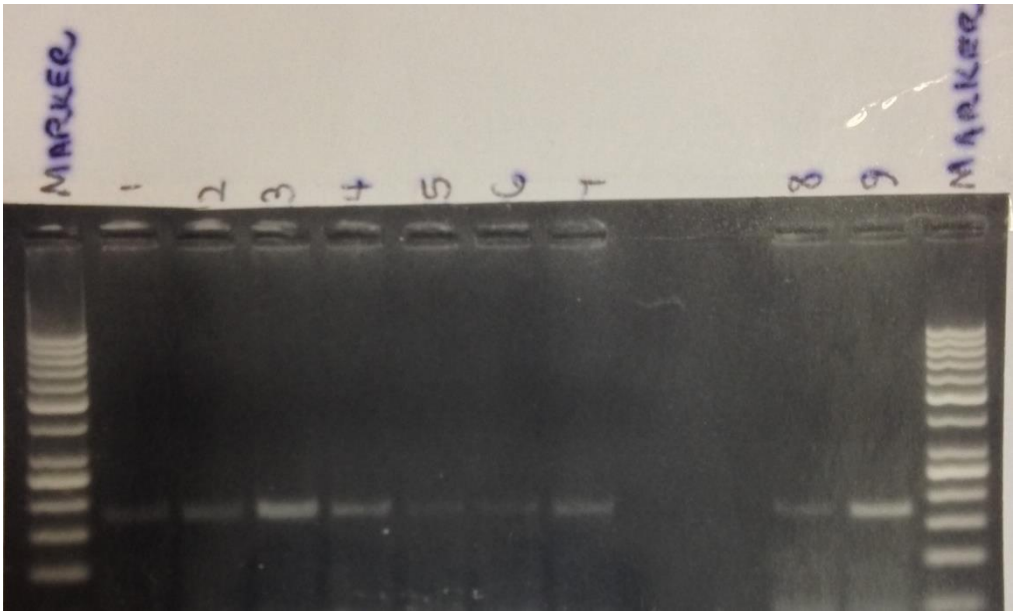


Figure 3.13 Photomicrograph of PCR gel electrophoresis of cases 1 to 9.
The unlabelled space between lane 7 and 8 represented a blank used. The molecular size of the PCR products was 173 base pairs (bp).

As illustrated in Table 3.4, four different types of codon 12 *KRAS* mutations were identified. The majority of mutations were GAT (35.7%) in which glycine (GGT) was substituted for aspartate (GAT), followed by GTT (21.4%) were glycine was substituted for valine (GTT). CGT were glycine was substituted for arginine was noted in 7.1% of cases and GCT in which glycine was substituted for alanine was noted in 3.6% of cases.

Table 3.4 KRAS mutation results of the study population

Type of mutation	Frequency	Percentage (%)	Glycine replaced by
GGT(wild-type)	9	32.1	Glycine
GAT (p.G12D)	10	35.7	Aspartate
GTT (p.G12V)	6	21.4	Valine
CGT (p.G12R)	2	7.1	Arginine
GCT (p.G12A)	1	3.6	Alanine
Total	**28**	**100.0**	

The electropherograms of the different *KRAS* sequences are represented in Figure 3.14 to 3.18. An example of the wild-type *KRAS* sequence identified in 9 cases is demonstrated in Figure 3.14. The forward sequence read GGTGCC while the reverse read ACCGCC. The black colour represented guanine, red represented thymine, blue represented cytosine and green represented adenine.

Figure 3.15 shows GAT mutation were in the forward (sense sequence) glycine (GGT) was replaced by aspartic acid (GAT). A green peak which represented adenine was noted over the second black peak, changing the sequence from GGT to GAT. Likewise, on the reverse (antisense) sequence an additional read peak (T) which represented the mutation was noted over the normal blue peak, confirming the mutation noted on the forward sequence. Correspondingly, the GCT mutation illustrated in Figure 3.16 showed a blue peak over the second black peak confirming change from GGT to GCT. The reverse sequence confirmed the mutation and read GCCAGC.

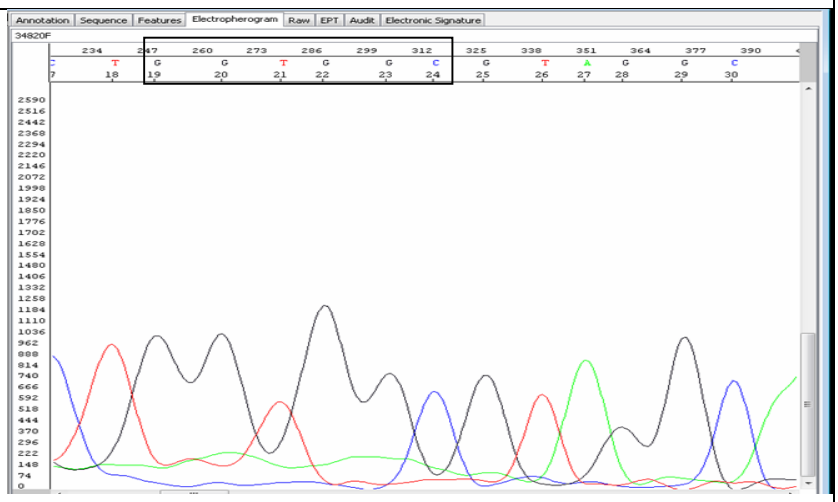
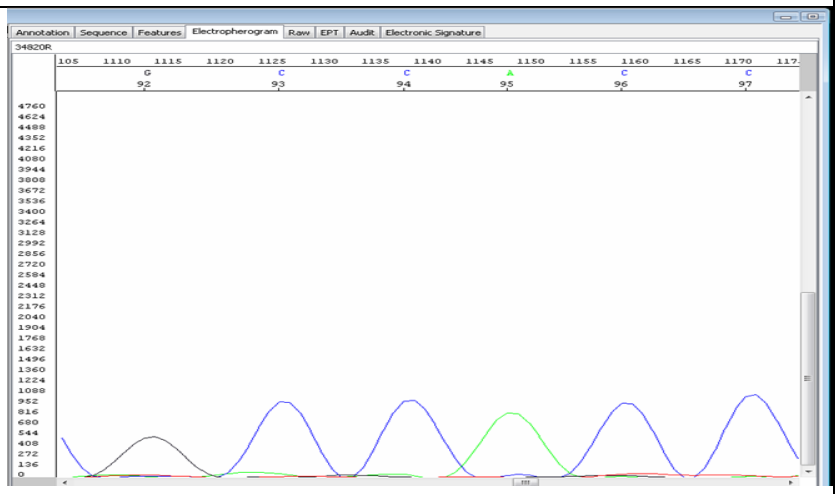
Region	Result	Electropherogram
Forward **19-24**	**No mutation**	
Reverse **92-98**	**No mutation**	

Figure 3.14 Electropherogram of the wild-type *KRAS* forward and reverse sequence.
The **forward** is the sense sequence which reads GGTGCC and the **reverse** is the antisense sequence which reads GCCACC.

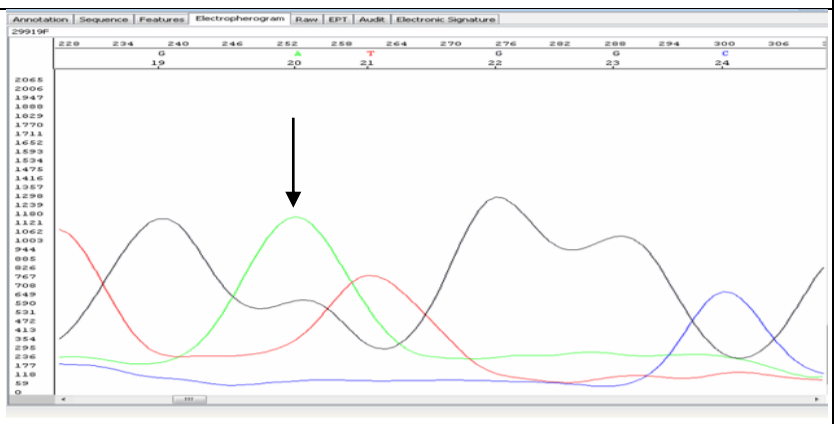
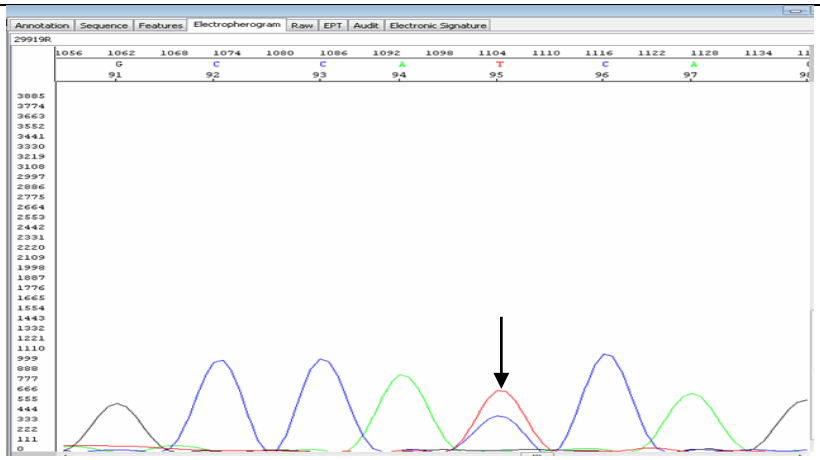
Region	Result	Electropherogram
Forward **19-24**	Mutation **GGT-GAT**	
Reverse **91-96**	Mutation **ACC-ATC**	

Figure 3.15 Electropherogram of GAT *KRAS* mutation seen in the majority of cases.
The **forward** (sense sequence) shows that glycine (GGT) is replaced by aspartic acid (GAT).
The **reverse** is the antisense sequence which confirms the mutation and it reads GCCATC instead of GCCACC.

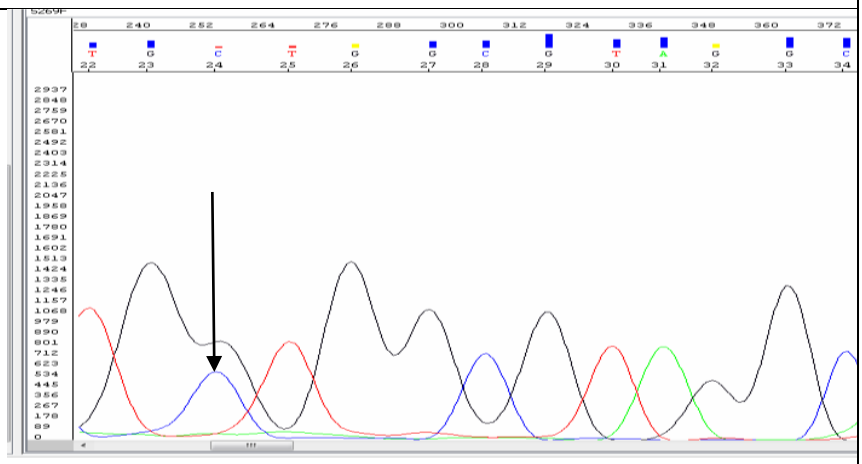
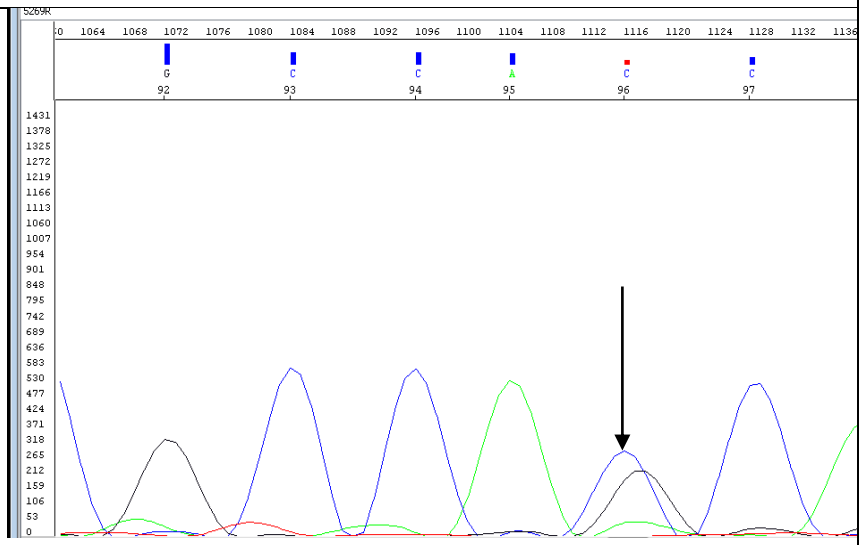
Region	Result	Electropherogram
Forward **23-28**	Mutation **GGT-G<u>C</u>T**	
Reverse	Mutation **ACC-A<u>G</u>C**	

Figure 3.16 Electropherogram of GCT *KRAS* mutation.

The **forward** demonstrates the sense strand showing substitution of glycine(GGT) for alanine (GCT) on codon 12 of the KRAS gene. The **reverse** demonstrates the antisense strand which confirms the mutation and reads GCCAGC.

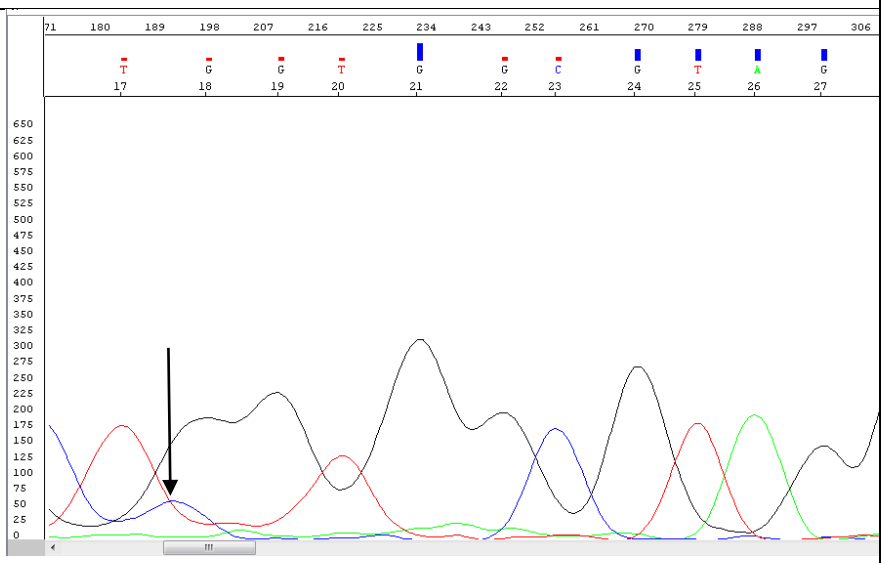
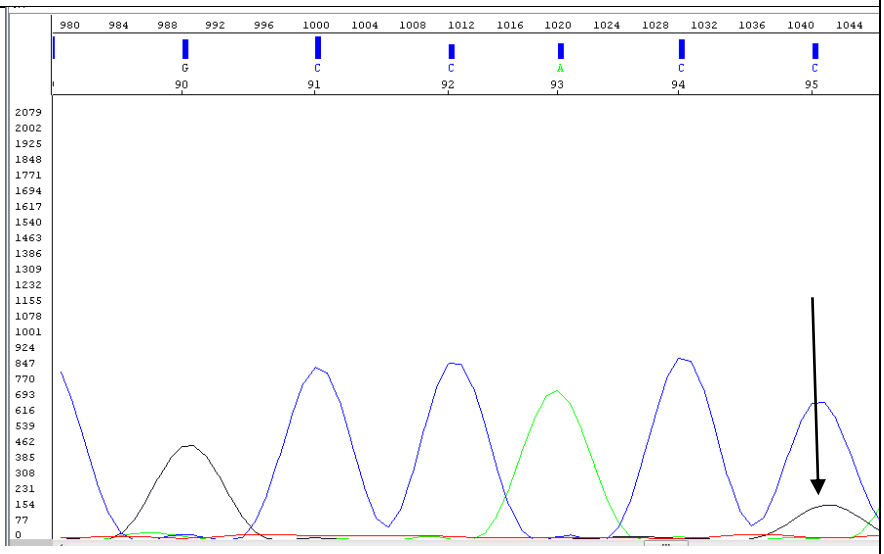
Region	Result	Electropherogram
Forward **17-24**	Mutation **GGT-CGT**	
Reverse **90-96**	Mutation **ACC-ACG**	

Figure 3.17 Electropherogram of CGT *KRAS* mutation.

The **forward** demonstrates sense strand showing substitution of glycine(GGT) for arginine (CGT) on codon 12 of the KRAS gene. The **reverse** demonstrates the antisense strand which confirms the mutation and reads GCCACG.

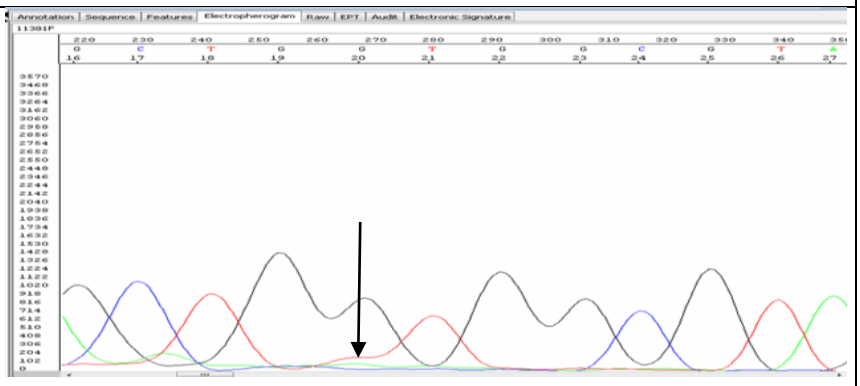
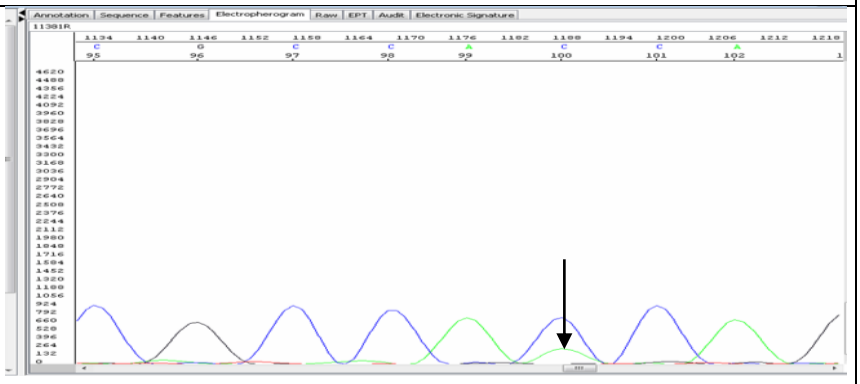
Region	Result	Electropherogram
Forward **19-24**	Mutation GGT - GTT	
Reverse **96-101**	Mutation ACC - AAC	

Figure 3.18 Electropherogram of GTT *KRAS* mutation.

The **forward** demonstrates the sense strand showing substitution of glycine(GGT) for valine (GTT) on codon 12 of the *KRAS* gene. The **reverse** demonstrates the antisense strand which confirms the mutation and reads GCCAAC.

3.4 Clinicopathological correlation of KRAS mutation status

As shown in Figure 3.19, patients with *KRAS* mutation were younger than patients with wild-type *KRAS*. On student t-test calculation (Table 3.5), the mean age of patients with *KRAS* mutation was 58.4 years (standard deviation [std. dev] 9.6) compared to 65.6 years (std. dev of 8.9) in patients without a mutation. However, there was no significant statistical significance as the p-value was 0.0596. The three patients whose ages were less than 50 years old, all had *KRAS* mutations.

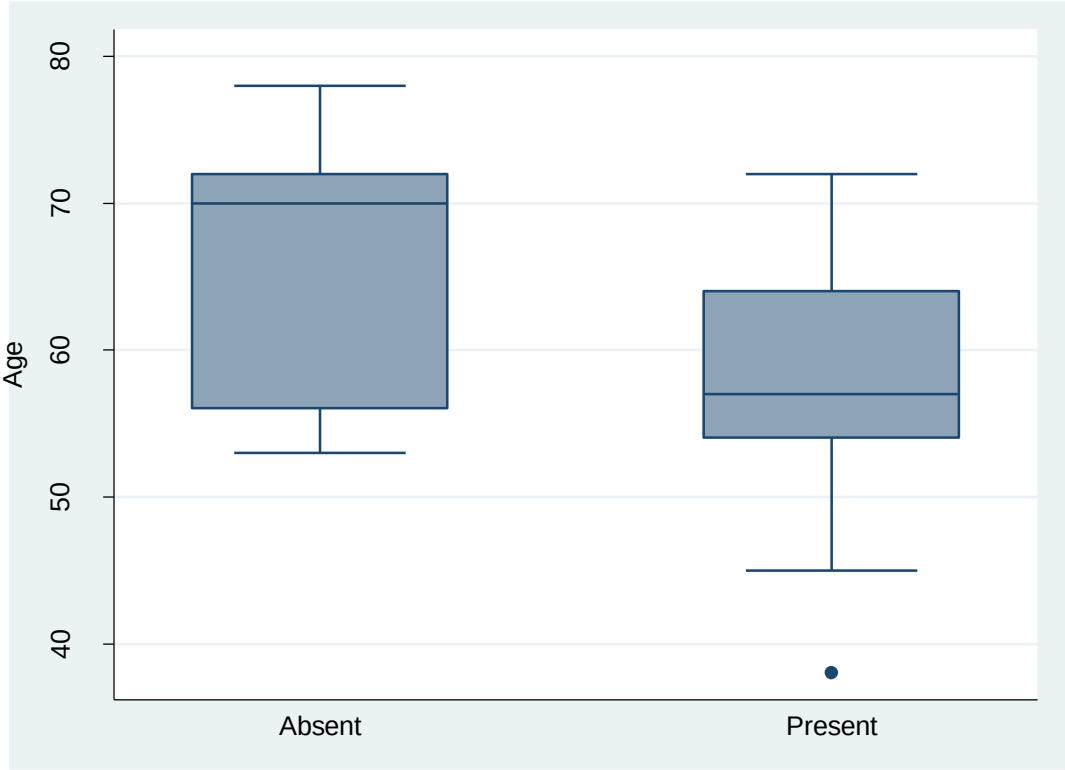


Figure 3.19 Boxplot of age by *KRAS* mutation.
The patients with *KRAS* mutation were younger than the patients with no mutation.

Table 3.5 Age by *KRAS* mutational status

Group	Observation	Mean	Standard Error	Standard deviation	[95% Confidence interval]	
KRAS wild-type	9	65.70	3.20	9.60	58.28	73.04
KRAS mutation	19	58.40	2.03	8.85	54.15	62.69
Combined	28	60.75	1.81	9.56	57.04	64.46
Difference		7.20	3.68		-0.32	14.81

The majority of female patients (72.7%) had a *KRAS* mutation compared to 27.3% with wild-type *KRAS* (Table 3.6). Similarly, a majority of male patients (64.7%) had a *KRAS* mutation while 35.3% had not mutation. On the Pearson chi2, the p-value was 0.657 hence there was no significant association between gender and *KRAS* mutational status. Likewise, there are no statistical significance between *KRAS* mutational status and site of tumour as the p-value was 0.312.

Table 3.6 Gender by *KRAS* mutation

Gender	***KRAS* wild-type (%)**	***KRAS* mutation (%)**	**Total**
Female	3 (27.3)	8 (72.7)	11
Male	6 (35.3)	11 (64.7)	17
Total	9 (32.1)	19 (67.9)	28

With regard to differentiation, the patient with well differentiated PDAC had wild-type *KRAS.* Among patients with moderately differentiated PDAC, 70.6% had a *KRAS* mutation whilst

29.4% had no mutation (Table 3.7). The proportions of patients with poorly differentiated PDAC were almost similar, with 70% having a *KRAS* mutation and 30% with wild-type *KRAS.* The p-value for grade of tumour by *KRAS* mutational status was 0.334, suggesting that there was also no association between grade of tumour and *KRAS* mutational status.

Table 3.7 Grade of tumour by *KRAS* mutation

Grade of tumour	***KRAS* wild-type (%)**	***KRAS* mutation (%)**	**Total**
Well differentiated	1 (100)	0 (0)	1
Moderately differentiated	5 (29.4)	12 (70.6)	17
Poorly differentiated	3 (30)	7 (70)	10
Total	9 (32.1)	19 (67.9)	29

The majority of patients (71.4%) demonstrating LVI, had a *KRAS* mutation compared to 28.6% with wild-type *KRAS.* Most patients (57.4%.) without lymphovascular space invasion also had *KRAS* mutation compared to 42.9% with wild-type *KRAS.* Lymphovascular space invasion was not associated with KRAS mutational status as the p-value was 0.486. Likewise, the majority of patients with perineural invasion (68.2%) had a *KRAS* mutation compared to only four patients without perineural invasion who had a *KRAS* mutation. Perineural invasion was not associated with *KRAS* mutational status as p-value was 0.944.

Of the three patients without PanIN, only one had *KRAS* mutation (Table 3.8). The number of patients with a *KRAS* mutation increased significantly with the grade of PanIN, from six patients with PanIN 2 to twelve patients with PanIN 3. Despite the aforementioned findings, there was no statistitical significant association between PanIN and KRAS mutation status as the p-value was 0.390. Likewise, the stage of tumour was not associated with KRAS mutation status as the p-value was 0.353. A summary of the frequency of KRAS mutational status by clinical and pathological parameters is illustrated in Table 3.9.

Table 3.8 Pancreatic intraepithelial neoplasia by *KRAS* mutation

PanIN	*KRAS* wild-type (%)	*KRAS* mutation (%)	Total
Absent	2 (66.7)	1 (33.3)	3
2	2 (25)	6 (75)	8
3	5 (29.4)	12 (70.6)	17
Total	**9**	**19**	**28**

Although good concentrations of DNA were obtained, direct sequencing results were too messy to read in 12 cases. Despite repeating direct sequencing on a different block of tumour, there were no readable results after sequencing. The cases that did not work were scattered throughout the years of the research period. Some cases from 2006 had very good results.

Table 3.9 Frequency of *KRAS* mutation presented by clinicopathological features

	n	%	*KRAS* wild-type (n = 9) n (%)	*KRAS* mutation (n = 19) n (%)	P value
Age(years)					0.39
<60	13	46.4	3 (33.3)	10 (52.63)	
>60	15	53.6	6 (66.7)	9 (47.4)	
Gender					0.657
Female	11	60.7	3 (33.3)	8 (42.1)	
Male	17	39.3	6 (66.7)	11 (57.9)	
Site					0.312
Head	26	92.9	9 (100)	17 (89.5)	
Body	2	7.1	0 (0)	2 (10.5)	
Differentiation					0.334
Well	1	3.6	1 (11.1)	0 (0)	
Moderate	17	60.7	5(55.6)	12(63.2)	
Poor	10	35.7	3(33.3)	7 (36.8)	
Perineural invasion					0.944
Present	22	78.6	7 (77.8)	15 (79.0)	
Absent	6	21.4	2 (22.2)	4 (21.1)	
Lymphovascular space invasion					0.483
Present	21	75	6 (66.7)	15 (79.0)	
Absent	7	25	3 (33.3)	4 (21.1)	
PanIN					0.390
Present	25	89.3	7 (77.8)	18 (94.7)	
I	0	0	0	0	
II	8	28.6	2(22.2)	6(31.6)	
III	17	60.7	5(55.6)	12(63.2)	
Absent	3	10.7	2 (22.2)	1 (5.3%)	
Stage					0.353
IB	3	10.7	2 (22.2)	1 (5.3)	
IIA	5	17.7	2 (22.2)	3 (15.8)	
IIB	17	60.7	5 (55.8)	12 (63.2)	
III	3	3	0 (0)	3 (15.8)	

CHAPTER 4

4 DISCUSSION

The current study investigated the frequency of *KRAS* gene mutations in PDAC, identifying mutations in 67.9% (19/28) of patients. PDAC is one of the most lethal malignancies, having a dismal 5-year survival rate of 5-10% even after resection (Hayashi et al., 2017; Felsenstein et al., 2018). The prognosis has not improved despite extensive research and development of targeted therapy (Liu et al., 2019). Although the only hope of treatment still remains surgical treatment, approximately 80% of patients present with metastasis and there are no early symptoms of PDAC (Partensky, 2013). Further, PDAC is resistant to current chemotherapy due to abundant tumour desmoplastic stroma (Partensky, 2013; Adamska, Domenichini & Falasca, 2017).

Studies have been performed in order to identify the main genes mutated in PDAC in the hope of producing drugs that target these genes (Lou, Subramanian & Steer, 2013). *KRAS* gene mutations have been implicated as a driver event in the development of PDAC (Smit et al., 1988). The aforementioned findings have resulted in a plethora of studies investigating the incidence of *KRAS* mutation not only in PDAC but in other tumours such as lung and colorectal adenocarcinomas (Benvenuti et al., 2007; Amado et al., 2008; Lièvre et al., 2008; Harbison et al., 2013; Shin et al., 2013; Nadal et al., 2014; Sinn et al., 2014).

KRAS gene mutations have been postulated as early event in the development of PDAC as *KRAS* mutations have been identified in 30% of PanIN 1A with progressive increase in frequency to 87% in PanIN 2 and 3 (Löhr et al., 2005). Correspondingly, *KRAS* mutations were also reported as an early event in colon carcinoma as *KRAS* mutations have been identified in adenomas and in invasive adenocarcinoma (Hershkovitz et al., 2014).

Several studies have demonstrated *KRAS* gene mutations in more than 90% of patients with PDAC (Smit et al., 1988; Hruban et al., 1993; Caldas et al., 1994; Bosman F. T., Carneiro F., Hruban R. H., 2010). As illustrated in Table 4.1, the majority of studies reported *KRAS* mutations in more than 80% of cases (Smit et al., 1988; Tada et al., 1990; Hruban et al., 1993;

Caldas et al., 1994; Schultz et al., 2012; Sinn et al., 2014; Hayashi et al., 2017). Few studies reported lower frequencies ranging from 41-68% (Shin et al., 2013; Sinn et al., 2014; Kwon et al., 2015).

Hayashi et al., (2017) detected *KRAS* mutation in 96% of cases in their study. Hashimoto et al., (2016) reported *KRAS* mutations in 92.8% of their cases, and similarly Nagawkar et al., (2016) reported high rates of KRAS mutations in 89.9% of patients in their study. In contrast lower incidences of *KRAS* mutation were reported by Kwon et al., (2015), who reported *KRAS* mutations in 42.2% cases in their cohort. Previously, Cunha et al., (2012) also reported a low frequency of *KRAS* mutations, occurring in only 41.1% of cases. Both studies postulated that *KRAS* mutations had ethnical variation with lower incidence in their population.

Similar to previous reported studies, all *KRAS* mutations in our study were identified on codon 12 (Hashimoto et al., 2016). Smit, et al., (1988) reported mutations on codon 12 of the *KRAS* in 28 of the 30 patients in their study. Hruban, et al., (1993) reported point mutations on codon 12 in 83% of tumours in their study. The high frequency of *K-RAS* point mutations on codon 12 was also demonstrated in a later study by Wei, et al., 2005. In their study, *K-RAS* mutations were noted in 83.3% (25 of 30) patients with 24 (80%) mutations identified on codon 12. *KRAS* codon 13 mutations were less common in previous studies, being reported in less than 1% of cases (Schultz et al., 2012; Shin et al., 2013). Similar to previous studies, codon 13 mutations were not identified in the present study (Oliveira-cunha et al., 2012; Hashimoto et al., 2016).

Four different types of codon 12 *KRAS* mutations were identified in our study. Likewise multiple previous studies reported four different types of *KRAS* codon 12 mutations (Smit et al., 1988; Hruban et al., 1993; Wei et al., 2005; Schultz et al., 2012; Kwon et al., 2015; Nagawkar et al., 2016). Three different types of codon 12 mutations were identified by Caldas et al., (1994), Oliveira-Cunha et al., (2012) and Hayashi et al., (2017). Slight higher numbers of codon 12 mutations were reported by Sinn et al., (2014) and Hashimoto et al., (2016) who identified six different mutations. Shin et al., (2013) reported eight different types of mutations.

The types of *KRAS* mutations identified in our study were GAT (p.G12D) in 35.7% of cases followed by GTT (p.G12V) in 21.4% of cases. Less common mutations were CGT (p.G12R) in 7.1% of cases and GCT (G12A) in 3.6% of cases. The findings of this study are in concordance with multiple previous studies shown in Table 6.1 were GAT mutations were the commonest type of mutation, ranging from 28-63% of cases (Smit et al., 1988; Tada et al., 1990; Hruban et al., 1993; Caldas et al., 1994; Schultz et al., 2012; Shin et al., 2013; Sinn et al., 2014; Hayashi et al., 2017). Similarly, p.G12D have been reported to be the most frequent type of *KRAS* mutation in colorectal carcinoma, being reported in 42.4% of cases (Kodaz et al., 2015).

Hayashi, et al., (2017) in their study noted p.G12D (GAT) mutations in 48% of their cases followed by G12V (GTT) in 32% and G12R (10%). In contrast, Kwon, et al., (2015) reported that the commonest mutation was p.G12C (TGT) in 11 cases followed by p.G12D (GAT) in 5 cases and lastly p.G12R (CGT) in two cases. Only one case had a *KRAS* mutation on codon 13. The commonest types of *KRAS* mutations reported by Wei, et al., (2005) included GTT (44%), and GAT (28%). The other reported mutations included CGT and TGT. Similar to a study by Kwon, et al., (2015) only one codon 13 mutation was identified.

Table 4.1 Frequency of KRAS mutations in PDAC in various studies.

Table 4.4 1

Study	n	Mutation%	Wild type %	Types of Mutation %									
				Codon 12*									Codon 13
				asp	val	arg	cys	ala	ser	phe	tyr	leu	
			GGT	GAT	GTT	CGT	TGT	GCT	TCT	TTT	TAT	CTG	
Smit et al., (1988)	30	93	7	32	29	4	36	0	0	0	0	0	0
Tada et al., (1990)	18	100	0	56	28	16	0	0	0	0	0	0	0
Hruban et al., (1993)	82	83	17	49	35	12	4	0	0	0	0	0	0
Caldas et al., (1994)	11	100	0	63.6[a]	36.4[a]	9.1	0	0	0	0	0	0	0
Wei et al., (2005)	30	83	17	28	44	16	8	0	0	0	0	0	4
Cunha et al., (2012)	68	41	59	25	0	21.4	46.4	0	0	0	0	0	0
Schultz et al., (2012)	170	80	20	35	27	8	0	0	4	0	0	0	0.5
Shin et al., (2013)	234	53.8	46.2	31.2	14.5	5.6	1.7	0.4	0.4	0	0	0.4	0.4
Sinn et al., (2014)	105	68	31.4	31.4	24.8	9.2	1.3	0.7	0	0	0	0	1.3
Kwon et al., (2015)	55	47.2	53	9	0	4	20	0	0	0	0	0	2
Hashimoto et al., (2016)	97	92.8	7	0	10	1.1	23.3	3.3	0	44.4	17.8	0	0
Nagawkar et al., (2016)	47	89.9	10.1	27	43	25	0	2	0	0	0	0	3
Hayashi et al., (2017)	100	96	4	48	32	10	0	0	0	0	0	0	0

[*Adapted from Hruban et al., (1993)*]

*asp, Aspartic acid; val, Valine; arg, Arginine; cys, Cysteine; ala, Alanine; ser, Serine; phe, Phenyalanine; try, Tyrosine; leu, leucine

[a]: some cases had more than one type of mutation

With regards to clinicopathological parameters previous studies reported higher incidences of PDAC were reported in males due to higher risk factors in males compared to females (Lou et al., 2019). The most important risk factor was smoking. In the current study, the majority of patients were male (60.6%) and 39.3% were female. Wei et al., (2005) reported almost similar percentages of males (60%) and females (40%) in their study. Similarly, in a study by Kim et al., (2019) most patients (63%) were male and a minority were female (37%). Nagawkar et al., (2016) also reported more males (57.4%) with PDAC than females (42.6%) in their study.

Pancreatic ductal adenocarcinoma has been reported as a disease of the elderly with a median age of 71 years (Partensky, 2013). The mean age of the study population in the current study was 60.6 years. Corresponding, the average age in a study by Wei et al., (2005) was 60 years which was similar to the current study. In a study by Hashimoto et al., (2016), the median age of the study population was slightly higher and was 69 years. Similarly, Nagawkar et al., (2016) reported an average age of 68.2 years.

PDAC is uncommon in patients less than 45 years with a reported incidence of less than 3% (Partensky, 2013). In our study only one patient (3.6%) was below age 45. It was postulated that PDAC in young patients occurs in patients who had significant history of smoking, underlying genetic predisposition and irradiated patients (Partensky, 2013). The current study did not investigate risk factors of patients.

The current study identified no statistically significant associations between *KRAS* mutation and clinicopathological characteristics examined. These findings were similar to previous reported studies (Oliveira-cunha et al., 2012; Hayashi et al., 2017). Hashimoto et al., (2016) found no association between *KRAS* mutational status with AJCC stage. Unlike in PDAC, *KRAS* mutational status was associated with clinical characteristics in colorectal adenocarcinoma, were Nadal et al., (2014) reported a statistically significant association with high tumour grade (Nadal et al., 2014).

At present, conventional chemotherapy is not effective in the treatment of PDAC. The only hope for a cure is surgery. Unlike colon and breast carcinoma, there are no targeted therapies for PDAC (Baechmann et al., 2017). Farnesyl and geranyltransferase inhibitors which directly inhibit KRAS pathway, have failed to treat patients with PDACs (Eser et al., 2014) and patients with *KRAS* mutated lung adenocarcinomas (Román et al., 2018).

Dual treatment with MAPK and PI3K inhibitors have had promising results in patients with *KRAS* mutation GAT (p.G12D) (Eser et al., 2014). If these drugs become available in South Africa, a significant proportion of patients will be treated because in our study the majority of mutations were GAT, occurring in 35.7% of patients. Gemcitabane has been reported by Baechmann et al. (2017) to have improved the survival of a 75-year-old female patient diagnosed with advanced stage PDAC in January 2011. She was alive and well in January 2016.

Since *KRAS* mutations are identified in the majority of cases of PDAC, detection of these mutations can be used as a tumour markers in specimens such as endoscopic brushings of duodenal secretions, in high risk patients (Biase et al., 2014). In the National Laboratory service, testing for *KRAS* mutations can be introduced routinely for cytology brushings to aid in diagnosis and early detection of PDAC. In tissue specimens *KRAS* mutational analysis can also be introduced routinely to identify patients who will benefit from novel therapies such as dual MAPK and PI3K inhibitors.

Currently, the best *KRAS* mutation test is next generation sequencing (NGS) as it cost effective, has a quick turn-around time, and not labour intensive. In addition, NGS has high sensitivity and large quantities of DNA can be analysed (Biase et al., 2014). Whereas DNA sequencing is labour intensive, expensive and has a significant failure rate which could negatively influence turn-around time (Biase et al., 2014). NGS is currently not available in the clinical setting and only available in academic centres only.

The limitations of this study included multiple factors. Firstly, there was a Big Dye sequencing failure rate of 30%. There were 12 cases with no readable result despite repeating the test three times on different tissue blocks and with good yield in the quality of DNA. Direct sequencing

has been reported to have low sensitivity in formalin fixed paraffin embedded tissues (FFPE) (Jancik et al., 2012; Kwon et al., 2015). In a study by Kwon, et al., (2015), direct sequencing was only successful in 55 of 72 samples whereas peptide nuclei acid-mediated PCR clamping was success in all 72 samples. A failure rate of 25% was reported for direct sequencing. Since only exon 2 mutations were investigated in this study, cases with exon 3 (codon 61) mutations were not targeted in this study. This may have resulted in fewer *KRAS* mutations identified.

Previously, Jancik, et al., (2012) reported in their study that direct sequencing missed 72% of KRAS mutations when compared to other methods. To improve sensitivity, laser microdissection was advocated. Furthermore, Schultz et al., (2012) identified 90 mutations using Thera Screen KRAS mutational kit that were missed by gene sequencing. Unfortunately, the most sensitive test currently, the droplet digital PCR is very expensive and could not be afforded in this study (Kim et al., 2019).

Microdissection could have improved DNA sequencing in the current study. The histology of PDAC in the present study showed a predominance of desmoplastic stroma compared to the malignant neoplastic cells which could have interfered with the test results. Wei et al., (2005) reported that microdissection of tumour was recommended for *KRAS* mutational analysis.

Secondly, there may have been false negative results. Some sequences may have had very low peaks and falsely interpreted as negative. In addition, Big Dye sequencing could have missed certain mutation resulting in false negative results. Negative cases could have been confirmed with another test to see if those cases were truly negative as has been detected in previous studies (Kwon et al., 2015).

Additional limitations of this study included the retrospective nature of the study. Additional clinical parameters such as risk factors and prognosis were not obtained. Lastly, very limited number of cases were included which resulted in limited statistical significance.

CHAPTER 5

5 CONCLUSION:

1. This study demonstrated *KRAS* mutations in 67.9% of patients in this cohort.
2. All mutations were on codon 12 of the *KRAS* gene.
3. The commonest codon 12 *KRAS* gene mutations were GAT followed by GTT.
4. There were no codon 13 mutations identified.
5. There was no statistically significance associated between *KRAS* gene mutation and clinicopathological characteristics.
6. DNA sequencing yielded a failure rate of 30%.

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7 APPENDICES:

ETHIC CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Sharon P Ngwenya

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M190686

NAME: (Principal Investigator)	Dr Sharon P Ngwenya
DEPARTMENT:	School of Anatomical Pathology
PROJECT TITLE:	The Prevalence of K-Ras Mutations in Pancreatic Adenocarcinoma at the Johannesburg Academic Hospital
DATE CONSIDERED:	04/05/2012
DECISION:	Approved unconditionally
CONDITIONS:	Renewal for 5 years Valid for the period 01 August 2019 - 31 July 2024 Previously M120490
SUPERVISOR:	Dr S Pather
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	03/07/2019

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **June** and will therefore be due in the month of **June** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 4 July 2019

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

KRAS REFERENCE SEQUENCE

KRAS coding DNA reference sequence number is NG_007524.1 from ncbi.nlm.nih.gov.

KRAS MUTATIONAL SCREENING:

REFERENCE SEQUENCE:

F Primer: AAGGCCTGCTGAAAATGACTG
R Primer: CAAAGAATGGTCCTGCACCAG

Forward:
AAGGCCTGCTGAAAATGACTGAATATAAACTTGTGGTAGTTGGAGCTGGTGGCGTAGGC

AAGAGTGCCTTGACGATACAGCTAATTCAGAATCATTTTGTGGACGAATATGATCCAAC

AATAGAGGTAAATCTTGTTTTAATATGCATATTA**CTGGTGCAGGACCATTCTTTG**

Reverse Complimentary:

CAAAGAATGGTCCTGCACCAGTAATATGCATATTAAAACAAGATTTACCTCTATTGTTG

GATCATATTCGTCCACAAAATGATTCTGAATTAGCTGTATCGTCAAGGCACTCTTGCCT

ACGCCACCAGCTCCAACTACCACAAGTTTATATT**CAGTCATTTTCAGCAGGCCTT**

Boxed in are codons/amino acids 12 and 13; Exon shaded

PCR Amplicon Size: 173bp

Known Mutations:

Codon 12: **G**GT > **A**GT/ **T**GT/ **C**GT (Gly > Ser / Cys / Arg)
Codon 12: G**G**T > G**A**T/ G**T**T/ G**C**T (Gly > Asp / Val / Ala)
Codon 13: G**G**C

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LETTER REGARDING TURNITIN

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03/09/2019

Re: Letter regarding Turnitin report for the MMed research by DR. S. Ngwenya 9700949K, entitled "The prevalence of *KRAS* mutation in pancreatic adenocarcinoma at the Johannesburg academic hospitals."

This letter serves to confirm that I have supervised the abovementioned research project as conducted by DR. Ngwenya.

I hereby affirm that I have perused the Turnitin report. The matched content derived predominantly from a published abstract that is based on this research report.

Yours sincerely



.....

DR. S. Pather