Prognostic Molecular Markers

In

Resected Ductal Pancreatic Carcinoma

A thesis submitted to the Faculty of Medicine of the University of Birmingham for the degree of Doctor of Medicine

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ABSTRACT

Previous studies of molecular prognostic markers following resection for exocrine pancreatic cancer have produced conflicting results. The aim of this study was to undertake a comprehensive analysis of potentially useful markers in a large multicentre patient population and compare these markers with standard pathological prognostic variables. Formalin fixed, paraffin-embedded specimens of pancreatic ductal adenocarcinoma were analysed from 157 patients (100 men and 57 women with a median [range] age of 60 [33-77] years) who had undergone pancreatectomy. Immunhistochemistry was used to detect expression of $p16^{INK4}$, p53, $p21^{WAF1}$, cyclin D1, c-erbB-2 and c-erbB-3. In a selected number of p53 positive and negative staining cases, mutational analysis was undertaken using DNA obtained from microdissected specimens. Mutations in codons 12 and 13 of the K-ras oncogene were detected by SSCP and sequencing following DNA extraction and amplification by PCR. The median [range] survival post-resection was 12.5 [3-83] months. Abnormalities of $p16^{INK4}$, p53, $p21^{WAF1}$, cyclin D1, c-erbB-2 and c-erbB-3 expression were found in 87%, 41%, 75%, 72%, 33% and 57% of cases, respectively. There was no significant correlation between expression of any of these markers and patient survival. K-ras mutations were found in 73 (75%) out of 97% cases with amplifiable DNA. The presence of K-ras mutation alone did not correlate with survival, but there were significant differences in survival according to the type of K-ras mutation (p=0.0007). Reduced survival was found in patients with GaT, cGT and GcT K-ras mutations compared to GtT, aGT and GaC mutations. In conclusion survival was associated with the type of K-ras mutation but not the expression of $p16^{INK4}$, p53, $p21^{WAF1}$, cyclinD1, c-erbB-2 and c-erbB3.

This work is dedicated to my wife and children for their love and understanding during its preparation and my weekly absence from home over many years.

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DECLARATION

I hereby declare that I personally carried out the work in this thesis. The work was undertaken in the laboratories of the university departments of surgery in Birmingham and Liverpool and at Clatterbridge Cancer Research Trust, Wirral, and Liverpool. The mutational analysis work on K-ras mutations was performed in collaboration with scientists at the department of Molecular Medicine in Norway at Haukeland University using specimens that I provided them with. This was done because they had a working protocol on K-ras mutation analysis. I carried out the rest of the laboratory work personally with occasional supervision from technical staff in the respective laboratories. I declare that this is original work that will be presented for an MD degree in Medicine at Birmingham University.

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CHAPTER 1 EPIDEMIOLOGY

1.1 Introduction

Pancreatic cancer is often a symptomatically silent disease and commonly presents at an advanced stage. The lack of currently available methods for cure emphasises the need to identify the risk factors that in turn may lead to a clue to the aetiology of this disease.

1.2 Incidence

Pancreatic Ductal Adenocarcinoma (PDAC) is the fourth commonest cause of cancer related death in the USA, accounting for just over 40,000 cases and an almost equal number of deaths (Jemal, A et al, 2009). In the USA the age adjusted mortality rate for PDAC between 200-2010 was 12.2 in a population of 100,000 in both sexes (SEER Cancer Statistics Review, 1975-2010, November, 2012). In 2008, the tumour accounted for about 8,000 deaths in England and Wales (Office For National Statistics, 2010). In a large epidemiological study of pancreatic cancer in the West Midlands between 1980 and 1984, Bramhall et al found the incidence was 10.1 per 10^5 population in men and 8.4 per 10^5 in women (Bramhall SR et al (1995). In Europe, about 30,000 cases of pancreatic cancer occur each year representing the sixth commonest cause of cancer related death (Parkin DM et al, 1999; Pisani P et al, 1999,). The incidence in the other developed countries is similar (Ahlgren JD, 1996; Swedish National Board of Health and Welfare (SNBHW), 1998; Kleeff J et al, 2000). World-wide approximately 185,000 new cases are reported annually but it is important to take into account that the percentage of cases lacking a histological confirmation varies widely, up to 85% in some areas (Parkin DM et al 1993, Porta M et al, 2000).

1.3 Risk factors for pancreatic cancer

Currently, there are no screening tests for the disease but there are a number of environmental and genetic risk factors that have been identified. Smoking has been reported to be associated with pancreatic cancer in about a third of cases in the United Kingdom (Parkin DM, 2010). Additionally, smokers have been reported to acquire the disease about ten years younger than smokers (Parkin DM, 2011). N-nitroso compounds in tobacco are postulated to be carried in the blood stream and then affect the pancreas gland (Iodice S et al, 2008). Hereditary Pancreatitis is associated with an earlier onset of PADC and where a family history also exists, the risk ratio has been reported at about fifty (Lowenfals AB, 2000). Other risk factors identified are pancreatitis (Lowenfels AB, 2000, Rebours V, 2011)), diabetes mellitus (Stolzenburg-Solomon, RZ, 2005), obesity (Aune, D et al, 2012), alcohol consumption (Michaud DSet al, 2001) and diet (Table 1.5; Larsson SC et al, 2011).

1.4.1 Genetic syndromes associated with pancreatic cancer

1.4.1.1 Familial pancreatic cancer (FPC)

This is rare, but inheritance may play a small part in its etiology. Studies have shown that about 10 per cent of patients with pancreatic cancer have a family history of the disease (Shi C et al, 2009; Permute-Wey J et al, 2009). The increased risk of pancreatic cancer in pancreatic cancer prone families is associated either as part of a known syndrome, where predisposition to pancreatic cancer is a one part of the cancer spectrum that these families develop. Alternatively it can occur as the only type of cancer that is inherited. To make a diagnosis of this syndrome, the following is required; At least two first degree relatives (parents or siblings) with the disease; One first degree relative found with the cancer at 50years or less and at least two or more second degree relatives with PDAC (Finch M et al, 1997; Bretnall TA et al, 2000; Klein AP et al, 2004). Klein and colleagues used this criteria and other data to develop a risk assessment model called PancPro (Wang et al, 2007) to assess an individual's risk.

1.4.1.2 Hereditary pancreatitis (HP) and pancreatic cancer

This is an autosomal dominant disorder with similar gender prevalence. Usually recurring abdominal pain in childhood is perhaps ignored or not fully investigated until chronic pancreatitis is diagnosed in early teens or even later in life (Finch M et al, 1997; Paolini O et al, 1998; Whitcomb DC et al, 1996). According to one report, individuals with chronic pancreatitis are many times more likely to develop pancreatic cancer when compared to the normal population (Chowdhury P and Rayford PL, 2000). In a French study from a single centre, a risk of 13.7 was reported (Madeura J et al, 1998) while an American study found a risk of 4 (Bansal P and Sonnenberg A, 1995) and a Swedish study found this to be 8 (Karlsson BM et al, 1997). There is no consensus, however with Bansal et al expressing

doubts as to whether there could be misdiagnosis of pancreatitis for cancer could (Bansal, P et al, 1995).

A lot of interest was generated when Whitcomb et al, reported a mutation in the cationic trypsinogen gene PRSS1 associated with hereditary pancreatitis with the hope that a better understanding of the disease could be explained (Whitcomb DC et al, 1996). Whitcomb et al suggested that the mutation appears to make the protein resistant to trypsin cleavage. The consequence of this process is a protein that is auto activated with inappropriate intracellular accumulation of the proteolytic enzyme trypsin and leading to digestion of the pancreatic tissue. The probable risk of developing cancer in one's lifetime has been suggested to be about 40% in elderly never smoking individuals (Lowenfels AB et al, 1997). In those individuals with non-hereditary pancreatitis, the risk of developing pancreatic cancer to the surprise of some investigators appears to be higher [4% at 20 years from the diagnosis of chronic pancreatitis (Lowenfels AB et al, 1993]. An added diagnostic problem is that inability to identify mutations in the PRSS1 gene in clinically suspected individuals does not in itself eliminate the diagnosis. In fact at least one report has stated that about a third of hereditary pancreatitis families test negative for the known mutations (Steinberg WM et al, 1999).

1.4.1.3 BRCA2 gene germline mutation and pancreatic cancer

Carriers of this mutation have a ten-fold higher risk of developing pancreatic cancer than the general population (Bretnall TA et al, 2000; Liede A et al, 2004). Mutations appear to occur often in Ashkenazi Jews (McClain, MR et al, 2005). The estimated risk of acquiring pancreatic cancer by the age of 75 is 7% in the carriers as compared to 0.85% in the general population (Ozcelik H et al, 1997). Bretnall et al have reported that loss of heterozygosity in the BRACA 2 gene is a later event in tumourigenesis. They explained this by the observation

that the pancreatic intraepithelial neoplasia lesions showed minimal to no loss of heterozygosity alone the different grades of the lesions (Bretnall TA et al, 2001).

1.5 Familial Cancer Syndromes

1.5.1 Familial atypical multiple mole melanoma syndrome (FAMMM) and CDKN2A/p16 germline mutations

This is a rare autosomal dominant inherited disorder associated with multiple atypical naevi, malignant melanomas (cutaneous or ocular) and caused by germline mutations in p16

Pancreatic adenocarcinoma is probably the second most common cancer in FAMMM families. In several chromosome 9p-linked FAMMM families, a mutation in the cell cycle inhibitor gene CDKN2A//p16/MTS1 has been found to co segregate with both melanoma and pancreatic cancer (BorgA et al, 2000). In a study among 200 members from nine Dutch families with FAMMM, the observed/expected ratio for pancreatic cancer was 13.4 to 1.0 (Bergmann W et al, 1990). Affected family members had an incidence of pancreatic cancer 29 times that of the general population. Using an in vitro assay, Goldstein et al made a distinction between FAMMM kindreds with a functionally defective p16 protein and the FAMMM kindreds without a defective p16 gene product (Goldstein AM et al, 2000). A 22-fold excess risk of pancreatic cancer (standardised incidence ratio 21.8; 95% CI, 8.7-44.8) was restricted to FAMMM families with impaired p16 function. Other reports which confirmed an increased risk of pancreatic cancer in melanoma-prone kindreds have also identified CDKN2A/p16/MTS1 germline mutations (Whelan AJ et al, 1995; Lal G et al, 2000). Not all patients with the germline CDKN2/p16/MTS1 mutations come from the classic FAMMM families. Twenty-one kindreds with familial pancreatic cancer without the FAMMM syndrome were screened for germline mutations in CDKN2A/p16/MTS1 and in the related CDK4 gene. A germline CDKN2/p16/MTS1 mutation was identified in only one family (5% of kindreds) and one of the affected carries also had a melanoma (Platz A et al, 1997).

1.5.2 Hereditary non-polyposis colorectal carcinoma (HNPCC): Lynch syndrome 11 and pancreatic cancer

The genetic abnormality in this disorder is that of inability to repair damaged DNA usually performed by the mismatch repair genes; hMSH2, hMLH1, nPMS1 and 2. Individuals with this condition are at risk of developing colon cancers, usually on the right side as well pelvic cancers in females and pancreatic cancer (Goggins M et al, 1998). Bretnall et al have reported that the actual number of hereditary pancreatic cancers that occur in this autosomal dominantly inherited tumours is less than that seen with mutations occurring in other genes, for example from the p16 and BRACA2 genes (Bretnall TA, 2000).

1.5.3 LKB1/STK11 and Peutz- Jeghers syndrome (PJS)

Peutz-Jeghers syndrome is rare and associated with numerous polyps in the gastrointestinal tract and pigmented lesions in and around the mouth. Implicated in this condition is an aberration in the LKB1/SK11 gene (Hemminki A et al, 1998, Hearle, N et al, 2006). Pancreatic cancer is also known to occur with this genetic abnormality (Hizawa K et al, 1993; Hearle et al, 2006). A review of 210 patients with Peutz-Jeghers syndrome reported that the risk of pancreatic cancer was increased 132 fold when compared with the general population (Giardiello FM et al, 2000). The exact risk of patients with PJS patients for pancreatic cancer development is unclear (Bretnall TA, 2000), however, in the report by Su et al**,** 53 PJS patients reported in four independent studies, averaged an 11% lifetime risk of developing pancreatic tumors (Su GH et al, 1999).

1.5.4 Ataxia telangectasia

This is an autosomal recessive condition with loss of function of the ATM gene. The ATM protein is essential for the signaling of DNA damage to p53 and loss may be associated with an increased risk of PDAC (Taylor AM, 1992).

1.5.5 Li-Fraumeni Syndrome (LFS)

LFS is a rare condition in which the genetic abnormality is a mutation in the p53 gene and has been reported to predispose to pancreatic adenocarcinoma (Hisada M et al, 1998).

1.6 Diabetes mellitus

The case for diabetes mellitus as a cause of pancreatic cancer has been debated since Marallie first reported this association in the late nineteenth century (Marallie et al, 1893). It is still not known whether diabetes is a true aetiologic factor or an early manifestation of the tumour (Warshaw AL et al, 1992; Anderson KE et al, 1996). Gullo et al have found that diabetes mellitus predates the diagnosis of pancreatic cancer by up to two years in more than 20% of patients (Gullo L et al, 1994) and it has also been reported that impaired glucose tolerance is present in up to 75% of patients with pancreatic cancer at diagnosis (Permert J et al, 1993). In these patients impaired glucose tolerance is characterised by peripheral resistance to high circulating levels of insulin (Permert J et al, 1993) and in cases where the primary tumour is

resected glucose tolerance improves despite a reduction in serum insulin levels (Permert J et al, 1993). The case that long standing diabetes is a risk factor for pancreatic cancer is supported by two large cohort studies analysing the association between diabetes and pancreatic cancer over 10 or more years (Chow WH et al, 1995; Calle EE et al, 1998). After an initial hospitalisation for diabetes mellitus, the patients had an increased risk of developing pancreatic cancer and this risk persisted for more than 10 years (Chow WH et al, 1995). This increased risk however was only seen in patients with non-insulin dependent diabetes or in those patients whose diabetes was diagnosed after the age of 40 years. Gapstur has recently reported on evidence for a positive dose-response relationship between post load glycaemia and pancreatic cancer mortality among individuals who were initially not diabetic (Gapstur SM et al, 2000).

1.7 Diet

Numerous studies have looked at various diets that may be implicated in pancreatic cancer but, these are varied and report a weak association (see Table 1.5).

1.8 Summary

The incidence of pancreatic cancer is rising and has a devastating mortality that almost equals its incidence. Older age groups are mainly affected uncommon below the age of 40 years and rare in children. Wide geographical and racial variations in incidence are seen. The highest rates age-adjusted rates are found in Western countries, in those of Polynesian extract and USA blacks, whilst the lowest incidences are found in India. The roles of alcohol and coffee in the pathogenesis of pancreatic cancer are controversial. The relation between diabetes mellitus and pancreatic cancer is also controversial and possibly may reflect pancreatic endocrine insufficiency secondary to the cancer rather than the other way round. Hereditary factors do play a role in pancreatic carcinogenesis and the extent of this contribution is under intense investigation. There is strong evidence implicating cigarette smoking and pancreatic cancer with a relative risk of about 2.0:1.0. Those who stop smoking for many years may have their risk of developing pancreatic cancer reduced so that this is comparable to non-smokers. Dietary habits may play a role in pancreatic cancer. Whereas a western type diet rich in animal protein, saturated and unsaturated fats increases the relative risk, a vegetarian type diet high in fruits, vegetables and pulses appears to exert a protective effect. Occupational exposure to carcinogens may play a small role in the aetiology of pancreatic cancer. The management of individuals with inherited predisposition for pancreatic cancer is controversial and is still not clear but options range from close observation to aggressive surgery. Some have suggested that screening should start at least ten years before the earliest age of onset of pancreatic cancer or when suspicious symptoms are found (Brentnall TA et al, 2000). Given the lack of more than a few hard aetiological factors that can explain the development of pancreatic cancer the future in its understanding lie in the identification of prognostic molecular markers with potential for screening, early diagnosis and the development of chemotherapeutic agents.

Table 1.1. Five-year survival rates from single institution

Institution	Authors	Year published	Overall 5-year survival rate (%)
Sloan Kettering	Geer, RJ et al	1993	24
Mannheim	Trede, M et al	1990	24
Johns Hopkins	Yeo, CJ et al	1995	21
Kyoto, Japan	Manabe, T et al	1990	14
Erlangen	Declore, R et al	1996	13
Padua	Declore, R et al	1996	12
Erlangen	Gall, FP et	1991	11
Mayo Clinic	Nitecki, SS et al	1995	6.8
Munich	Roder, JD et al	1992	6
Amsterdam	Allema, JH et al	1995	0

Adapted from Finch MD et al (1999) in " Pancreatic Diseases: towards the year 2000". C.D Johnson and C.W. Imrie (Eds).

Table 1.2. Pancreatic cancer statistics per 100,00 (Cancer Research 2010)

Pancreatic cancer sta	males	females	Persons	Country	year
New cases A year	4189	4274	8463	UK	2010
Incidence rate per 100,000	10.6	8.4	9.4	UK	
Number of deaths per year	3875	4029	7901		2010
Mortality rate per 100,000	9.8	7.7	8.6	UK	
One year survival	17.4%	19.1%	18.2%	England	2005-9
5 year survival	3.6%	3.8%	3.7%	England and Wales	2007
10 year survival	2.9%	2.7%	2.8%	England and Wales	

Table 1.3. SEER 2002-10 Age - adjusted incidence rate per 100,000 population (USA)

Race/Ethnicity	Male	Female
All races	13.9	10.9
White	13.8	10.7
Black	17.6	14.3
Asian/pacific	10.4	8.9
American Indian	11.4	10.5

Table 1.4 Genetic conditions associated with pancreatic adenocarcinoma

Disease	**(chromosome)**	
Familial Pancreatic cancer	(4q32 -34)	Lowenfels AB et al, 2000
Hereditary Nonpolyposis colon cancer	(2, 3)	Tersmette, AC et al 2001
Familial adenomatous polyposis	(5q12-21)	Murphy , KM et al,2002
Hereditary pancreatitis	(7q35)	Ghadirian, P et al, 2003
Familial atypical malignant melanoma	(9p21)	Vasen, HF et al, 2000
BRACA 2	(13)	Murphy KM et al, 2002
Peutz- Jeghers syndrome	(19q)	Su, GH et al 1999
Ataxia telangiectasia	(11q)	Maisonneuve, P et al, 2003
Li – Fraumeni syndrome	(17p13.1)	

Adapted from (Jpn J Clin Oncol 2004; 34(5). All these diseases are associated with the formation of pancreatic cancer to a varying degree.

Table 1.5. Cigarette smoking and pancreatic cancer

Reference	Year	Cohort Study	Mortality ratio
Falk et al	1988	Louisiana whites males	
		1 to 5 cigarettes/day	1.5
		16-25 per day	1.9∗
		>25 per day	2.03∗
Gold et al	1985	Baltimore both sexes	
		ever smoked	1.2 to 1.4
		never quit smoking	1.7 to 2.7∗
Heuch et al	1983	Norwegian males	1.13to 2.13
Hirayama et al	1977	both sexes	1.83
Doll and Peto	1976	British male physicians	1.6∗
Cedarlof et al	1975	Swedish males	3.1∗
		Swedish females	2.5∗
Hammond et al	1966	US men (age 45- 64 yrs.)	2.69∗
		US men (age 65 -79yrs)	2.17∗
Wynder et al	1973	US Hospital-based	
		Males	2.67
		Females	2.1
Best	1966	Canadian veterans	2.1
Kahn et al	1966	US veterans	1.83∗

∗ Significant at $p<0.05$ Adapted from Ahlgren, JD (1996).

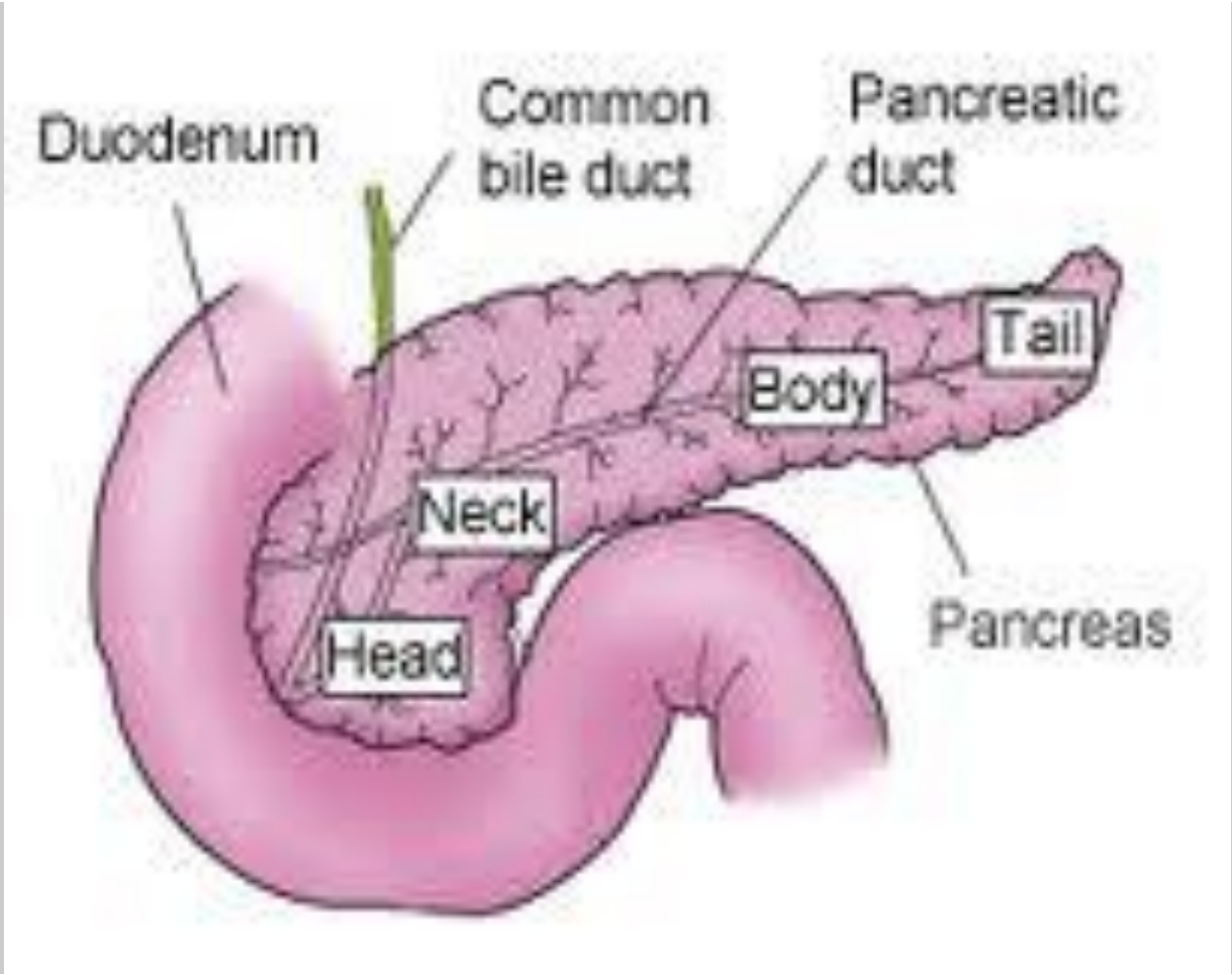
Table 1.6. Possible protective foods for pancreatic cancer

Reference	Diet
Fernandez, E et al, 1996	
Gold EB, 1995	
Shibata, S et al, 1994	
Thouez JP et al, 1990	**Fruits and vegetables**
Ohaba, S, et al, 1996	
Ji, BT, et al, 1991	
Lyon, JL, et al, 1993	
Bueno de Mesquita, HB, et al, 1990	
Falk, RT et al, 1988	
Ghadirian, P, et al, 1991	
Olsen, GW, et al, 1991	
Mills, PK, et al, 1993	
Watanapa, P, et al, 1997	**Japanese foods**
Muscat.JE, et al, 1996	**Plant protein**
Adlercreutz ,H et al, 1996	**Soy**
Stephens, FO, 1997	**Other Legumes**

Chapter 2 Pathology of pancreatic cancer

2.1 Introduction

Pancreatic tumours were first reported by Bigsby (1835) and later by Mondiere (1836). Malignant epithelial tumours account for over 90% of primary non-endocrine pancreatic tumours (Cubilla AL and Fitzgerald PF, 1978). Benign neoplasms are usually of the islet cell variety and are uncommon. Primary pancreatic sarcomas and lymphomas are occasionally reported (Kloppel G, 1984). Ductal adenocarcinoma has an extremely poor prognosis even in the resected cases, however an accurate histological diagnosis is important because there are rare types of tumours that have a good prognosis following resection. The histological type and stage of pancreatic tumours must be established at presentation to allow for the assessment of prognosis and appropriate treatment. Pre-malignant lesions are now classified by the term “pancreatic intraepithelial neoplasia” (Pan IN), mucinous cystic neoplasia (M CN) and intraduct pancreatic mucinous neoplasia (IPMN).



Anatomy of Pancreas gland

Pancreatic Intraepithelial Neoplasia (Pan IN)

The better understanding of the progression of precursor lesions to invasive neoplasia in several organs has resulted in a number of standard classification systems (Haggitt RC, 1994; Ellis IO, 1999). For example, a histologic classification system for dysplasia arising in inflammatory bowel disease established a basis for a number of clinical, pathologic and molecular studies that in turn have led to a better understanding of neoplastic progression in these patients (Rabinovitch PS et al, 1999). Knowledge of well-defined premalignant lesions of some tumours has resulted in screening of several tumours with improved outcome (Boyes DA et al, 1970; Kronborg O et al, 1996). Precursor lesions of the pancreas have previously been reported by different names e.g. lesions, metaplasia, hyperplasia, dysplasia and neoplasia (Bretnall TA et al, 1994; Brockie E et al, 1998; Klimstra D and Longneker DS, 1994). To address this problem a group of pathologists with an interest in pancreatic disease met in Utah, USA in 1999 (Kern SE et al, 2001). A modification of the Pan IN terminology originally proposed by Klimstra and Longnecker (Klimsrta D and Longnecker, 1994) was agreed upon (Figure 2.1). This new terminology took into account the growing evidence to support a progression model for pancreatic cancer. The classification system is based on clinical and morphologic observations and the evidence available from molecular genetic studies of duct lesions and their associated infiltrating adenocarcinomas (Caldas C et al, 1994; Digiuseppe JA et al, 1994; Day JD et al, 1996; Goggins M et al, 2000). The stepwise nature of the grading system correlates with mutational events found in duct lesions such that the genetic alterations in the higher grades of Pan IN approach those found in the infiltrating adenocarcinomas (Hruban RH et al, 2000, Hruban , RH et al, 2004, Singh, M et al, 2007)). The system also applies to duct lesions that involve the smaller calibre ducts and takes into account that some ducts may have changes of more than one Pan IN grade. In these cases, it was agreed that the lesions should be graded on the highest component of the lesion.

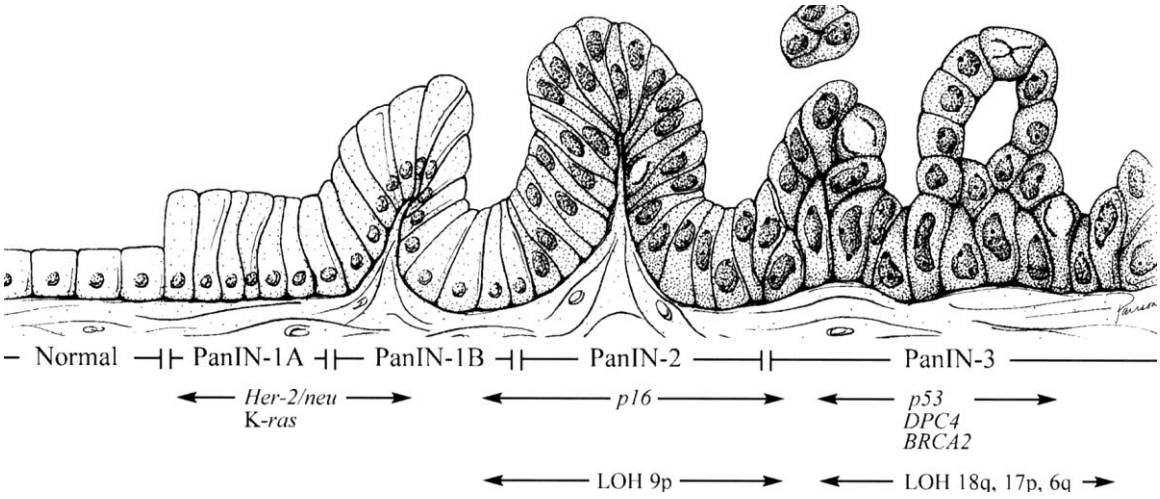


Figure 2.1. Progression model for pancreatic cancer (Wilentz RE et al, 2000). The progression from low grade Pan IN to a higher grade Pan IN is associated with acquisition of genetic changes.

2.3 Classification of pancreatic cancer

Human pancreatic tumours are classified as exocrine or endocrine with the former further classified according to the UICC/TNM classification (Sobin LH and Wittekind Ch, 1997).

2.3.1 Ductal carcinoma of the pancreas

PDAC is believed to arise from the ductal epithelium of the pancreas (Cubilla AL et al (1976); Kozuka S et al (1982), although some studies suggest a possible origin from transdifferentiated pancreatic acinar cells (Longnecker DS et al, 1980). Alternatively, a hamster model has suggested that the cell of origin may actually derive from the endocrine portion of the pancreas, or even multipotent stem cells (ref). Carcinoma of duct cell origin is the most common malignant tumour of the pancreas and the general term 'carcinoma of the pancreas' refers to this. Sixty-five percent of carcinomas are located within the head of pancreas. The remaining tumours are situated within the body (15%), tail (10%) or multiple subsites within the pancreas (10%). Tumours of the body and tail are usually clinically silent until large and usually present late with severe back pain, anorexia and dramatic weight loss. When they typically obstruct the common bile duct and present with obstructive jaundice often before distant spread has occurred.

Macroscopically, the tumour is usually a grey white, schirrous, nodular growth that is poorly demarcated with irregular edges. The cut surface may show scattered red or brown areas of necrosis or haemorrhage and locules of turbid grey or brown liquid may be present. In most cases, obstruction and dilatation of proximal main pancreatic duct is present resulting in

secondary chronic pancreatitis with atrophy in the remainder of the gland. In such cases the tumour margins can be indistinguishable and it is often difficult to differentiate between a carcinoma and chronic pancreatitis. Mucinous non-cystic adenocarcinoma of the pancreas appears grossly mucinous and sometimes the tumour may form a papillary mass within an extremely dilated main pancreatic duct presenting as an intraductal carcinoma.

2.3.2 Histological features

Over 80% of exocrine pancreatic cancers show ductal differentiation and are termed pancreatic ductal adenocarcinoma (PDAC). They are characterised by the presence of glandular structures or in poorly differentiated cases, a cord-like or diffuse pattern. Glands are of a variable size and shape with columnar epithelium and irregular nuclei. Mucin is produced in a variable amount. With poorly differentiated carcinomas signet-ring cells with intracytoplasmic lumina occur and a sarcomatous pattern may be seen. Frequently there is an intense desmoplastic stromal reaction. They must be distinguished from carcinomas of the intrapancreatic bile duct, ampulla of Vater or duodenal mucosa that are frequently resectable and tend to have a better prognosis.

2.3.3 Variants of pancreatic ductal adenocarcinoma (PDA)

There are several uncommon tumour types, which may be considered to be variants of PDAC.

2.3.3.1 Squamous and adenosquamous cell carcinomas

These tumours account for 1% and 2% of cases respectively and contain purely squamous or a mixture of adenocarcinoma and squamous components. Both types are generally more aggressive than PDAC.

2.3.3.2 Small cell carcinomas

Occur in approximately 1% of cases and are composed of sheets of small basophilic cells, which may result in this tumour being mistaken for lymphoma. Small cell carcinoma cells however, demonstrate typical neuroendocrine features including positive staining for calcitonin and chromogranin a similar to that seen in small cell carcinomas of the lung. These tumours have an extremely poor prognosis.

2.3.3.4 Pancreaticoblastoma /Infantile tumours

These tumours are an extremely rare variant of PDAC seen predominantly in infants and children under the age of seven years. Macroscopically, cystic changes may be observed. This tumour is composed of epithelial tissue with acinar cell differentiation, squamoid cell nests and occasional neuroendocrine cells, sometimes with a pronounced mesenchymal component and has a more favourable prognosis.

2.3.3.5 Acinar cell carcinoma of the pancreas

Acinar cell carcinomas are most commonly seen in elderly patients and are rare accounting for 1-2% of cases. Grossly, they are large, soft tumours with areas of necrosis. Microscopically, they are characterised by typical groups of polygonal acinar cells with abundant eosinophilic granular cytoplasm in which 1-amylase, lipase, trypsin and chymotrypsin can be demonstrated by immunohistochemistry. High serum lipase levels may be seen and may result in subcutaneous fat necrosis. In poorly differentiated tumours, poorly formed acini can be misdiagnosed as endocrine tumours. Very rarely, cystic appearances are observed known as acinar cell cystadenocarcinoma. Some cases are associated with the paraneoplastic syndrome characterised by polyarthralgia, subcutaneous and interosseous fat necrosis and eosinophilia associated with an extremely poor prognosis.

2.4 Peri-Ampullary Tumours

Ampullary epithelial neoplasms include benign (5%) and malignant (95%) tumours and account for up to 36% of surgically resectable pancreaticoduodenal tumours (Yamaguchi K and Enjoji M 1987). Peri-ampullary tumours include tumours of the ampulla of Vater the intra-pancreatic bile duct, duodenal tumours involving the papilla and tumours of the head of the pancreas involving the ampulla. It is very important to distinguish peri-ampullary cancers from carcinomas of the pancreas as a larger proportion are resectable and the prognosis tends to be better (Yamaguchi K and Enjoji M 1987, Talbot I et al 1988).

Macroscopically, tumours may be classified as; intramural protruding, exposed protruding and ulcerated (Yamaguchi and Enjoji 1987). Initially peri-ampullary tumours spread locally and are often small when diagnosed as early compression of the distal bile duct results in jaundice. Resectability rates are therefore much higher, approximately 80% when compared with ductal carcinoma of the pancreas (Ihse I et al, 1993). Histologically, adenomatous areas are often observed in the majority of malignant tumours and similarly foci of carcinoma frequently observed within adenomas, which has led to hypothesis that an adenoma-carcinoma sequence may exist (Scarpa A et al 1994). Kimura and Ohtsubo (1988) however suggest alternative pathogenetic mechanisms for the development of benign and malignant ampullary tumours as a proportion of cancers do not show any detectable adenomatous areas.

2.5 Cystic tumours of the Pancreas

There are four different types of these tumours with relatively similar features on the cell of origin, biology and on imaging. The tumour arises in any part of the gland and is slow growing usually attaining a large size before detection. Cystic tumours represent a particular diagnostic problem and the differentiation between pseudocysts, serous (usually benign) and

mucinous (potentially malignant) tumours is difficult but very important, as their management is different. Patients usually present with an abdominal mass although they may present with pressure effects for example obstructive jaundice. Management is by surgical exploration and resection if technically feasible.

2.5.1 Serous cystadenomas

Serous cystadenomas are glycogen rich microcystic adenomas that are usually benign. They may be associated with other neoplasms and chronic disorders, the best recognised of which is the Von-Hippel Lindau syndrome. Histologically they are well-circumscribed multicystic tumours most commonly seen in the proximal pancreas, which have a mean diameter of 7 centimetres and a range of 2-25 centimetres. Macroscopically serous cystadenomas consist of a collection of small cysts sometimes microscopic in size, lined by a low cuboidal epithelium. Microcystic areas are separated by vascular fibrous stroma, which may be calcified producing a sunburst or stellate pattern on CT. The epithelial cells are rich in glycogen and no mitotic activity, cellular atypia or dysplasia is observed within the epithelium. Routine staining for mucin, chromogranin, neuropeptides, enteroendocrine peptides and carcinoembryonic antigen (CEA) are all routinely negative (Sarr MG et al, 2001). Almost all serous cystadenomas are benign and follow a benign course over many years although malignant change is occasionally seen (Pyke et al 1992; Kamei et al 1992). They are composed of multiple small (<2cm) cystic areas that resemble a bunch of small grapes and appear like a honeycomb-like appearance grossly and on imaging. A starburst appearance with a centrally located calcified scar caused by a calcification of the central fibrous stroma is virtually pathognomonic but occurs in only about a third of patients. The characteristic findings of stromal hypervascularity with predominance of small cystic areas, combined with an indolent course, lack of metastases or local invasion and an appropriate

clinical setting, allows the diagnosis of serous adenoma to be made with 95% accuracy. There are few reported cases and management should be conservative in most cases.

2.5.2 Mucinous Cystic Neoplasms

Mucinous cystic neoplasms are very rare and represent a broad spectrum of related neoplasms ranging from benign cystadenomas with a real potential for malignant transformation to cystadenocarcinomas with an aggressive metastatic potential (Sarr MG et al, 2000). A fifth of patients may have a history of pancreatitis while the rest are found during investigation for non-specific abdominal symptoms that usually are from a local mass effect. It is important to differentiate this tumour from other cystic disorders because of its premalignant potential. The presence of papillary fronds or septae with the cystic structures or an eccentric solid component within the wall of the cystic mass on ultrasonography or CT scanning is pathognomonic. When present, calcification is peripheral unlike the central location with serous cystadenomas.

Macroscopically, mucinous cystic neoplasms are different from their benign serous counterparts as cystic areas are often larger (>2cm) and contain less than six separate cysts. Most mucinous cystic neoplasms appear as multilocular tumours with smooth glistening surfaces that vary from a few centimetres to 25cm in diameter with a mean size of 8-10 cm. The cyst lumen often contains a thick sometimes-bloodstained mucoid material. Some are unilocular and some may contain large whitish nodules, which project into the lumen. The malignant tumours are usually larger than the benign ones; show a marked female preponderance and approximately two-thirds are located within the body and tail of the pancreas.

Microscopic examination of the wall of the cyst reveals three distinct layers: an inner epithelium, a middle zone of mesenchymal stroma and an outer layer of hyaline connective tissue. They are lined by a tall mucin secreting columnar epithelium, the cells of which often stain positive for CEA and somatostatin supporting their origin from ductal or stem cells. These neoplasms are notorious for containing multiple areas of discontinuous epithelium and differing degrees of differentiation and dysplasia within the mucinous epithelium. Within the same tumour, variations within the spectrum of cystic neoplasms may be seen with areas of atypia, dysplasia, carcinoma in situ and overt invasive carcinoma (Warshaw A et al 1990). Albores-Saavedra et al (1990) considered true benign mucinous tumours to be very rare, but difficulties in interpretation arose as the tumour may have a single focus of malignant invasive epithelium with the remaining areas appearing benign. Therefore a simple biopsy of the wall of a presumed mucinous cystic neoplasm may not be adequate to make the diagnosis or to exclude malignant transformation.

Some reports from the Mayo Clinic (Sarr et al, 2000) and Johns Hopkins Hospital (Wilentz RS et al, 1999) support an aggressive resection approach to tumours and more importantly shed new light on the natural history of mucinous cystic neoplasms suggesting that they are premalignant conditions, but this has not gained wide acceptance (Sarr et al, 2000). Sarr et al (2000) have proposed three groups of these tumours. Mucinous cystadenoma (65%) that are benign, proliferative cystic mucinous neoplasms (30%) with some dysplasia and mucinous cystadenoma (<10%) that have stromal invasion.

2.6 Intraductal papillary mucinous tumour (IPMT)

IPMT represents a spectrum, which varies from a generalised dilatation of the main pancreatic duct to a more segmental dilatation of the pancreatic ducts (Hruban et al, 2004). This tumour has been referred to by various names including mucin hypersecreting tumour

(Furukawa T et al 1992), intraductal mucin hypersecreting neoplasm (Rickaert F et al, 1991), mucin producing tumour (Yamada M et al 1991)**,** intraductal papillary neoplasm (Conley CR et al 1987), intraductal mucinous papillary tumour (Nagai et al, 1995) and duct ectatic mucinous cystadenoma (Itai Yet al, 1986). The incidence appears to be rising as reported in some series and this may be due to an increased awareness of the condition (Sugiyama M et al, 1998; Traverso LW et al, 1998).

This tumour is found most commonly occurs within the head of the pancreas and produces thick viscid mucus resulting in dilatation of the main pancreatic duct. Approximately 30-50% of patients have associated malignancy within the areas of mucinous ductal ectasia strongly suggesting that mucinous dysplasia is premalignant in a similar way to mucinous cystic neoplasms of the pancreas. The tumour grows along the duct before invading the parenchyma of the gland and distant metastases may occur. Microscopically IPMT consists of mucinous cell dysplasia of the dilated ductal segment but a spectrum of changes from mucinous hyperplasia to severe dysplasia to carcinoma in situ to invasive cancer may be observed in different segments of columnar epithelial cells. Lesions of IPMT cause pain probably because the mucin that is produced or papillary growth results in ductal obstruction. Clinically, IPMT usually presents with symptoms suggestive of idiopathic chronic pancreatitis but in an older age group (mean, 68 years) and usually with the similar risk factors (Sugiyama M et al, 1998; Traverso LW et al, 1998). Jaundice secondary to ductal obstruction and pancreatitis are common in mucinous papillary tumours of the pancreas and prompt surgical treatment results in good results (Tibayan F et al, 2000). In a study of 33 cases, Traverso et al (1998) found that malignancy is common (42%) and there was an association with alcohol abuse and jaundice. The prognosis was found to be better than in pancreatic cancer but about a fifth of patients developed recurrence of symptoms in residual pancreatic remnants and this was in both benign and malignant cases.

2.7 Grading of pancreatic tumours

Tumour grading is a histological method for determining the tumour's inherent biology and predicting its aggressiveness. A major problem in grading pancreatic tumours is that the degree of differentiation is often variable in different parts of the tumour. The amount of gland (duct) formation and mucin production is the basis of grading (Kloppel G et al, 1982) but this is subjective. Well-differentiated tumours display a high degree of gland formation while poorly differentiated tumours show little. Several studies have shown a correlation between histologic grade and survival by both univariate analysis and multivariate analysis (Eskelinen M et al, 1991).

A semi quantitative system for the histological grading of pancreatic ductal adenocarcinoma was described by Kloppel et al (1985) in which the entire neoplasm was classified according to the highest grade of its components, regardless of their prevalence (Table 2.2). Most recently, Luttges J et al (2000) studied the possibility of supplementing tumour grade with proliferative activity as assessed by immunohistochemistry. They found that either the number of mitoses or the growth fraction do not seem to discriminate tumours of varying prognosis. The traditional cellular or structural differentiation appears to be superior particular on low power magnification over irregular glands (Lutteges J et al, 2000).

The Japanese grade tumors according to the most predominant structures histologically (Japanese General Rules for Surgical and Pathological Studies in Cancer of the Pancreas (1996). In contrast however, the UICC recommends that PDA should be assigned the least favourable grade of G1-G4 (Table 2.4) and includes carcinomas with undifferentiated areas

next to areas of glandular differentiation. The International Pancreatic Cancer Study Group, (IPCSG) recommends that in addition to the predominant grade observed, any additional grades present should also be stated and it is therefore essential that sections from different parts of the tumour be examined to enable an accurate assessment of tumour grade (Pour P et al 1994). Histologically well-differentiated tumours have small acini and a fibrous stroma and may be difficult to distinguish from chronic pancreatitis. Perineural and vascular invasion are common, diagnostic of malignancy and associated with a poorer outcome. Although there is a case for a more objective grading system, grading itself is of relatively low prognostic value compared with other predictors of outcome such as stage

2.8 Staging of Pancreatic Tumours

Staging is classification of the anatomical extent of a tumour (Sobin LH, 2001). After the tumour is removed this assessment is further extended by considering the observed (R0) or the extent (R1/R2) of the residual tumour (Hermanek P and Wittekind C, 1994) as shown in (Table 2.3). Clinical stage and the individual staging variables e.g. tumour size and lymph node status have also been separately associated with prognosis (Eskelian MJ et al, 1999). In most specialist units pre-operative staging is routinely carried out by spiral CT scanning, endoscopic ultrasonography, diagnostic laparoscopy and laparoscopic ultrasound (Durup-Scheel-Hincke J et al, 1999). Pierre Denoix working at the Institute Gustave Roussy in France introduced the TNM classification between 1943 -1952. In pancreatic cancer, the TNM classification relies on tumour size (T), lymph node involvement (N) and the presence of distant metastases (M). This staging system has since has undergone several modifications. The present UICC TNM classification of adenocarcinoma of the pancreas (Sobin LA and Fleming ID, 1997) comprises four stages. Stage I represents tumours with no extra pancreatic spread (T1, N0, M0); stage II those tumours with direct extension into

surrounding structures (T2 or T3, N0, M0); stage III any tumour with regional lymph node metastases (N1-3, N1, M0); and stage IV those tumours with distant metastases (T1-3, N0-1, M1).

The UICC T category considers tumour size, peripancreatic extension and venous invasion while the Japanese T considers tumour size only. The latter is supplemented by a separate S (serosal invasion), Rp (retro peritoneal invasion), and V (venous invasion) classifications. In the UICC system the first and second lymph node stations only are considered as regional lymph nodes while the Japanese include other intra-abdominal nodes. This staging system emphasises lymph node involvement causing a tumour of whatever size and degree of loco-regional infiltration to be classified as stage II. Loco-regional infiltration of the tumour is underestimated, classifying a tumour as stage II even when it invades adjacent organs or large vessels. A further problem is that correlation of stage-prognosis in this system reveals no statistical difference between patients in stage II and II (Kawarada Y and Isaji S, 1998).

2.8.1 TNM residual tumour classification (R classification)

The absence or presence of residual tumour after treatment influences prognosis and may be described by the symbol R. The definitions of the R classification are (RX- presence of residual tumour; R0- No residual tumour; R1- microscopic residual tumour and R2- macroscopic residual tumour). Following surgical treatment there are three areas that should be considered before definition of the R classification: distant metastases following adequate staging for example liver metastases and peritoneal deposits; macroscopic local or regional tumour remaining following resection; histological assessment of resection margins. In a series of 130 resections for pancreatic ductal adenocarcinoma 76 were considered R0 resections and 54 R1 or R2. None of the patients with R1 or R2 disease were alive at two years but the five-year survival in the patients staged as R0 was 36% (Trede M et al, 1990). An acceptable long-term prognosis can be expected in R0 patients only. Although there is a

correlation between stage and R classification, the differences cannot be explained in stage alone (Hermanek P and Wittekind C, 1994).

The Japanese Pancreatic Society system considers a larger number of distinctly more refined parameters and stresses involvement of the adjacent structures to the pancreas [(other organs, large vessels and retroperitoneal tissue, playing down the role of lymph node invasion (Japanese Pancreas Society, 1982)]. Kobari et al (1996) compared the two major systems in over 1600 patients who underwent resection and reported that the Japanese system may offer improvements over the UICC system. The JPS system showed better survival for each individual stage compared to the UICC system when applied to the identical groups of patients. This paradoxical effect needs to be kept in mind when considering survival data from the Japanese since a direct stage-for stage is inappropriate due to this paradoxical effect. The two major staging systems are poor at describing early lesions of the pancreas. Early carcinoma of the pancreas is variously defined as "small carcinoma", "early carcinoma" or T1a/pT1a. All define the tumour as being less than or equal to 2 cm in size and most limit the tumour to the boundaries of the pancreas (Tsuchiya R et al, 1986). This definition of early carcinoma excludes lymph node and distant metastases yet lymph node metastases and spread beyond the pancreas may have occurred in more than 40-80% of such "early" tumours (Tsuchiya R et al; 1986, Nagakawa T et al, 1993). Consequently only about 49% of small ductal adenocarcinomas fit the pT1aN0Mo classification and small carcinomas make up only about 10% of all resected tumours and early lesions only about 5% (Moosa AR and Levin B, 1981). The two staging systems are unsatisfactory in determining prognosis in pancreatic cancer.

2.9 Benign tumours of the exocrine pancreas

2.9.1 Intraductal Papilloma

These are rare tumours of the pancreas which occur within the pancreatic duct in the head or body of the pancreas. They may be multiple and usually present in patients over 50 years of age with symptoms resembling pancreatitis. Macroscopically they are intraductal masses which may obstruct the main pancreatic duct. Histologically, they are papillary neoplasms lined by mucus-secreting epithelium which may contain foci of dysplasia but the tumours are benign (Morohoshi T et al 1989).

2.9.2 Tumours of the endocrine pancreas

These tumours account for approximately 5% of pancreatic tumours.. They have been known by a variety of names including carcinoid tumours, APUD cell tumours, APUDOMAS, islet cell tumours or endocrine cell tumours but it is now believed that they are derived from a common bipotential cell. Insulinomas are the commonest variety accounting for up to 50%, whilst gastrinomas account for 20% and the rarer functioning tumours only 5%; the remaining 25% are non-functioning tumours. About 25% of islet cell tumours are associated with the multiple endocrine neoplasia type 1 (MEN-1) syndrome of which half are malignant. The remaining 75% are sporadic and of these, 70% are malignant. Tumours may be derived from any one component of the pancreatic islets including alpha cells (glucagon-secreting); beta cells (insulin-secreting); delta cells (somatostatin-secreting); PP cells (pancreatic polypeptide-secreting); G cells (gastrin-secreting) and VIP (vasoactive intestinal peptide) derived from nerve fibres. Several hormones may be produced by a tumour but symptoms

are invariable due to one dominant hormone**.** They run an unpredictable course and only curative resection has been shown to alter the outcome of the disease (O`Shea D et al, 1996). Neuroendocrine tumours of the pancreas are evenly distributed throughout the gland, affect males and females equally and present most commonly in the fifth or sixth decade. They are usually encapsulated, firm and normally compress the surrounding tissue rather than infiltrate it. Malignant neuroendocrine tumours however, invade the normal pancreatic tissue and may show lymph node metastases. Gastrinomas and glucagonomas are more likely (60%) to be malignant than insulinomas (10%). Histologically, tumours may be divided into four categories. Type A is the carcinoid pattern consisting of typical nests and cords of cells; type B tumours show ribbons of cells; type C appear histologically as tumour cells in tubular and rosette formations and type D are undifferentiated tumours which form sheets of cells with no characteristic pattern. Many tumours however, show combinations of these histological types. Benign and malignant tumours are difficult to distinguish histologically as malignant tumours may show little anaplasia and benign tumours are not always encapsulated. Larger tumours are more likely to be malignant but the only absolute indicators of malignancy are invasion of the normal surrounding pancreas or the presence of metastases. Local complications include gastrointestinal bleeding and obstruction and involvement of the superior mesenteric and portal veins. Adenocarcinoma of the islets generally follows a more indolent course than adenocarcinoma of the exocrine pancreas and although half the patients have liver metastasis at the time of diagnosis, the median survival time is 3.5 years compared to 4-6 months in unresected PADC. A classification system based on clinicopathological features has been suggested but its clinical value has yet to be seen (Schind IM et al, 2000). The non-functioning tumours are not associated with an obvious hormonal syndrome. Most of them are found in the head of the pancreas and are malignant (Cheslyn-Curtis S et al, 1993; Eckhauser FE, 1986). They are identical to the functional tumours but usually present

at an advanced stage. Selective angiography and CT scanning are the main localisation methods (Geoghegan JG et al, 1994).

Results from medical and surgical treatment are encouraging, showing reduction of hormonal excess and increased survival (Hellman P et al, 2000).

2.10 Primary pancreatic lymphoma

Primary pancreatic lymphoma is a rare form of extra nodal lymphoma (less than 0.5% of pancreatic cancers) that arises from the pancreatic parenchyma (Boni L et al, 2002). They may present as primary or secondary lymphomas. The tumour is often bulky on CT with an average diameter of eight centimeters and surrounding peripancreatic lymphadenopathy often with involvement of adjacent organs. Primary pancreatic lymphoma includes only those patients with stage I or II disease according to the modified Ann Arbor staging system. The diagnosis must be confirmed by CT or USS guided biopsy or cytology of pancreatic juice obtained at ERCP. Most of the patients present with abdominal pain, and CT guided percutaneous biopsy may be useful in making the diagnosis (Bouvert M et al, 1998). The tumour appears to have some response to adjuvant treatment. Berhns et al (1994), in a small series found that the mean survival following chemotherapy was 13 months (n=2), 22 months with radiotherapy (n=5) and 26 months for those who had chemotherapy.

2.11 Summary

It is important to define a tumour, classify it and assess whether it is one of the entities that have a more optimistic outlook. Even amongst the common tumours there is a difference in prognosis according to cell type, whilst in the unusual types of tumours there is an entirely different pattern of prognosis. The clinician needs to be aware of these variants in order to avoid failing to treat a patient with a potentially curable lesion for whom a simple surgical procedure can be life saving. Histological typing and sub typing of primary pancreatic cancers, in addition to staging and grading, should form the basis for treatment selection and prognostic assessment. The two major staging systems do not accurately predict prognosis in pancreatic cancer and they are likely to be replaced by other systems, possibly related to the genetic changes of this aggressive tumour. The chapter explored the different pathological entities of some precursor lesions with different names referring to the same condition. Since 2004, clarity has now established three main precursor lesion; pancreatic intraepithelial neoplasia (Pan IN), which is the commonest, mucinous cystic neoplasia (MCN) and intraductal pancreatic mucinous neoplasia (IPMN). This will inevitably standardize reporting and the development of appropriate treatment strategies.

Table 2.1. Histological classification of tumours of the pancreas

Origin	Frequency	Histological Type	Clinical Features
Exocrine (95%)	80%	DuctalAdenocarcinoma (PDA)	long term survival rare
	2%	Mucinous non cystic	prognosis worsens with content of signet rings
	<1%	Squamous Cell Carcinoma	generally more aggressive than PDA
	2%	Adenosquamous Carcinoma	Generally more aggressive than PDA
	5%	Anaplastic (Undifferentiated)	worse prognosis
	3%	Mucinous	female preponderance, more favourable prognosis tha
	<1%	Intraductal (Papillary)-	more favourable prognosis than PDA
	rare	Pancreatoblastoma	childhood, more favourable prognosis than PDA
	1%	Small Cell Carcinoma	extremely poor prognosis
	(5%)	Acinar cell carcinoma	may be associated paraneoplastic syndrome, poor pro
Endocrine (2.5%)		Insulinoma	10% malignant
		VIPoma	50% malignant
		Gastrinoma	60% malignant
		Glucagonoma	60% malignant
		non-functioning	60% malignant
Other (2.5%)		Lymphoma	responsive to chemotherapy

Table 2.2. UICC TNM clinical classification of exocrine pancreatic carcinoma (Sobin LH and Wittekind Ch, 2003)

T – Primary Tumour

TX Primary tumour cannot be assessed

T0 No evidence of primary tumour

Tis Carcinoma in situ

T1 Tumour limited to the pancreas, 2cm or less in greatest dimension

T2 Tumour limited to the pancreas, more than 2cm in greatest dimension

T3 Tumour extends directly into any of the following: duodenum, bile duct. Peripancreatic tissues[1]

T4 Tumour extends directly into any of the following: stomach, spleen, colon, and adjacent large vessles[2]

N – Regional Lymph Nodes

NX Regional lymph nodes cannot be assessed

N0 No regional lymph node metastases

N1 Regional lymph node metastases

- N1a Metastases in a single regional lymph node
- N1b Metastases in multiple regional lymph nodes

M – Distant Metastasis

MX Distant metastasis cannot be assessed

M0 No distant metastasis

M1 Distant metastasis

Table 2.3. R classification

RX	**Presence of residual tumour cannot be assessed**
R0	**No residual tumour**
R1	**Microscopic residual tumour**
R2	**Macroscopic residual tumour**

TNM 7

Table 2.4. Histopathological Grading

GX	Grade of differentiation cannot be assessed
G1	Well differentiated
G2	Moderately differentiated
G3	Poorly differentiated
G4	Undifferentiated

Chapter 3 Molecular biology of pancreatic cancer

3.1 Introduction

Cancer is caused by uncontrolled cell division leading ultimately to the formation of a tumour and this has been shown to be a multi-step process requiring the accumulation of several genetic changes. When a cell experiences stress e.g. chemical, heat or DNA damage it can respond in several ways to counteract any damage incurred. Inhibitory factors are produced at the appropriate checkpoint, which can cause arrest of the cell cycle allowing the damage to be repaired and cell cycle to resume or, if the damage is too great cause the onset of apoptosis (programmed cell death). Cancer can be considered to be a result of numerous failed attempts to adequately control the cell cycle process and/or instigate apoptosis.

Genetic alterations predispose an individual to cancer and can be inherited or acquired. In inherited predisposition the individual already has a genetic lesion e.g. point mutation and because cancer is due to the accumulation of genetic lesions, the individual is at an increased risk of developing cancer as fewer additional steps are required for cancer formation and progression. Acquired genetic alterations can be attributed to environmental carcinogen factors (Soussi T, 1996). Two main categories of genes whose alteration or change may lead to cancer are oncogenes and tumour suppressor genes, (Steele RJ et al, 1998). An oncogene is a gene whose overexpression can predispose a cell to transformation. Proto-oncogenes, the wild type of the gene encode proteins that respond to cellular growth or survival factors (e.g. growth factors and growth receptors) and are involved in cell cycle progression. Mutations within these genes can confer malignant properties on the cell that can enhance cell proliferation and survival. The oncogenic genotype is dominant; therefore mutations arising in just one of the alleles can be sufficient for the onset of overexpression. In a few cases mutations in oncogenes are inherited, but more frequently they are acquired (Steele RJ et al, 1998). Tumour suppressor genes in their wild state suppress uncontrolled or unregulated

growth and are associated with the cell cycle check points. For a mutant gene to become apparent both alleles need to be altered either by mutation or loss. The molecular biology of pancreatic cancer is becoming clearer. The disease is caused by inherited and acquired mutations in cancer related genes. A study of premalignant genetic lesions has led to the now accepted Pan IN (Pancreatic intraepithelial neoplasia) classification to explain the tumour progression model (Hruban RH et al, 2001, Singh, M et al, 2007)). The premalignant changes seen in colorectal cancer seem to be similar to carcinogenesis in pancreatic cancer and this adeno-carcinoma sequence has led to a better understanding of pancreatic cancer formation (Hruban RH et al, 2000). The stepwise accumulation of mutations in a number of genes leads to a disturbance of the cell cycle. In pancreatic cancer there is over expression of growth factors and growth factor receptors including EGF, TGFα, EGFR, amphiregulin, c-erbB 2-4, αFGF, βFGF, TGFR, TGFβsl-3 and TGFβRs. Inactivation of the tumour suppressor genes (TS) p53, p16, SMAD4 (Hahn SA et al, 1995) and activation of dominant oncogenes K-ras and cyclin D1 all play a role in tumourigenesis. Most recently, global genomic sequencing for genetic changes in pancreatic cancer has been carried out by Jones, S et al (2008). More than twenty thousand genes were screened in 24 different pancreatic specimens and about sixty mutations were found in each sample and mainly affecting 12 overlapping signalling pathways (Carter, H et al, 2010). The five pathways were; apoptosis, DNA damage repair, G1/S phase cell cycle progression, cell-cell adhesion and invasion. Other pathways include the signalling cascades; embryonic. MAPkinase and TGF-B signalling (Jones, S, et al, 2010

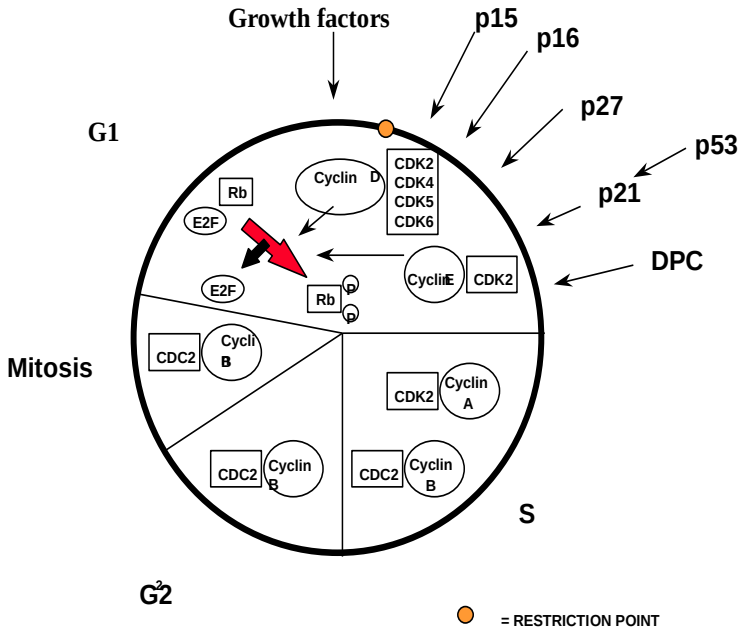


Figure 3.1: The cell cycle: Normal cell division is checked at numerous stages before it is allowed to continue.

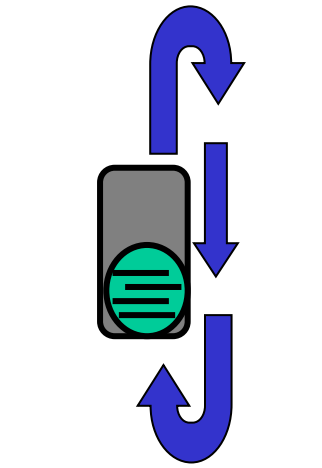
M: mitosis
G1: Gap1
G2: Gap2
S: DNA synthesis

3.2 Growth Factors and their Receptors

Epidermal growth factor receptor (EGFR) and the erb-B family of growth factor receptors (c-erbB-2, c-erbB-3 and c-erbB-4) are all frequently overexpressed in pancreatic cancer and in epithelial proliferative states such as chronic pancreatitis. They are all transmembrane

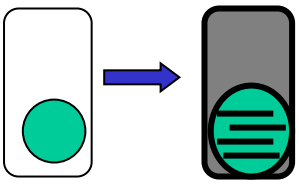
tyrosine kinase receptors with a similar sequence. The action of these oncogenes is shown in Figure 3.2. (Magee et al, 2001) There are essentially 3 different types of actions that can occur. Initially in the autocrine phase, cancer cells appear to release substances that act on its own cell surface. In the paracrine type of action, factors produced from the surrounding area of a normal or malignant cell appear to stimulate surrounding or distant cells. In Proxicrine action, substances from islet cells of the pancreas are transported via the portal system where translocation takes place upon contact. Translocation is where the growth factor couples itself to the nucleus and is able to influence DNA synthesis or change of genetic function (Raper SE et, 1987). How the cells respond to these stimuli is dependent on a number of factors. Clearly the quantity of the ligand substrate which may be under or overexpressed as well as the receptor is crucial, adequate function of these ligands, effectiveness of downstream signalling pathways and finally the feedback loop to all these responses

Figure 3.2 Signalling pathways



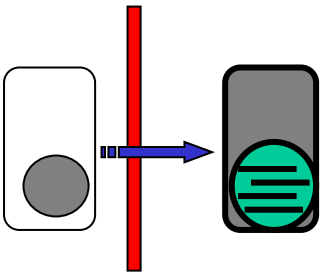
AUTOCRINE SIGNALLING

EGF/EGF-R
FGF/FGFR
PDGF/PDGF-R platelet derived growth factor
HGF/MET hepatocyte growth factor
VEGF vascular endothelial growth factor
KGF/KGFR keratinocyte growth factor



PARACRINE SIGNALLING

TGF-β/ TβR-I, TβR-II



PROXICRINE SIGNALLING

Insulin, IGF-I, IGF-II/IGF-IR, IGF-IIR

3.2.1 Epidermal growth factor (EGFR) family

This family of type 1 growth factor receptors comprises four transmembrane tyrosine kinases: the epidermal growth factor receptor (EGFR), c-erbB-2, 3 and 4 (Carraway KL and Hartley LC, 1994). EGFR is activated by a family of peptide ligands that includes EGF, TGFα, heparin-binding EGF-like growth factor, betacellulin and amphiregulin (Prigent SA and Lemoine NR 1992). EGF is a polypeptide of 53 amino acids which stimulates proliferation and differentiation of a wide variety of cell types through the EGFR (Ullrich A and Schlessinger J, 1990). The EGFR is encoded by c-erbB-1 proto-oncogene activity. In the normal pancreas, EGFR is only expressed in the islets of Langerhans. The EGFR gene however is overexpressed in 95% of ductal adenocarcinomas and human pancreatic cell lines due to an increase in gene transcription (Barton CM et al, 1991a). EGFR binds EGF and TGFα with high affinity and both of these ligands are overexpressed in pancreatic cancer (Yamanaka Y et al, 1992). The overexpression of EGFR has been shown to increase the production of EGF and TGF*a* promoting autocrine and paracrine loops that promote cell proliferation (Korc M et al, 1992). TGFα is expressed at low levels in the ductal epithelium of the normal human pancreas but is overexpressed at high levels in 95% of ductal adenocarcinomas (Barton CM et al, 1991b). Whilst EGF is not detectable in the normal pancreas, it is found in 12% of pancreatic cancers (Barton CM et al, 1991). High levels of EGFR, EGF and or TGFα correlated with reduced patient survival which is also associated with overexpression of amphiregulin (Yokayama M et al, 1995).

3.2.2 c-erbB-2

The c-erbB-2 proto-oncogene encodes a 185-kDa transmembrane glycoprotein (Akiyama T et al, 1986). There are a number of ligands for the c-erbB-2 receptor, including heregulin, gp30 and NEU-differentiation factor (NDF) but the full contribution of the c-erbB-2 to growth stimulation in pancreatic cancer remains unknown (Leung HY and Lemoine NR et al, 1992a). c-erbB-2 is overexpressed in bladder, breast, oesophageal and gastric cancers where it appears to have a role in lymph node metastases (Mellon JK et al, 1996; Hardwick RH et al, 1997). In invasive ductal adenocarcinomas of the pancreas and ampullary tumours c-erbB-2 are overexpressed in 20% and are usually due to c-erbB-2 gene amplification (Hall PA et al, 1990).

3.2.3 c-erbB-3

This is the third member of the EGF receptor-related family of growth factor receptors (Kraus MH et al, 1989). The c-erbB-3 gene encodes a Mr 180,000 transmembrane polypeptide which shares close structural similarity with the EGF receptor and c-erbB-2 but it has no known natural ligands. In the normal pancreas c-erbB-3 is expressed in the islets of Langerhans but has been detected in up to 90% of pancreatic and ampullary cancers (Thangarajan R et al, 1993; Lemoine NR et al, 1992b).

3.2.4 c-erbB-4

This is a 180 kDa transmembrane tyrosine kinase whose extracellular domain is similar to that of c-erbB-3. The cytoplasmic kinase domain exhibits a high degree of similarity with EGFR and c-erbB-2 (Plowman GD et al, 1993). The heregulins and betacellulin can all

activate c-erbB-4 (Tzahar et al, 1994; Riese DJ et al, 1996). c-erb B-4 is predominately expressed in normal skeletal muscle, heart, pituitary, brain and cerebellum as well as breast cancer cell lines. In a recent study of c-erbB-4 expression in pancreatic cancer using quantitative RT-PCR and immunohistochemistry, no significant difference was found between patients with c-erbB-4 positive tumours and those with C-erb-4 negative tumours (Graber HU et al, 1999).

3.2.5 TGFβs and TGFβ receptors

The Transforming growth factor-β family of cytokines actively influence cell division, cellular differentiation and cell death (Heldin C et al, 1997; Massague J, 1998). Members include TGF-β s; bone morphogenetic (BMPs) and activins, all of which are found in PDAC (Li CY et al, 1995). TGF β normally exists in the extracellular space as a complex with the latency-associated protein (LAP) and the latent TGF-β binding protein (LTBP). Once released from this complex, TGF-B binds to a teterodimeric transmembrane receptor consisting of 2 components – TBR-1 and TBR-11. Binding of TGF- β to TBR-11 forms a complex that binds to TBR-1. This enables phosphorylation and activation of TBR-1 by TBR-11. TBR-1 phosphorylates the cytoplasmic proteins Smad2 and Smad3, which are termed receptor regulated or R- Smads. These R-Smads associate with Smad4 (common mediator Smad or Co-Smad) as shown in figure 3.3. The co-smad/R-Smad heterocomplex translocates to the nucleus where it can co-operate with a host of co-factors leading to the up- or down-regulation of transcriptional activity. This transcriptional regulation appears to be mediated by chromatin remodelling by histone acetylases (HATs) and histone deacetylases (HDACs). Evidence has shown that TGF- β signalling through Smad4 upregulates p21cip1/waf1 (Li cy et al, 1995) thereby arresting the cell in G1 and that TGF- β mediated growth inhibition is mediated through pRb-E2F trans repression (Kleeef J et al, 1999). There

are also inhibitory SMAD proteins (1-Smads, Smads6 and Smads7) that abrogate Smad pathway signalling and these have been shown to be elevated in pancreatic cancer samples when compared to normal pancreatic tissue and such overexpression may confer TGF- β resistance and enhanced tumorigenicity (Kleef J et al, 1999; Kleef J et al, 1999). They bind to specific cell surface receptors and are able to down regulate transcription factors, decrease phosphorylation of target proteins and inactivate cell cycle regulatory enzymes. The end targets are the G1 cyclins and cyclin dependent kinases. The presence of mutant p53 has been correlated with the loss of TGFβ-1 responsiveness in malignant epithelial cell lines. TGFβ-1 may also induce the universal cyclin inhibitor p21 by a p53 independent pathway (Bladydes JP et al, 1995, Datto MB et al, 1995). The three isoforms TGF β -1-3 have been shown to be overexpressed in pancreatic cancer. Survival data from 60 patients with pancreatic cancer showed significantly decreased survival time with the overexpression of these isoforms (Friess H et al, 1993; Lu Z et al, 1997). In a more recent study, however, TGF β -1, which was expressed in 31% of cancers, was significantly associated with increased survival (Friess H et al, 1993; Lu Z et al, 1997). These contradictory findings deserve further investigation. The TGF β receptors 1-3 are all found in normal pancreas, but in cancer there is overexpression of TGF β -2 receptor only (Friess H et al, 1993)

3.2.6 Fibroblast growth factors

Fibroblast growth factors (FGFs) belong to a large group of polypeptide growth factors that include the two main groups, FGF (αFGF) and FGF (βFGF). These growth factors are highly abundant in the basement membrane and extracellular matrix of a variety of tissues. FGFs influence cell differentiation, tissue homeostasis, regeneration and repair, cell migration and growth. Binding to specific transmembrane receptors (which have intracellular tyrosine kinase activity) is dependent on the presence of heparin sulphate proteoglycans (on the cell

surface or in the extracellular matrix) for which they have a high affinity. The intense desmoplastic stromal reaction that is seen in pancreatic cancer and the histological prevalence of perineural invasion supports the role of FGFs in the progression of this tumour.

3.3 Insulin-Like Growth Factor-1 and its Receptor

This is a polypeptide that binds and activates both the IGF-I and Insulin receptors (Frosch RG et al, 1985). Additionally, both receptors appear to promote cell division in different cells but it has been reported that it is the latter receptor that is mainly involved (Frosch RG et al, 1985). Both receptors activate insulin receptor substrate-1, an important regulatory protein that mediates the growth promoting and metabolic effects of the IGF-I and insulin (Myers MG et al, 1994). IGF-I acts in a paracrine and autocrine manner to enhance pancreatic cancer cell growth in vivo.

3.4 Tumour suppressor genes

Tumour-suppressor genes are genes that, when activated, convey transforming properties. The genes are inactivated either by homozygous deletion (deletion of both copies of the gene) or by loss of one allele (one copy of the gene). Loss of function can be associated with uncontrolled cell growth and proliferation, decreased apoptosis, and malignant transformation.

3.4.1 p53

The p53 gene is 21Kb in length and is located on the short arm of chromosome 17 (17p). It spans 11 exons and a 2.8Kb mRNA encodes a 393 amino acid protein. The protein is comprised of four functional domains. (1) a transcriptional activation domain in the acidic terminus (0-80aa); (2) a DNA- binding domain (80-150aa); 3) an oligomerisation domain (150-300) and 4) a basic C terminus.

3.4.1.1 p53 in normal cells

The normal p53 protein referred to as the wild type has a short half-life and is found in the nucleus of cells. Karstan et al postulated a form of feedback loop with the MDM2 protein that is itself stimulated by normal p53 resulting in the destruction of the latter. Additionally, he suggested that the p53 protein is like a gatekeeper for the entry of cells into the cell cycle at the G1/S transition and is implicated in the repair of DNA that has been damaged prior to its entry in the replication process (Kastan MB et al, 1991). During times of damage to DNA, the cell increases the concentration of p53 protein which in turn forms bonds with DNA leading to the production of other genes responsible for aborting cell growth and destruction of damaged cells also known as apoptosis. Additional functions of the p53 protein include its role as a transcription factor, cell growth and normal function of the cell by regulation of other genes. The timing and combination of these functions results in a controlled cell cycle.

P53 upregulates p21cip1/waf1 (El Diery WS et al, 1993) that acts upon the retinoblastoma pathway to inhibit retinoblastoma phosphorylation thus arresting the cell cycle in G1 and Bax (Miyashita T et al, 1994) that promotes apoptosis.

3.4.1.2 p53 and cancer

Mutations that occur in the p53 protein prevent binding of the protein to DNA and as a result the up regulation of the MDM protein does not occur with the mutated p53 protein has a much longer half-life than wild-type p53 protein. Accumulation of p53 protein is used as a surrogate marker for the presence of mutant p53, although an increase of the protein may also be found in stressful conditions. More than half of tumours in have mutations in the p53 gene (Lane DP et al, 1991; Hollstein M et al, 1994). Early work by Barton et al, found that In PADC the gene is inactivated in approximately 65% of cases usually by missense point mutations (Barton CM et al, 1991). Other work by Apple et al suggested that the genetic aberrations demonstrate loss of the wild type allele and appears to be a late event in cancer formation (Apple SK et al, 1999). Additional reports that mutation in the gene shows an in exact number of chromosomes referred to as aneuploidy makes the case that normal p53 is important for normal cell function. (Harada T et al, 2002; Gorunova L et al, 1998). Germline mutations in p53 have been described as Li Fraumeni syndrome, predisposing to many cancers but pancreatic cancers are a rare finding (Strong LC et al, 1987; Li FP et al 1988). The majority of mutations are found in exons5, 6 and 8 which is the location for DNA binding. This so called hot-spot region was subsequently shown to be the DNA binding region of the molecule. Mutations in this region of the molecule, by disrupting DNA binding, affect the ability of the p53 protein to regulate genes involved in cell cycle arrest. Hence the high proportion of tumours with mutations in this region of the gene. Generally, because p53 is a tumour suppressor gene both copies of the allele need to be lost or their function altered

in order for its suppression role to be lost. Many tumours therefore as well as possessing point mutations in the p53 gene also show loss of heterozygosity (LOH) i.e. the other, wild type allele of the gene has been lost through chromosomal changes. Mutant p53 however can exert harmful effects on the cell even when there is still one remaining wild-type allele present. This function of the mutant allele is exerted either through a transdominant effect of the mutant over wild type when tetramerisation of mutant p53 molecules alongside wild-type molecules causes inactivation of the wild-type functions. In addition, it has been shown that mutant p53 molecules can exhibit gain of function that may lead to increased oncogenic potential.

3.4.1.3 p53 and adjuvant treatment

When cells are exposed to either radiation or cytotoxic agents the levels of wild type p53 protein rise 5-60 fold (due to increased stability of the protein rather than increased protein synthesis). It follows that tumours with functioning p53 may carry a better prognosis than those expressing the mutant protein when exposed to radiation or DNA damaging agents.

3.4.2 p16

The p16 (MTS1/INK4A/CDKN2) tumour suppressor gene is found on the short arm of chromosome 9 (9p21) and encodes for a 16-kDa protein (Serrano M et al, 1993) that plays a key role in controlling the G1 checkpoint (or restriction point, R) of the cell cycle (Figure 3.1). The retinoblastoma gene product (pRb) is an active transcriptional repressor when bound to transcription factors such as the E2F family. Inactivation of the pRb by phosphorylation (mediated by the complex formed by CDK4, 6 and cyclin D) causes release of E2F and subsequent transcription of genes important for DNA synthesis. The p16 protein prevents the association of CDK4 and 6 with cyclin D and the subsequent phosphorylation of

pRb. This growth suppression by p16 requires functional retinoblastoma protein (Medema R et al, 1995). Loss of p16 expression occurs in up to 85% of pancreatic cancer cell lines and xenografts (Caldas C et al, 1994) due to homozygous or heterozygous deletion (Naumann M et al, 1996) and usually occurs late in the tumour progression model (Caldas C et al, 1994; Rozenblum E et al, 1997; Wilentz RE et al, 1998). The p16INK4A locus also encodes the tumour suppressor ARF (alternative reading frame). Both p16INK4A and p19ARF share a common exon 2, however ARF has a different frame resulting in a non-identical protein product. ARF's role as a tumour suppressor is dependent on its ability to sequester MDM2 in the nucleolus thereby potentiating p53 mediated G1 arrest (Weber JD et al, 1999). Loss of the p16INK4A locus in pancreatic cancer is inevitably accompanied by loss of the ARF reading frame. This loss of heterozygosity is often followed by hypermethylation and inactivation of the p16INK4A promoter on the remaining allele. ARF does not share the same promoter as p16INK4A, potentially allowing ARF to still be produced. The exact role of ARF in pancreatic cancer is not fully understood.

3.4.3 SMAD4 (DPC4) and the TGF-β signaling pathway

Previously, the DPC4 gene was named after the finding of mutations in hepato-biliary – pancreatic tumours (Hahn HA et al, 1996). The name has since changed to SMAD4 which is named after the protein family *Drosophila melanogaster* MAD (mothers against decapentaplegic) protein and the *Caenorrhabditis* elegans protein SMA (small body size). Loss of an allele at chromosome 18q in has been observed with high frequency, about 90% of PADC and in approximately half of these tumours, homozygous deletions are located at chromosome 18q21.1 (Hahn SA et al, 1995). DPC4 has not been reported in early lesions such as flat, papillary or atypical intraduct neoplasias but is inactivated at a high frequency in

severely atypical neoplastic lesions and from this, it can be concluded that its influence on tumourigenesis is a late event (Biankin AV et al; 2001Wilentz et al, 2000). The protein product is believed to play a role in signal transduction from the transforming factor- B (TGF-β) superfamily of cell surface receptors (Hahn SA et al, 1995; Dai JL et al, 1998; Wilentz JP e al, 2000). Smad proteins interact with these receptors, become phosphorylated and then complex with the DPC4 (Smad4) (Montgomery E et al, 2001). Activated SMAD complexes are then translocated to the nucleus where binding to DNA stimulates the transcription of nearby genes (Montgomery E et al, 2001). Immunohistochemical labelling for the DPC4 gene product has been reported to correlate with gene expression (Wilentz et al, 2000). The 3′ end of the gene is highly conserved and reports of mutations within this region support the idea that the C-terminus region is important for tumour suppressor function (Hahn SA, 1995). There is an 85% homology in amino acid sequence between the SMAD4 protein and the Drosophila melanogaster (Mad) protein in exons 1, 2 and 11 and a 75% homology in exons 8, 9 and 10. Drosophila that have homozygous mutations in the Mad gene exhibit defects in midgut morphogenesis with a similar phenotype being produced with mutations in the dpp gene which codes for a member of the TGF-β superfamily (Massague J, 1996). TGF-β is a potent inhibitor of cellular proliferation and many cancer cells show a diminished responsiveness to TGF- β induced growth inhibition which could be explained by the loss of the SMAD4 gene (Kloppel G et al, 2000). Similarity with the Caenorhabditas elegans proteins sma-2, sma-3 and sma-4 has also been reported. These proteins form part of the post-receptor transforming growth factor beta (TGF−β) signalling pathway and it is suggested that SMAD4-induced tumour suppression acts along a TGFB-like signalling pathway. Most recently Kloppel et al (2000) studied the restoration of the SMAD4 gene in human pancreatic carcinoma cells in vivo and found that it did not restore sensitivity to TGF-β. Inspite of this finding, the SMAD4 gene has an important function in angiogenesis and in

decreasing expression of vascular endothelial growth factor. The results of Koppel's work suggest an angiogenic switch pathway previously unknown in the SMAD4 gene. Finally, the SMAD4 (protein) has only been reported in mammals in (Cook T et al, 2000) and mutations in pancreatic cancer occur with high frequency (Jones, S et al, 2008).

3.4.4 p21 ^{WAF1}

p21 ^{WAF1} is a cyclin-dependent kinase inhibitor and a downstream target and effector of p53 which is overexpressed in pancreatic cancer. The 21-kDa product of the WAF1 gene forms part of a quaternary complex along with cyclin/CDKs and the proliferating cell nuclear antigen (PCNA) in normal cells but not transformed cells and is a universal inhibitor of CDK activity (Xiong Y et al, 1993). One consequence of p21 binding to and inhibiting CDKs is to prevent CDK-dependent phosphorylation and subsequent inactivation of the Rb protein, which is essential for cell cycle progression at both G1 and G2 checkpoints. The negative regulatory action of p21^{WAF1} on the cell cycle permits sufficient time for repair to extensive DNA damage to be completed. Functional p21 is essential for p53-mediated G1 arrest due to WAF1 inhibition of both CDK activity and PCNA-dependent DNA replication (Xiong Y et al, 1993). Thus p21 overexpression causes growth suppression consistent with its role as an

inhibitor of CDKs and a tumour suppressor gene. Induced expression of p21 ^{WAF1} by p53 directly or by p53-independent mechanisms results in as much as 20 to 100 fold increase depending on the cell type and mode of induction. In preneoplastic lesions of the pancreas p21 overexpression occurs early (Biankin AV et al, 2001). In pancreatic cancer p21 has been reported to be overexpressed with increased frequency (Biankin AV et al, 2001). Kinetic analysis of p53-dependent induction indicates that p21 ^{WAF1} expression begins to rise coincident with the accumulation of p53 in response to DNA damage. Failure to do so is associated with failure of G1 arrest and with inappropriate onset of apoptosis (Agarwal ML et al, 1995). Overexpression of p21^{WAF1} is an early event in the development of pancreatic intraepithelial neoplasia

3.4.5 BRCA2

BRCA2 is a tumour suppressor gene that has been identified on chromosome 13q12, which along with BRCA1 has been implicated in the pathogenesis of carcinoma of the breast (Wooster R et al 1994). Its role is that of regulation of repair mechanisms for DNA. (Venktaraman AR, 2002). Inherited mutations have been reported to increase the risk of pancreatic cancer (Ozcelik H et al, 1997). In the non-hereditary pancreatic cancers, the BRCA2 gene has been estimated to occur in just fewer than 10 percent is (Goggins M et al, 1996). Schutte reported a single case of a homozygous deletion in pancreatic cancer within the region of the BRC2 locus suggesting that a tumour suppressor gene might be located within this region (Schutte M et al, 1995).

3.4.6 APC (Adenomatous Polyposis Coli gene)

This gene is located at chromosome 5q21 and was first identified in patients with familial adenomatous polyposis. At present there is a discrepancy of the results between Western groups that have found no evidence of gene mutations or loss of protein expression of the APC gene (McKie AB et al, 1993) and those of some Japanese authors who reported inactivating mutations in a small number of cases, predicting a truncation of the gene product (Horii A et al, 1992). The significance of this discrepancy has not been explained yet and whether it represents some form of ethnic variation remains to be seen. Studies with pancreatic cancer cell lines support the absence of APC gene mutations in pancreatic cancer as all had a full length of the APC protein (Smith KJ et al, 1993).

3.5 Oncogenes

These are genes that, when activated by mutation or overexpression, possess transforming properties.

3.5.1 K-ras

The Ras family of proto-oncogenes include three human genes, Harvey (H-ras), Kirsten-ras (K-ras) and N-ras in which the highest incidences of mutations are found in the early genesis of pancreatic cancer (Caldas C and Kern SE, 1995). The Ras proteins are located on chromosome 12p and encode for small GTP-binding proteins that are involved in cell proliferation, survival and migration (Shields JM et al, 2000).

3.5.1.1 K-ras in normal cells

K-ras responds to chemical substances that encourage division of cells by firstly binding to GTP. This creates an environment for further stimulation of other substances which cascade information further downstream and one of these being the RAF/MAPK pathway (Magee et al 2001). A number of complex processes occur culminating in the eventual activation of the of molecular translocation to the cell membrane and in part phosphorylation of RAF. Upon completion of the signalling process, the GTP is destroyed and therefore switching off the signalling pathway of K-Ras (Maggee et al, 2001; Figure 3.3)

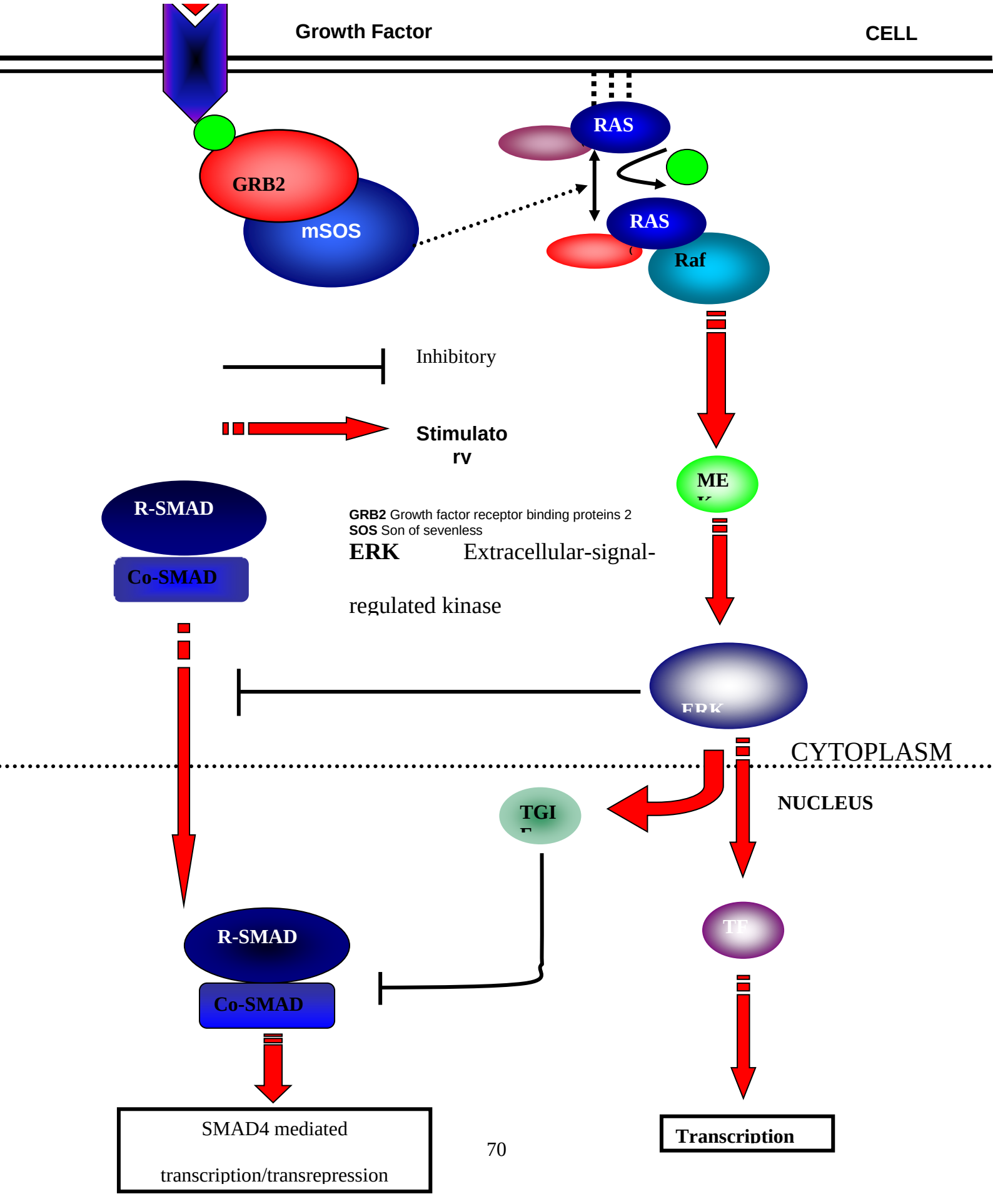
3.5.1.2 K-ras in cancer

Most of the commonly reported mutations in the K-ras gene are found in codon 12 and range from being very uncommon N-ras and H-ras genes (about 20%) and almost in all cases in the K-ras gene (Smit V et al, 1988; Lemoine NR et al, 1992; Caldas C and Kern SE, 1995; Maruta H and Burgess AW, 1994). Fewer mutations are also seen in other codons, usually in 13 and 61. Finkelstein et al have described the location of these mutations; the aspartic acid mutation (GAT), valine (GTT), arginine (CGT) and cysteine (TGT) (Finkelstein SD et al, 1994). K-ras mutations affect the ability of the K-RAS protein to metabolise GTP by hydrolysis. The result of this process is that K-Ras protein is activated with constant stimulation of other targets in a cascading manner.

In general, human epithelial cells are not sensitive to oncogene transformation by mutated Ras while this is not the case with mice fibroblasts which are readily activated with the addition of other either a tumour suppressor gene or an additional oncogene (Serrano M et al, 1997). This senescence is thought to be a defence mechanism against oncogenic stress. Whether the observed expression of $p21^{CIP1}$, whose frequency mirrors that of K-Ras mutation in the pancreatic cancer model is part of this defense mechanism or directly linked to the cell

cycle by working as an assembly factor for the cyclin D1/CDk4 complex, is unclear (Biankin AV et al, 2001). These mutations are well defined and reliably detected by DNA assays. K-ras mutations can be detected in cancer tissue, blood, pancreatic secretions and stool. In benign and premalignant diseases of the pancreas, mutations are seen in a very small proportion of cases but the risk of progression to malignancy is low in the absence of other genetic events (Tada M et al, 1996; Moskaluk CA et al, 1997). Inspite of the high mutation frequency in human pancreatic cancer, mice which harbour a latent allele of K-ras, G12D which is capable of spontaneous activation in vivo, develop multiple onset lung tumours but not PADC. This further demonstrates the species differences of Ras functions (Johnson L et al, 2001)

Figure 3.3: RAF/MAPK pathway



3.5.2 Cyclin D1

Cyclin D1 is a cell cycle regulator, which may act as an oncoprotein and is overexpressed in ductal pancreatic cancer. It forms part of the enzyme complexes that are active in G1 phase of the cell cycle and inactivate pRb by phosphorylation. These enzyme complexes (CDKs) contain two components, a regulatory subunit- the cyclin and the catalytic subunit the cyclin dependent kinase (cdk) **(**Kato JY et al, 1993). The CDKs can be activated by binding a cyclin and the phosphorylation of a conserved threonine by the CDK-activating kinase (CAK). Inactivation of the active cdk-cyclin complex can occur by binding of the CDK inhibitory subunits (CKIs) (Scher CJ et al, 1995). There are two classes of CKIs; the INK4 kinase inhibitors, p15, p16, p18, and p19 which specifically regulate complexes of cyclin D1, D2 and D3 with CDK4 and 6 and a second group p21, p27 and p57 inhibit all G1 cyclin/cdk complexes. The primary regulator of CDK activity is the cyclin subunit whose levels oscillate during cell cycle. The G1 cyclin complexes consist of D-type cyclins (D1, D2 and D3) complexed with either cdk4 or 6, cyclin E is complexed with cdk2 and cyclin A is complexed with cdk2. The appearance of D type cyclins is tightly linked to growth factor exposure and their downstream cell cycle effects are due to inactivation of pRb by phosphorylation. Cyclin D1 has been implicated in the pathogenesis of a number of cancers including oesophageal, lung, head and neck, bladder and also sarcomas. Overexpression of cyclin D1 leads to phosphorylation of pRb and thus deregulated (and increased) E2F activity. Also activation mutations of cdk4 have been identified in certain tumours such as melanoma. Thus both cyclin D1 and cdk4 can act as oncoproteins by inactivating pRb.

3.5.3 DCC (Deleted in Colorectal Carcinoma)

This is a complex gene consisting of 29 exons spanning 1.4 megabases and is situated on chromosome 18q close to SMAD4. Some early reports found abnormalities in the expression of the DCC gene in pancreatic cancer (Simon B et al, 1994; Hohne MW et al, 1992) but subsequent work found that deletion of the DCC locus was uncommon (Barton CM et al, 1995). More work is needed and in particular the definitive sequence has yet to be carried out**.**

3.6 DNA Mutation Mismatch Repair (MMR) genes

The DNA mismatch repair genes code for proteins that ensure the smooth DNA replication. Single base pair mutations that occur during division of DNA are identified and then repaired (Goggins M et al, 1998; Wilentz RE et al, 2000). Inherited mutations in DNA mismatch repair genes are associated with an increased risk of cancer (Goggins M et al, 1998; Wilentz RE et al, 2000). Moreover, there is an association with Lynch syndrome, as discussed elsewhere in the thesis which is characterised by an increased risk of developing colorectal, breast and about 4% in pancreatic cancer (Aarnio M et al, 1995; Goggins M et al, 1998).

3.7 Apoptotic factors

Apoptosis is a central regulator of homeostasis in normal tissue. Damaged cells are removed without an immune response and apoptosis balances cell proliferation under normal physiological conditions. There are numerous apoptotic pathways; proapoptotic (Bcl-2, Bcl-xl and Mcl-1) and anti-apoptotic (Bax, Bcl-xs) proteins have been reported in pancreatic

cancer (Miyamoto Y et al, 1999). The bcl-2 family of apoptotic genes includes bcl-2, bcl-x, bax and bak plus others, most having four conserved domains. The first two domains are important for homo- and hetero-dimerisation and the fourth is important for normal function. Bcl-2 is an anti-apoptotic factor and sometimes is referred to as a cooperating oncogene. By itself it is unable to transform cells, but when activated in the presence of the oncogenes, bcl-2 is vital to malignant transformation. Bcl-x exists as two isoforms: bcl-xL is the longer form and functions as an apoptotic inhibitor (Boise LH et al, 1993) and bcl-xS functions as an apoptosis promoter. Bax is a promoter of apoptosis and it has been shown that the ratio between bax and bcl-2 can be important in determining cell survival. An excess of bax homodimers promotes cell death whereas an excess of bcl-2 homodimers will inhibit apoptosis.

3.7.1 Tumour angiogenesis

Angiogenesis is essential for tumour growth and metastasis. Angiogenic factors include FGF and FGF, which stimulate locomotion and proliferation of endothelial cells and TGFα, which has an effect on endothelial cell proliferation. These and other angiogenic factors can be produced by the tumour as well as by endothelial and stromal cells.

3.7.2 Angiogenin

Angiogenin (ANG), an inducer of vascularisation is a 14,000MW polypeptide. ANG may interact with endothelial cells via a cell surface receptor and extracellular matrix (ECM) such as proteoglycans (Hu G F et al, 1994). ANG has been shown to bind to actin on the

endothelial cell surface and this complex may lead to the activation of several protease cascades. In patients with pancreatic cancer high levels of mRNS expression and high levels of serum ANG were significantly associated with poorer survival (Shimoyama S et al, 1996).

3.7.3 Vascular Endothelial Growth Factor (VEGF) Platelet derived Endothelial Cell Growth Factor (PD ECGF)

VEGF is a very potent and selective endothelial cell mitogen on the surface of pancreatic cells (Korc M, 2003), which has been shown to be associated with tumour progression and metastases in a variety of gastrointestinal malignancies. VEGF is a 38-46 kDa dimeric N-glycoprotein which is chemotactic as well as mitogenic for endothelial cells in vitro, induces angiogenesis in vivo and increases the permeability of the vascular endothelium. In humans four different isoforms have been identified ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$). In pancreatic cancer the predominant species are $VEGF_{121}$ and $VEGF_{165}$ (Fujimoto K et al, 1998).

PG-ECGF is a 55kDa polypeptide, which exists in vivo as a homodimer. PD-ECGF stimulates chemotaxis of endothelial cells and therefore indirectly induces angiogenesis (Kuniyasi H et al, 1999).

3.8 Stromal factors and adhesion molecules

3.8.1 Urokinase plasminogen activator (uPA) and its receptor (uPAR)

Plasminogen is an inactive proenzyme that can be converted to plasmin by urinary or tissue plasminogen activator (uPA and tPA). This has been implicated in pericellular proteolysis during cell migration and tissue remodeling. This enzyme is initially released from cells as an inactive proenzyme (pro-uPA) that can be cleaved by and activated by protease (Schmitt M et al, 1992). Pro-uPA and uPA bind to a specific cell surface receptor and following

ligand binding the uPA receptor increases the enzymatic activity of uPA itself. Plasminogen is converted to plasmin by uPA leading to degradation of fibrin, type IV collagen, fibronectin and laminin. Plasmin also activates latent collagenases such as procollagenase (matrix metalloproteinase – MMP1) and pro-stromelysin (MMP3) (Lim YT et al, 1996; Baramova EN et al, 1997). The activation of several growth factors such as hepatocyte growth factor, TGFβ, FGF and VEGF is mediated by uPA, uPAR and plasmin. The resultant cellular activation and ECM proteolysis enhance the ability of pancreatic cancer cells to invade and metastasise. As in other tumours, a concomitant overexpression of uPA and uPAR was associated with significantly worse survival times compared to those patients with no tumour expression of either uPA or uPAR or neither (Cantero D et al, 1997).

3.8.2 E-cadherins and matrix metalloproteinases (MMPs)

E-cadherin is a transmembrane glycoprotein responsible for homotypic binding and morphogenesis of epithelial tissues, which is localised to the epithelial junction complex. In cancer decreased or absent expression of E-cadherin is associated with a decrease in cellular and tissue differentiation and higher metastatic potential. Transfection of E-cadherin has been shown to inhibit motility and invasiveness of cancer cells and the imbalance with the expression of the natural inhibitions of MMP`s (TIMPs) is weakly associated with pancreatic cancer progression.

The MMPs are a family of zinc-containing proteolytic enzymes that breakdown extracellular matrix proteins. One of the first steps of cancer invasion is the breakdown of the basement membrane, which is composed of predominately type IV collagen. The level of MMP

enzyme activity has been shown to correspond to tumour grade, regional lymph node metastases and distant metastases. MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are type 1V collagenases and are overexpressed in pancreatic cancer (Bramhall SR et al, 1996; Bramhall SR et al, 1997). The expression of these metalloproteinases has been shown to directly correlate with invasion and metastasis in pancreatic cancer. A recent study assessed the expression of E-cadherin and MMP-2 and MMP-9 using in-situ hybridisation in pancreatic cancer (Kuniyasi H et al, 1999). Expression of E-cadherin in pancreatic cancer was inversely correlated with tumour progression and the development of metastases. Patients with cancers that had an expression ratio of MMP: E-cad <3 had a significantly better prognosis than those with ratio of >3. Inactivation of the adenomatous polyposis coli (APC) is observed at early stages of intestinal tumour formation, whereas loss of E-cadherin is usually associated with tumour progression. Smit has demonstrated in an Apc mouse model that because both proteins compete for the binding to beta-catenin, an essential component of the Wnt signalling pathway, and a reduction of E-cadherin levels could influence both tumour initiation and progression (Smits R et al, 2000).

3.8.3 Expression profiling

It is well known that preoperative diagnosis for pancreatic cancer does not usually have the luxury of histological diagnosis as compared to other tumours. Resections for chronic pancreatic may sometimes turn out to be malignant on postoperative histology and the collorary is the same for pancreatic cancer resections. This then raises the question as to the need for accurate pre-operative diagnosis particularly given the high risk nature of these major operations. It was with these concerns in mind that Young et al suggested that perhaps genetic markers could be useful in the differentiation of these different disease states by the introduction of “expression profiling” (Young RA, 2000). The transcriptional profile or

transcriptome represents the entire mRNA population of a cell at a single time point. From this transcriptome a "DNA microarray" can be made. DNA microarrays can be stored on glass slides or nylon membranes. It is then possible to analyse the relative up- or down-regulation of genes in a particular disease state compared to normal. The hope is that this knowledge may be used in diagnosis and possibly outcome of disease (Golub TR et al, 1999; Alizadeh A A et al, 2000).

3.9 Summary

Several genetic alterations have now been identified in the causation of pancreatic adenocarcinoma. These fall broadly into oncogenes e.g. K-ras, tumour suppressor genes e.g. p53, p21, p16, SMAD4, growth factor receptors e.g. c-erb family and other genes responsible for maintenance of the genome e.g. BRCA2, microsatellite instability. Given this knowledge, it has been possible to postulate the cancer progression model with the potential for early diagnosis, screening and perhaps selection for chemotherapeutic agents. It has now been shown that K-ras mutations frequently occur early, whereas changes in the expression of the p16 gene appear in intermediate lesions. The inactivation of the p53, DPC4 genes occur late in the neoplastic progression model.

Table 3.1. Frequency of major genetic alterations in pancreatic cancer

Gene	(ref)	Normal	PanIN1A	PanIN1B	PanIN2	PanIN3	Carcinoma
K-ras	(1)		0%	38%	44%	87%	_
	(2)	3%	30%	31%	73%	_	90-100
INK4a	(3)	0%	30%	27%	55%	71%	100%
P53	(3)	0%	_	_	_	12%	40%
	(4)	0%	_	35%	_	36%	40%
	(5)	0%	0%	0%	20%	57%	47%
DPC4	(6)	_	0%	0%	0%	31%	55%

(1)Terhune PG et al, 1998, (2) Biankin AV et al, 2001, (3) Moskaluk CA et al, 1997, (4) Apple SK et al, 1999, (5) DiGiuseppe JA et al, 1994, (6) Wilentz RE et al, 1998.

Adapted from Schneider G and Schmid RM (2003) "Genetic alterations in pancreatic carcinoma". Molecular Cancer; 2:15.

Chapter 4 Prognostic molecular markers in Pancreatic cancer

4.1 Introduction

Pancreatic cancer is a very aggressive tumour with the majority of patients presenting with unresectable disease. The minority who undergo resection have a longer median survival of 18-24 months with a 5-year survival rate of less than 10% (Gudjonsson B et al, 1987; Bramhall SR et al, 1995). Resectability rates of pancreatic cancer have steadily improved, mainly because of improved postoperative care and the concentration of pancreatic resections in experienced centres (Neoptolemos JP et al, 2001, Riall TS et al, 2005). Moreover, the currently available chemotherapy and radiotherapy have not shown improved survival in advanced disease but a number of trials studying the efficacy of various forms of adjuvant therapy are in progress (Ghaneh et al, 2001, Abbott, DE et al, 2010). It is important therefore to have reliable prognostic factors to assess the efficacy of the increasingly numerous surgical and adjuvant therapies advocated in an attempt to improve survival rates in patients with pancreatic cancer. Prognostic factors are used to assess the risk of systemic disease, recurrence or death after primary treatment and as a guide to the use of adjuvant therapy.

Clinical prognostic variables used in many staging systems of solid tumours include tumour stage, tumour size, grade and lymph node involvement. Current staging systems in use include the UICC/TNM classification in the West and the JPS system in Japan but as discussed in chapter 2, they are not comparable. This causes difficulties when comparing survival data due to 'stage system migration'. The serum concentrations of tumour markers such as CA19-9, CA242 and MUC1 may have prognostic significance in advanced disease but not in patients with small tumours. Biological factors provide further prognostic information but although not effectively used in a routine clinical setting, they improve

understanding of cancer and development of new treatments. The clinical factors that have been studied as shown in this chapter are unsatisfactory in clinical the management of this lethal tumour and the two major staging systems are inadequate. The molecular biology of pancreatic cancer is now better understood and consistent genetic changes include overexpression of growth factors and growth factor receptors (EGF, TGF alpha, amphiregulin, c-erbBs 2-4,aFGF, βFGF, TGFR, TGFβRs); inactivation of tumour suppressor genes (TS), p53, p16, p21, SMAD4 and activation of dominant oncogenes (K-ras and cyclin D1). The possible use of these molecular markers for prognosis in pancreatic cancer has been suggested.

4.2 Tumour suppressor genes

4.2.1 p53

Mutations in the tumour suppressor gene p53 contribute to the development of up to half of all human cancers (Harris CC, 1993); Greenblatt MS et al, 1994). A high degree of p53 expression has been reported in several human cancers (Lipponen P et al, 1993; Joypaul BV et al, 1994; Auvinen A et al, 1994; Hardwick RH et al, 1997). The correlation between p53 expression and prognosis however varies in different studies and in different forms of cancer. In pancreatic cancer, a high level of p53 expression has been demonstrated in up to 75% of tumours (Barton et al 1991; Zhang et, 1994; Rozenblum et al, 1997; Apple SK et al, 1999). Results of p53 overexpression in pancreatic tumours are conflicting. Lundin et al (1997) found no association between p53 overexpression and tumour stage, histology, age, sex or survival in a study of 133 patients with pancreatic cancer. Other studies have demonstrated similar results (Zhang et al, 1994; Dergham et al, 1997a; Ruggeri et al, 1997; Makinen et al, 1998). Three small studies have reported reduced survival (Yokohama et al, 1994; Weyrer et al, 1996; Linder et al, 1997; Sessa F et al, 1998). Yokohama et al (1994) reported a

marginally significant association in 69 patients. DiGiuseppe et al (1994) in a study of 48 patients reported an association with poor prognosis although it was not significant and in the study by Linder et al (1997), a different cut off level of 1% p53 immunoreactivity was used. In a study of both p53 and Bcl-2 in the same patients, Bold et al, (1999) found that patients whose tumours stained positive for p53 and Bcl-2 had a longer survival period when compared to tumours that stained negative for both markers. Two studies investigated 58 patients of whom 28 had surgery alone and the other 30 had adjuvant chemotherapy. Patients with p53 positive tumours (shown by immunostaining) who had chemotherapy had a significantly better survival times than those who did not (Nio Y et al, 1998). This is probably a reflection of the fact that tumours containing a mutated p53 are usually radio resistant and/or chemo resistant (Dergham St et al, 1998). Patients with combined p53 positive and p21 negative tumours had significantly poorer survival and those of this group who had chemotherapy had a non-significant trend for improved survival (Nio Y et al, 1999). Immunhistochemistry has been used to examine premalignant lesions for p53 inactivation. DiGiuseppe et al found immunoreactivity in 12% of 17 cases of histologically high grade lesions (carcinomas in situ) while lower-grade lesions did not show immunoreactivity to p53 (DiGiuseppe JA et, 1994). This suggests that p53 gene inactivation is a late event in genetic progression in pancreatic ducts. The wide variation in p53 status seen with IHC can be partially explained by methodology; fresh specimens will give a more accurate picture than specimens that have had a long delay between extraction and processing. A more accurate approach is the use of microdissection to obtain samples containing only cancerous cells, followed by direct sequencing. Rozenblum et al (1997) for example found p53 mutation in 31 out of 41 (76%) samples. Apple et al, 1999 used IHC on 15 cases of PDAC and found that 13 out of 15 (87%) invasive carcinomas stained for p53, compared to 3 out of 15 (20%) dysplastic areas. Only one out of 50 samples showed positive p53 staining in normal or

hyperplastic cells. In contrast, 11 out of 15 (73%) dysplastic areas stained positive for the mutant K-RAS gene product p21. p53 mutation therefore represents a later genetic event in PDAC than K-RAS mutation (Apple SK, 1999).

4.2.2 p16

Approximately 85% of all pancreatic cancers have mutated or deleted p16 gene (Caldas C et al, 1994; Liu Q et al, 1995; Naumann M et al, 1996). The p16 gene has been shown to be genetically mutated or its expression abrogated in duct lesions (Wilentz RE et al, 1998; Moskaluk CA et al, 1997). Its inactivation can be detected occasionally as early on in flat intraductal neoplasia (including PanIN-1-A). Wilentz et al (1998), using immunohistochemistry for the p16 gene product, showed that 30% of flat duct lesions without significant atypia, 55% of papillary duct lesions without significant atypia, and 71% of papillary duct lesions with significant atypia had loss of expression of the p16 gene product. Bartsch D et al (1996) reported that the survival of patients whose tumours contained CDKN2 mutations was significantly less than that of patients whose tumours contained no mutations (median survival times of 8.5 months and 17.0 months respectively; $p<0.01$). Naka T et al (1998) in an immunohistochemical study of 32 cases of pancreatic cancer, found that 19 out of 32 (59%) stained positive for p16 protein and there was a significant correlation between p16 negativity and poor prognosis.

4.2.3 p21

p21 overexpression is an early event in the development of pancreatic intraepithelial neoplasia (Biankin AV et al, 2001). In a study of 451 Pan IN lesions present in the pancreas of 60 patients, p21 overexpression was found in 9% of normal ducts 9%, in 16% of patients with Pan IN-A lesions, 32% in Pan IN-B lesions, 56% with PanIN-2 lesions, 80% in patients with PanIN-3 and 85% of patients with invasive carcinoma ($p<0.01$). In the same study, p53 and cyclin D1 occurred predominantly in PanIN-3 lesions (Biankin AV et al, 2001). p21 overexpression was found to be independent of p53 and DPC4/Smad4 expression within invasive carcinoma and PanIN-3 lesions demonstrating that it is an early event in the development of Pan IN appearing before aberrations in p53, cyclin D1 and DPC4/Smad4 expression. This may possibly be explained by increased Ras activity either through activating K-ras mutations or by c-erbb-2 overexpression (Biankin AV et al, 2001). Previous work by Coppola D et al (1998) observed a lack of correlation between p53 alterations and $p21^{WAF1}$ expression in human pancreatic cancer, a finding that is consistent with the reported TGF-ß1 induction of $p21^{WAF1}$ through a p53-independent mechanism (Coppola D et al, 1998). Published studies so far have not shown any prognostic value in p21 expression as demonstrated with immunohistochemistry (Okada N et al, 1995; Coppola D et al, 1998; Ruggeri BA et al, 1997). A study of 75 cases by Dergham et al (1997) however reported a better survival of patients with an earlier clinical stage who overexpressed the p21 protein. They also reported that there was improved survival with adjuvant treatment in tumours that were p21 positive and p53 negative (Dergham ST et al, 1997). The role of chemotherapy in these patients may be important; in one study using immunostaining patients with a combination of p53 positive (implying the presence of mutant p53) and p21 negative tumours demonstrated poorer survival following resection and adjuvant chemotherapy (Nio Y et al, 1999). In another study median survival in patients with resected pancreatic cancer who

received adjuvant chemo radiation with $p21^{WAF1}$ positive tumours was significantly longer than those with negative staining (25 months versus 11 months p=0.01) (Ahrendt SA et al, 2000).

4.2.4 BRCA2

Carriers of this mutation have a ten-fold higher risk of developing pancreatic cancer than the general population (Bretnall TA et al, 2000). The penetrance of the mutation seems to be low and there are likely environmental factors involved resulting in a rather late onset of the disease. In addition, the exact incidence of BRACA2 germline mutations in the general population has not yet been determined and any screening programme needs more information in order to be cost effective. Mutations appear to occur often in Ashkenazi Jews (617delT). The estimated risk of acquiring pancreatic cancer by the age of 75 is 7% in the carriers as compared to 0.85% in the general population (Ozcelik H et al, 1997). Mutation analysis of different grades of precursor lesions [pancreatic intraepithelial neoplasia (Pan IN)] for allelic imbalance in germline BRCA2 mutations in carriers with pancreatic cancer revealed loss of heterozygosity in one grade 3 Pan IN and in none of 13 grade 1 Pan IN lesions. These findings indicate that LOH at the BRCA2 gene locus is not likely to be an early event in tumourigenesis unlike several other genes in inherited cancer syndromes [for instance APC gene in familial adenomatous polyposis (Goggins M et al, 2000)].

4.3 Oncogenes

4.3.1 cyclin D1

A study of 82 pancreatic cancers demonstrated overexpression (by immunostaining) of cyclin D1 in 65% of tumours and this was associated with shorter survival for these patients, but not independently of tumour stage and grade (Gansauge F et al, 1998). In another study (n=16 patients) a median survival of 6.5 months was reported in patients with higher levels of cyclin D1 compared to 15.5 months in those with low expression (Kornmann M et al, 1998). In a more recent study of 129 patients, there was no correlation between cyclin D1 overexpression and survival (Biankin AV et al, 2002). Cyclin D1 and DPC4 /SMAD4 overexpression or loss was seen in 85% of invasive carcinomas but in only 14% of PanIN-2 lesions. With respect to adjuvant treatment, the inhibition of cyclin D1 in addition to suppressing the growth of pancreatic cancer cells enhances their responsiveness to cisplatinum and this could possibly be due to the altered expression of several chemo resistance genes (Kornmann M et al, 1999).

4.3.2 K-ras

Kras mutations have been reported to occur with a very high frequency in pancreatic cancer with point mutations found in 70-90% of cases, the most common of which result in the substitution of the wild type glycine residue (codon 12) by cysteine, arginine, valine or aspartic acid (Smit et al, 1998; Lemoine NR et al, 1992a; Finkelstein et al, 1994). This high frequency of mutations is not found in other tumours. Mutated K-ras is present in both invasive and the earlier non-invasive cells. Evidence for this comes from a number of studies. Sugioe et al performed microdissection from tissue adjacent to areas of unequivocal malignancy and were able to demonstrate K-ras mutations in all of the 17 carcinoma-in – situ lesions, and 19 out of 21 (90%) of the atypical hyperplasia lesions (Sugio K et al, 1997). Additional evidence for this came from a larger study on operative specimens in which a

third of early ductal lesions were found to have a K-ras mutation (Luttges J et al, 1999). These two studies are also supported by work done using a hamster model of experimental PDAC using N-nitroso-bis (2-oxopropyl) amine (BOP) (Fujii H et al, 1990; Cerny WL et al, 1992). BOP induces pancreatic cancer characterized by a transition at codon 12 of K- RAS (GGT to GAT), causing a substitution of aspartate for glycine. This is the most frequently occurring K-ras mutation in human PDAC and furthermore the same experimental model gives rise to K-ras mutation in early lesions (described in the report as hyperplasia, papillary hyperplasia and carcinoma – in- situ). A number of investigators have micro dissected the duct lesions of the pancreas and analysed them for K-ras mutations (Yanagisawa A et al, 1993; Caldas C et al, 1994; Wilentz RE et al, 1998). The first genetic evidence for a progression model for these duct lesions was provided by Caldas et al (Caldas C et al, 1994; Yanagisawa A et al, 1993). They noted that activating point mutations in codon 12 of the K-ras gene occur in about half of the non papillary duct lesions, but were present in the vast majority of papillary and more advanced lesions. It is this progression in K-ras mutational frequency that suggests that K-ras is not necessarily the first change, or gatekeeper required for pancreatic ductal neoplasia (Kinzler KW et al, 1997).

The presence or the type of K-ras mutations in pancreatic tumours has not been shown to be associated with patient survival (Hruban RH et al, 1999; Dergham ST et al, 1997). Hruban et al (1999) studied 82 cancers and found mutations in 63 (83%). There was a higher prevalence of mutations in 72 patients (88%) who had smoked cigarettes at some time in their life; 67 patients (86%) in current smokers and 78 patients (89%) in ex-smokers. The presence of mutations did not correlate with survival (Hruban RH et al, 1999). Scarpa (1994) reported differences in the pattern of Kras mutations seen in the Japanese as compared to Europeans and also regional variations (Scarpa A et al, 1994), raising the possibility of ethnic or environmental differences. K-ras mutation analysis in Europeans showed

differences amongst countries; all mutations in an Italian population (80.5%) involved the second base and were G to A transitions or G to T transversions while in a Spanish population (73%) different bases were involved (Scarpa A et al, 1994). In a study of 51 Japanese and 34 Chinese patients, K-ras point mutations were found in 94% and 71% respectively and in addition the patterns of mutations was different (Song et al, 2000). The GAT mutation appeared more in the Japanese and there was also a higher ratio of transitions to transversions as compared to the Chinese. It is likely that environmental and social factors may contribute to these differences (Song et al, 2000). Given that the risk of pancreatic cancer is significantly elevated in patients with chronic pancreatitis, a study was carried out to determine the frequency of K-ras mutations in chronic pancreatitis. DNA samples from 21 normal pancreatic tissues, 26 chronic pancreatitis tissues and 24 pancreatic cancers were analysed for K-ras mutations. None of the DNA samples from normal or chronic pancreatitis exhibited a K-ras mutation at codons 12 or 13. In contrast 17 of the 24 pancreatic cancer were found to have a K-ras mutation (Hsiang D et al, 1997). DNA from the pancreas of intraoperative resected specimens of 60 patients with chronic pancreatitis and 11 patients with histologically confirmed pancreatic cancer was evaluated by PCR amplification. In none of the 60 samples of chronic pancreatitis (CP) could K-ras mutations be found (Ort M et al, 1998). Boadas however found K-ras mutations in 8 of 49 cases (16%) of CP studied (Boadas J et al, 2001). Mutant K-ras was detected in pancreatic juice from 20 out of 54 patients (37%) with CP and on long term follow up (mean 78 months) there was no evidence of the development of PDAC (Furuya N et al, 1997). Even more surprisingly some reports also suggest the presence of mt K-ras in patients with no pancreatic disease at all (Luttges J et al, 1999). This undermines the use of mt K-ras alone as a marker of pancreatic pathology. Moreover, it implies that the pancreas is in some way resistant to K-ras mutation that would normally induce death or that there may be another, as yet unknown, pre-existing genetic

event that allows K-ras mutation to be tolerated. The simplest method of detecting K-ras mutations is to amplify K-ras DNA using the polymerase chain reaction (PCR) and then sequence the product. However, neoplastic cells will normally comprise only 25% of the pancreatic tumour mass, the remainder being composed of the desmoplastic response. Hence microdissection of suspected tumour tissue is essential for accurate genetic analysis. To improve sensitivity various techniques have been developed to enrich mutant sequences e.g. restriction fragment length polymorphism (RFLP) and the amplification refractory mutation system (ARMS) based on mutation specific primers in combination with PCR. Clinical trials using vaccinations against mutant K-Ras protein are in progress (Z'graggen Z et al, 2000). Vaccinations using either directly injected peptides corresponding to the mutant codon 12 or through insertion of mini-gene cassettes that express mutant K-RAS peptides that reflect the mutation found in host cells (Gjertsen MK et al, 1995; Khleif SN et al, 1999).

4.4 Growth factor receptors

4.4.1 c-erbB-2

The c-erbB-2 proto-oncogene has been reported to be overexpressed in a number of human tumours (Yokota J et al, 1986; Gullick WJ et al, 1987; Scorilas A et al, 1995; Hardwick RH et al, 1997; Morote J et al, 1999). Few studies exist on c-erbB-2 overexpression and survival in pancreatic cancer. C-erbB-2 is overexpressed in about 20% of invasive pancreatic cancers and this is usually attributed to gene amplification. . In precursor lesions c-erbB-2 has been reported to be expressed in flat duct lesions, papillary duct lesions without atypia and atypical duct lesions in 82%, 86% and 92% of cases respectively (Day et al, 1996). Hall reported c-erbb-2 overexpression in 17 (19%) of 87 cases of pancreatic cancer studied (Hall PA, 1990). In a series of 79 pancreatic tumours Dugan MC et al (1997) studied c-erbB-2 expression and correlated this to histological grade and survival. They found a lower expression of c-erbB-2 in poorly differentiated parts of the tumour as compared to the well differentiated and poorly differentiated areas but no difference in survival. In a recent study of 154 patients with pancreatic cancer, Safran H et al (2001) reported that thirty patients (21%) demonstrated cerbB-2 overexpression by immunohistochemistry but this did not correlate with survival. Similar results have been found by Novotny J et al (2001) in 57 cases studied. In contrast, Lei S et al (1995) in a small series of 21 pancreatic cancers found c-erbB-2 overexpression in 10 (47.6%) of cases and this was closely and inversely related to survival. This study was supported by Okada et al (1995) who found a correlation between survival and serum levels of c-erbB-2.

4.4.2 c-erbB-3

The c-erbB-3 gene has no known natural ligand but shares a similar structure to EGF receptor and c-erbB-2. In normal pancreas c-erbb-3 is only seen in the islets of Langerhans but has been detected in up to 90% of pancreatic and ampullary cancers (Lemoine NR et al, 1992). Freiss et al (1995) found the c-erbB-3 overexpression in 47% (27 of 58) cases and these were found to correlate with decreased patient survival.

4.4.3 TGFBs and TGFB receptors

The three isoforms of TGFβ have been shown to be overexpressed in pancreatic cancer. Survival data from 60 patients with pancreatic cancer showed a significantly decreased survival time with overexpression of these isoforms (Friess H et al, 1993**;** Lu Z et al, 1997). In a more recent study however, TGFβ-1, which was expressed in 31%, was not significantly associated with survival (Coppola D et al, 1998). The TGFβ receptors are all found in normal pancreas but in cancer there is an overexpression of TGFβ-2 receptor only (Friess et al, 1993b)

4.4.4 Fibroblast growth factors

Both αFGF and βFGF are overexpressed in pancreatic cancer tissue at the mRNA and protein levels (Yamanaka Y et al, 1993; Vickers SM, 1999). Expression of βFGF and FGFR but not αFGF is associated with poor prognosis (Yamanaka Y et al, Ohta T et al, 1995).

4.5 Apoptotic factors

Several recent studies have looked at these factors in pancreatic cancer. In a study of 60 patients tumour expression of bax immunostaining was associated with significantly longer survival compared with patients who had negatively staining tumours (Friess H et al, 1998). Bcl-2 also has prognostic significance (Friess H et al, 1998) but several studies have shown that expression of the bcl-xL is significantly associated with poor survival (Friess H et al, 1998; Miyamoto Y et al, 1999; Evans JD et al, 2001). This is surprising since bcl-xL inhibits apoptosis and suggests reduced anti-apoptosis by heterodimerization.

4.5.1 Vascular Endothelial Growth Factor (VEGF) Platelet derived Endothelial Cell Growth Factor (PD ECGF)

VEGF and PD-ECGF are frequently co-expressed in human cancers. Intratumoural micro vessel density (MVD) is a prognostic marker in several tumour types including breast cancer. Two studies in pancreatic cancer (a relatively hypovascular tumour) did not show prognostic value for MVD; the expression of PD-ECGF however was associated with a significantly reduced survival (Fujimoto K et al, 1998; Ellis LM et al, 1998). These latter studies also showed no prognostic value for VEGF expressing tumours but a third study found a significantly shorter survival for those patients with VEGF positive tumours (Ikeda N et al, 1999).

4.5.2 SMAD4 (DPC4-deleted in pancreatic carcinoma, locus 4)

Immunohistochemical labelling for the SMAD4 gene product has been reported to correlate with gene expression (Wilentz et al, 2000). Loss of DPC4/Smadd4 expression cosegregated with resectability ($p<0.0001$) and was associated with improved survival after resection ($p<0.0001$), whereas resection did not improve survival in patients whose tumour expressed

DPC4/SMAD4 (p=5). They concluded that preoperative assessment of this gene has a potential as a prognostic maker in patients with pancreatic cancer given that resection did not benefit those patients whose cancers expressed DPC4/Smad4.

4.6 E-cadherins and matrix metalloproteinases (MMPs)

The expression of metalloproteinases has been shown to directly correlate with invasion and metastasis in pancreatic cancer. A recent study assessed the expression of E-cadherin and MMP-2 and MMP-9 using in-situ hybridisation in pancreatic cancer (Kuniyasi H et al, 1999). Expression of E-cadherin in pancreatic cancer was inversely correlated with tumour progression and the development of metastases. Patients with cancers that had an expression ratio of MMP: E-cad <3 had a significantly better prognosis than those with ratio of >3. Inactivation of the adenomatous polyposis coli (APC) is observed at early stages of intestinal tumour formation, whereas loss of E-cadherin is usually associated with tumour progression. Smit has demonstrated in an Apc mouse model that because both proteins compete for the binding to beta-catenin, an essential component of the Wnt signalling pathway, and a reduction of E-cadherin levels could influence both tumour initiation and progression (Smits R et al, 2000). In addition the loss or reduced function of E-cadherin may affect tumour formation by altering its cell-adhesiveness.

4.7 DNA Mismatch Repair Genes

In a small series of nine pancreatic carcinomas Han H-J et al (1993) found microsatellite instability in six pancreatic ductal adenocarcinomas.

4.8 DNA Ploidy and cell proliferation

The content of DNA in the nuclei of tumour cells has been correlated with survival in pancreatic cancer. There seems to be agreement that diploid tumours have slightly better survival than aneuploidy tumours, but there is no real effect on survival (Linder S et al, 1994; Takada T et al, 1995; Berczi C et al, 1999). Correlation with liver metastases in pancreatic cancer has been reported with increased levels of Ki-67, a marker of proliferating tumour cells (Ferrara C et al, 1999).

Table 4.1. Immunohistochemical studies of molecular factors in pancreatic cancer

Study	Year	No	Factor	Expression	Survival Median (mths)	5year (%)	P value
Gansauge et al	*1998*	*82*	*cyclin D*	+	*10*	-	
				-	*18*	-	*<0.01*
Friess et al	*1998*	*60*	*bax*	+	*12*	-	
				-	*5*	-	*<0.04*
Friess et al	*1998*	*74*	*bcL-xL*	+	*5*	-	
					12	-	*<0.05*
Evans et al	*1998*	*24*	*bcL-xL*	+	*6*	-	
				-	*20*	-	*<0.002*
Dong et al	*1998*	*57*	*EGF+R*	+	*10*	-	
				-	*17*	-	*<0.002*
Lei et al	*1995*	*21*	*c-erbB-2*	+	*7*	-	
				-	*19*	-	*<0.01*
Friess et al	*1995*	*58*	*c-erbB-3*	+	*9*	-	
				-	*13*		*<0.04*
Friess et al	*1995*	*60*	*TGFβ-1*	+	*9*	-	
				_	*12*	-	*<0.05*
			TGFβ-2	+	*7*	-	
				-	*13*	-	*<0.001*
			TGFβ-3	+	*7*	-	
				-	*12*		*<0.01*
Coppola et al	*1998*	*42*	*TGFβ-1*	+	*13*	*23*	
				-	*5*	*4*	*<0.05*
Yamanaka et al	*1993*	*78*	*βFGF*	+	*9*	-	
				-	*16*	-	*<0.001*
Bramhall et al	*1996*	*50*	*MMP-2 –3 & timp1*	+	*50*		*<0.0001*
Seo Y et al	*2000*	*142*	*vegf*	+	*132*		
				-	*10*		*< 0.0001*

EGFR+R=epidermal growth factor and receptor; TGFß = transforming growth factor beta; bFGF=basic fibroblast growth factor.

4.9 Summary

The molecular alterations found in pancreatic cancer, loss of control at key points in the cell cycle together with the over expression of growth factors help to explain the poor prognosis associated with this tumour. In addition angiogenic factors and apoptotic genes have significant correlations with survival and/or treatment responsiveness in pancreatic cancer. The effect of oncogene mutations and tumour suppressor gene loss on the biology of pancreatic cancer is very complex and is still not fully understood in pancreatic cancer. There is now the potential to repair the genetic defects and to develop pharmacological agents that counter the resulting changes. Pancreatic cancer is the only gastrointestinal malignancy with a consistent genetic profile and this places scientists in a position to take advantage of the recent advances in molecular biology techniques to improve diagnosis and treatment. Given the inadequate staging systems available at present, the genetic changes in pancreatic cancer may provide a basis for prognostic markers.

4.10 Research aims

Previous studies of molecular prognostic markers following resection for exocrine pancreatic cancer have produced conflicting results. The aim of this project was to undertake a comprehensive analysis of potentially useful molecular markers in a large, multicentre patient population and to compare these markers with standard pathological prognostic variables. Formalin-fixed, paraffin-embedded specimens of pancreatic ductal adenocarcinoma were analyzed from 157 patients who had undergone pancreatectomy in the United Kingdom and Sweden. Immunohistochemistry was used to detect expression of $p16^{INK4}$, p53, $p21^{WAF1}$, cyclin D1, erbB-2 and erbB-3. Mutations in p53 and K-ras were detected using Sequence Specific Conformational Polymorphism (K-ras only) and sequencing following DNA extraction and amplification by PCR.

CHAPTER 5: IMMUNOHISTOCHEMICAL STUDIES

5.0 Immunohistochemistry

The aim of this part of the study was to employ immunohistochemistry to study the significance of growth factor overexpression including receptors (c-erbB-2 and c-erbB-3), p53, p21 and cyclin D1 overexpression and p16 loss in resected ductal pancreatic adenocarcinoma using formalin-fixed paraffin-embedded glass slides. The data produced was analysed in conjunction with the Union International Contre Le Cancer system using univariate and multivariate analysis. Immunohistochemistry is an inexpensive method to detect altered protein products. Many investigators use IHC as a screening test prior to DNA sequencing, and staining is often considered a surrogate marker for gene mutation in the absence of confirmatory DNA studies. Other methods for screening tumours to identify those likely to contain mutations involve detection of altered electrophoretic mobility patterns of DNA sequences containing point mutations e.g. Sequence Specific Conformational Polymorphism (SSCP).

5.1 Introduction

Recent molecular studies have identified a high frequency of genetic abnormalities in growth factor regulation (e.g. c-erbB-2 and 3), inactivation of tumour suppressor genes (e.g. p53, p21, p16) and activation of dominant oncogenes (e.g. cyclin D1) in pancreatic cancer formation. Currently used staging systems are poor at predicting the biological behaviour of pancreatic cancer, particularly with “small” or “early” pancreatic tumours. Therefore accurate prognostic systems are required to assess the outcome of different forms of treatment currently under investigation. Previous immunohistochemical studies of molecular markers in pancreatic cancer are few with small numbers of patients and usually studying expression of a single gene. Immunohistochemistry is a method where localisation and

visualisation of cellular or tissue components (antigen) *in situ* by use of specific antibody: antigen reactions and has become important in the diagnosis of tumours. As well as being important diagnostically, the expression of several antigens expressed by immunohistochemistry correlates with the prognosis of a number of cancers and may influence treatment (Gaskell DJ et al, 1989). The principle of the method was first described by Marrack (1934). The avidin biotin complex (ABC) technique and the similar streptavidin biotin complex (StrepABC) were the most commonly used methods. The principle of the ABC method is based upon the high affinity that Streptavidin, a large bacterial protein has for biotin, a low molecular weight vitamin found in egg yolk

5.2 Avidin-biotin indirect

The high affinity of avidin for biotin is exploited in immunohistochemistry. Avidin has four binding sites for biotin although the molecular orientation of these binding sites is such that fewer than four molecules will actually bind. A secondary antibody that contains a covalently attached biotin molecule is able to occupy these unbound sites, hence allowing attachment of the avidin-biotin complex. Labelling of the avidin-biotin complex with a peroxidase molecule enables the use of a chromagen reaction, involving hydrogen peroxidase, to complete the immuno-labelling process. If the primary antibody used in this method is derived from mouse or rabbit, the secondary antibody must be directed against mouse or rabbit immunoglogulins respectively. The third step involves the application of a pre-formed avidin-biotin complex labelled with horseradish peroxidase. This complex binds to the biotinylated secondary antibody, which in turn is bound to the primary antibody. A substrate-chromagen solution containing 3, 3'- diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide is then applied to the sections for eight minutes. As a result of these steps, the staining patterns in the section reflect the presence or absence of the antigen of interest.

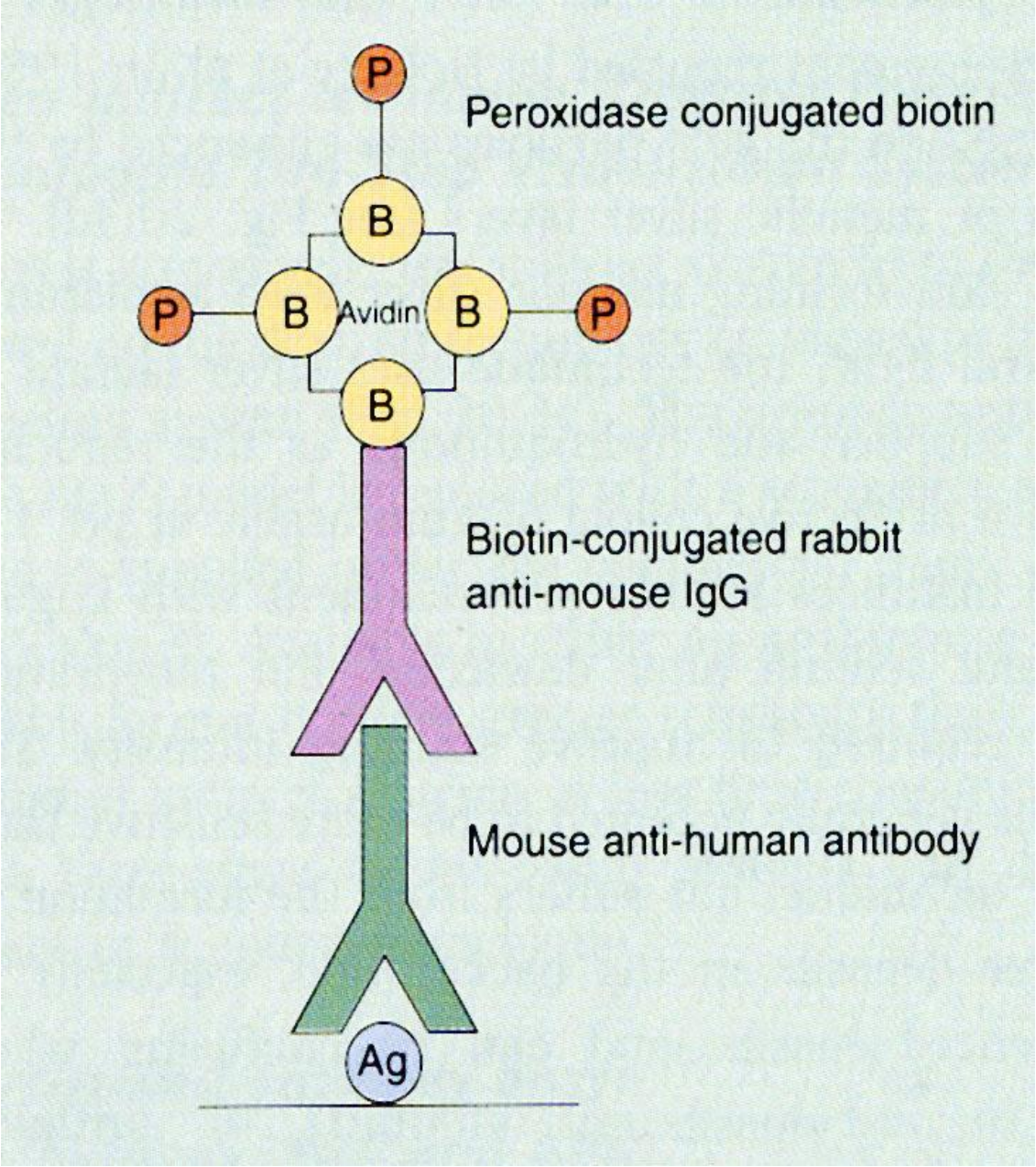


Figure 5.1: Avidin- Biotin Complex.

5.3 Patients

Three centres contributed archival pathological specimens from one hundred and fifty seven patients who had undergone resection for pancreatic ductal carcinoma with curative intent. There were 69 patients from the University Hospital of Lund, Lund Sweden operated upon between 1969-1984; 66 patients who had undergone resection between 1984 and 1995 at the Queen Elizabeth Hospital, Birmingham, UK; and 22 patients who had undergone resection between 1990 and 1997 at the Royal Liverpool University Hospital, Liverpool, UK. Thirteen patients (8%) had adjuvant treatment as part of the ESPAC trial (Neoptolemos JP et al, 2001). Archival formalin fixed paraffin embedded blocks were retrieved from the respective pathological departments. Survival and clinico-pathological data were obtained from hospital records, cancer registries and in a few cases by contact with the general practitioner of the patient.

Table 5.1 Swedish clinical data

Case	Age	Clear margins	Grade	Stage	Tumor size (cm)	Lymph node	Adjuvant treatment	Survival (Days)
1	70	Yes	Poor	3	2.5	Yes	None	7
2	60	Yes	Poor	3	3.5		None	135
3	60	Yes	Moderate	2	2.0		None	420
4	73	Yes	Moderate	3	4.5		None	161
5	46	Yes	Well	1	2.0		Dxt	269
6	59	Yes	Poor	3	3.0		None	**0**
7	47	Yes	Moderate	3	3.5		None	104
8	38	Yes	Moderate	3	4.0		None	154
9	56	Yes	Poor	3	4.0	Yes	None	327
10	48	Yes	Poor	3	3.0		Dxt	473
11	65	Yes	Poor	3	2.0		None	461
12	77	Yes	Poor	3	3.0		None	670
13	65	Yes	Poor	1	5.0		None	402
14	59	Yes	Poor	1	3.0		None	389
15	46	Yes	Poor	1	2.0		None	498
16	45	Yes	Poor	3	3.0		None	390
17	67	Yes	Poor	1	4.0		None	53
18	60	Yes	Moderate	3	2.5		None	131
19	68	Yes	Well	1	5.5		None	120
20	58	Yes	Moderate	2	2.0	No	None	252
21	72	Yes	Poor	3	4.5	Yes	None	206
22	64	Yes	Moderate	3	4.0	Yes	None	511
23	53	Yes	Poor	1	4.0	No	None	637
24	56	Yes	Moderate	2	2.0	No	None	210
25	68	Yes	Moderate		3.5		None	210
26	62	Yes	Moderate	1	1.5	No	None	390
27	61	Yes	Poor	2	5.0	No	None	210
28	56	Yes	Poor	1	3.0	No	None	533
29	59	Yes	Moderate	2	3.0	No	None	13
30	71	Yes	Poor	3	4.0	Yes	None	342
31	72	Yes	Moderate	1	3.0	No	None	1120
32	70	Yes	Poor	1	3.5	No	None	716
33	75	Yes	Moderate	2	6.0	No	None	403
34	56	Yes	Poor	3	5.0	Yes	None	210
35	46	Yes	Moderate	3	4.0	Yes	None	137
36	66	Yes	Moderate	2	3.0	No	None	60
37	64	Yes	Poor	3	3.5	Yes	None	29
38	53	Yes	Moderate	3	3.5	Yes	None	204
39	69	Yes	Poor	3	2.0	Yes	None	210
40	68	Yes	Poor	3	4.0	Yes	None	112
41	73	Yes	Poor	2	4.0	No	None	30
42	58	Yes	Moderate	3	2.0	Yes	None	92
43	67	Yes	Moderate	3	4.0	Yes	None	347
44	49	Yes	Well	1	3.0	No	None	1308
45	65	Yes	Poor	1	3.5	No	None	454
46	76	Yes	Poor	2	2.5	No	None	219
47	56	Yes	Moderate	3	3.0	Yes	None	440
48	54	Yes	Poor	2	4.0	No	None	181
49	77	Yes	Moderate	2	1.5	No	None	337
50	59	Yes	Moderate	1	4.0	No	None	150
51	71	Yes	Moderate	1	2.0	No	None	1769
52	55	Yes	Well	3	1.0	Yes	None	234
53	68	Yes	Well	3	6.0	Yes	None	125

54	51	Yes	Moderate	3	4.0	Yes	None	798
55	63	Yes	Moderate	1	2.0	No	None	458
56	74	Yes	Well	3	5.0	Yes	None	157
57	59	Yes	Moderate	3	2.0	Yes	None	316
58	67	Yes	Well		5.0		None	157
59	61	Yes	Poor	3	5.0	Yes	None	150
60	65	Yes	Moderate	3	4.0	Yes	None	195
61	72	Yes	Moderate	3	4.0	Yes	None	37
62	74	Yes	Poor	1	5.0	No	None	1278
63	60	Yes	Moderate	2	5.0	No	None	330
64	49	Yes	Moderate	3	5.0	Yes	None	267
65	43	Yes	Poor	3	8.0	Yes	None	90
66	65	Yes	Poor	2	2.5	No	None	900
67	65	Yes	Poor	3	2.0	Yes	None	240
68	76	Yes	Moderate	3	2.0	Yes	None	77
69	66	Yes	Moderate	1	4.0	No	None	330

Blank spaces indicate that data was not available.

Table 5.2 United Kingdom clinical data

Case	Age	Clear margins	Grade	Stage	Tumour Size (cm)	Lymph node	Adjuvant treatment	Survival (Days)
70	47	Yes	Poor	3	4.0		None	367
71	60	No	Well	3	7.0		None	63
72	42	Yes	Moderate	3	3.0		None	305
73	54	Yes	Moderate	1	1.0		None	451
74	61	Yes	Moderate	3	3.0		Dxt/Chem	450
75	48	Yes	Poor	3	2.0		None	34
76	63	Yes	Moderate	2	1.5		None	197
77	74	Yes	Moderate	2	3.5		Dxt/Chem	393
78	71	Yes	Poor	2	3.4		Dxt	293
79	50	Yes	Moderate	3	3.0		None	118
80	65	Yes	Well	3	2.0		Dxt/Chem	313
81	51	Yes	Moderate	2	3.1		Dxt/Chem	1827
82	74	Yes	Moderate	3	1.0		Dxt/Chem	190
83	48	Yes	Poor	2	2.0		None	760
84	49	Yes	Moderate	3	3.0		Dxt/Chem	229
85	46	Yes	Poor	3	2.5		Dxt/Chem	300
86	48	Yes	Well	2	1.0		Dxt	1060
87	68	Yes	Well	2	5.0		None	2485
88	74	Yes	Moderate	3	3.0		None	1206
89	64	Yes	Moderate	2	2.0		None	170
90	40	Yes	Moderate	1	1.0		-	353
91	60	Yes	Moderate	2	2.0		None	924
92	60	Yes	Poor	3	3.0		Dx	106
93	69	Yes	Well	2	2.0		Dxt/Chem	1113
94	45	Yes	Poor	2		No	None	

95	65	Yes	Moderate	3		Yes	None	767
96	48	Yes	Moderate	3		Yes	None	88
97	55	Yes	Poor	1		No	None	509
98	52	Yes	Poor	2		No	None	103
99	53	Yes	Well	3		No	None	193
100	69	Yes	Moderate	3		Yes	None	167
101	57	Yes	Moderate	3		No	None	23
102	65	Yes	Poor	2		No	None	450
103	63	Yes	Moderate	3		No	None	245
104	53	Yes	Moderate	3		Yes	None	493
105	63	Yes	Poor	1		No	None	475
106	65	Yes	Moderate	3		Yes	None	568
107	51	Yes		3		Yes	None	50
108	59	Yes	Moderate	3		Yes	None	443
109	62	Yes	Moderate	2		No	None	547
110	71	Yes	Well	2		No	None	915
111	73	Yes	Well	1		No	None	398
112	68	Yes	Well	2		No	None	822
113	68	Yes	Well	2		No	None	9
114	56	Yes	Poor	3		Yes	None	155
115	55	Yes	Moderate	2		No	None	323
116	64	Yes	Moderate	2		No	None	260
117	62	Yes	Poor	3		No	None	257
118	64	Yes	Poor	3		Yes	None	225
119	61	Yes	Poor	3	3.0	No	None	205
120	50	no	Poor	3	2.0	Yes	None	162
121	66		Moderate	3		No	None	
122	69		Moderate	1		No	None	357
123	36		Well	1	4.5	No	None	138

124	71		Well	3	2.0	No	None	912
125	53		Moderate	1		Yes	None	489
126	73		Well	1	5.0	No	None	24
127	76		Moderate	1		No	None	569
128	45		Poor	1	4.0	No	None	540
129	48		Moderate	1		No	None	229
130	45		Moderate	1	1.2	no	None	353
131	61		Poor	1		No	None	393
132	56		Moderate	1	3.0	No	None	259
133	57		Moderate	1		No	None	171
134	62		Poor	1	5.0	No	None	132
135	58			1		no	None	78
136	51		Poor	1	3.0	No	None	314
137	60		Well	1	0.7	No	None	235
138	53		Moderate	1	2.5	No	None	598
139	58		Poor	1	3.0	No	None	236
140	59			1		no	None	16
141	58		Moderate	1	3.5	Yes	None	1366
142	59		Well	1	2.8	No	None	1465
143	50	No	Moderate	3		Yes	Dxt/Chem.	114
144	63	Yes	Poor	1	2.0	No	Chem.	798
145		Yes	Poor	3	4.0	Yes	Chem	212
146	62	-	Moderate			No	Chem	722
147	30	No	Poor	3	2.5	Yes	Dxt	180
148	66	Yes	Poor	3	2.5	Yes	Dxt	33
149	51	Yes	Moderate		2.0		Chem	249
150	53	Yes	Poor	3	6.0	No	None	241
151	64	No	Moderate			Yes	Dxt	191
152		**Yes**	**-**	**3**	**3.5**	**No**	**None**	**1055**

153	60	No	Poor	3	3.5	Yes	None	187
154	58	Yes	Moderate	3	3.5		None	167
155	56	Yes	Moderate	3	2.5		Chemo	
156	51	No	Moderate	3	-	Yes	Chem	
157	60	No	Poor	3	5.0	yes	Chem	

Table 5.3 Swedish Immunohistochemical data

Case	P53	P16	P21	C-erbB-2	C-erbB-3	Cyclin D1	Survival (Days)
1	Neg	Neg	Neg	Neg	Pos	Pos	7
2	Neg	Neg	Pos	Neg	Neg	Pos	135
3	Neg	Neg	Neg	Pos	Neg	Pos	420
4	Pos	Pos	Neg	Pos	Neg	Pos	161
5	Neg	Neg	Neg	Neg	Pos	Pos	269
6	Pos	Neg	Neg	Neg	Neg	Neg	**0**
7	Pos	Neg	Neg	Pos	Neg	Pos	104
8	Neg	Neg	Pos	Neg	Neg	Neg	154
9	Neg	Neg	Neg	Neg	Neg	Pos	327
10	Neg	Neg	Neg	Neg	Pos	Pos	473
11	Neg	Neg	Pos	Neg	Pos	Pos	461
12	Neg	Neg	Pos	Pos	Pos	Pos	670
13	Neg	Pos	Neg	Neg	Pos	Pos	402
14	Neg	Neg	Neg	Neg	Pos	Pos	389
15	Neg	Neg	Neg	Neg	Neg	Pos	498
16	Neg	Neg	Pos	Neg	Pos	Neg	390
17	Neg	Neg	Neg	Pos	Pos	Pos	53
18	Pos	Neg	Neg	Neg	Neg	Neg	131
19	Pos	Neg	Neg	Neg	Pos	Pos	120
20	Neg	Pos	Neg	Neg	Neg	Pos	252
21	Pos	Neg	Neg	Neg	Neg	Pos	206
22	Pos	Neg	Neg	Neg	Pos	Pos	511
23	Neg	Neg	Pos	Pos	Neg	Pos	637
24	Neg	Neg	Neg	Neg	Pos	Neg	210
25	Neg	Neg	Neg	Neg	Neg	Pos	210
26	Neg	Neg	Neg	Neg	Pos	Pos	390
27	Pos	Neg	Pos	Neg	Pos	Pos	210
28	Pos	Neg	Neg	Neg	Pos	Neg	533
29	Pos	Neg	Neg	Neg	Neg	Neg	13
30	Neg	Neg	Neg	Pos	Neg	Neg	342
31	Pos	neg	Neg	Neg	Pos	Pos	1120
32	Pos	pos	Neg	Neg	Pos	Pos	716
33	Neg	Neg	Neg	Neg	Neg	Neg	403
34	Pos	Neg	Neg	Neg	Neg	Pos	210
35	Pos	Neg	Pos	Pos	Pos	Pos	137
36	Neg	Neg	Neg	Pos	Neg	Pos	60
37	Neg	Neg	Neg	Pos	Neg	Pos	29
38	Neg	Pos	Neg	Pos	Neg	Pos	204
39	Neg	Neg	Neg		Pos	Neg	210
40	Neg	Neg	Neg	Pos	Pos	Pos	112

41	Neg	Neg	Neg		Pos	Pos	30
42	Pos	Neg	Neg	Pos	Pos	Pos	92
43	Pos	Neg	Neg	Pos	Pos	Pos	347
44	Neg	Neg	Neg		Pos	Pos	1308
45	Neg	Neg	Pos	Pos	Neg	Neg	454
46	Pos	Neg	Neg	Pos	Pos	Pos	219
47	Pos	Neg	Neg	Pos	Pos	Neg	440
48	Neg	Neg	Neg	Neg	Pos	Pos	181
49	Neg	Neg	Neg	Neg	Pos	Pos	337
50	Neg	Neg	Neg	Neg	Neg	Pos	150
51	Pos	Neg	Neg	Neg	Neg	Pos	1769
52	Pos	Neg	Neg	Neg	Neg	Pos	234
53	Neg	Neg	Neg	Neg	Neg	Pos	125
54	Neg	Pos	Neg	Neg	Pos	Pos	798
55	Neg	Neg	Neg	Neg	Neg	Neg	458
56	Neg	Pos	Pos	Pos	Pos	Pos	157
57	Pos	Neg	Neg	Pos	Pos	Pos	316
58	Neg	Neg	Neg	Neg	Neg	Neg	157
59	Neg	Neg	Neg	Neg	Pos	Pos	150
60	Neg	Neg	Neg	Neg	Pos	Pos	195
61	Neg	Neg	Neg	Pos	Pos	Pos	37
62	Neg	Pos	Neg	Neg	Neg	Pos	1278
63	Pos	Pos	Neg	Neg	Pos	Pos	330
64	Neg	Neg	Neg	Pos	Pos	Pos	267
65	Neg	Neg	Neg	Neg	Neg	Pos	90
66	Neg	Neg	Neg	Neg	Neg	Neg	900
67	Neg	Neg	Pos	Neg	Neg	Pos	240
68	Pos	Pos	Neg	Neg	Neg	Pos	77
69	Neg	Neg	Neg	Neg	Pos	Pos	330

Table 5.4 United Kingdom immunohistochemistry data

Case	P53	P16	P21	c-erbB-2	c-erbB-3	Cyclin D1	survival
70	Pos	Neg	Neg	Pos	Neg	Pos	367
71	Neg	Neg	Neg	Neg	Pos	Pos	63
72	Neg	Neg	Neg	Neg	Pos	Pos	305
73	Pos	Neg	Neg	Neg	Pos	Pos	451
74	Pos	Neg	Neg	Neg	Pos	Pos	450
75	Pos	Neg	Neg	Neg	Pos	Neg	34
76	Neg	Neg	Neg	Neg	Neg	Neg	197
77	Pos	Neg	Pos	Neg	Neg	Pos	393
78	Neg	Pos	Neg	Neg	Pos	Neg	293
79	Neg	Neg	Neg	Neg	Neg	Pos	118
80	Neg	Neg	Neg	Neg	Pos	Pos	313
81	Neg	Neg	Neg	Neg	Pos	Neg	1827
82	Pos	Neg	Neg	Neg	Pos	Pos	190
83	Pos	Neg	Neg	Neg	Pos	Neg	760
84	Neg	Neg	Neg	Pos	Neg	Pos	229
85	Neg	Neg	Neg	Neg	Pos	Pos	300
86	Pos	Neg	Neg	pos	Pos	Pos	1060
87	Neg	Neg	Neg	Neg	Pos	Pos	2485
88	Pos	Neg	Neg	Neg	Neg	Pos	1206
89	Neg	Neg	Neg	Neg	Neg	Pos	170
90	Neg	Neg	Neg	Neg	Neg	Pos	353
91	Neg	Neg	Neg	Neg	Pos	Pos	924
92	Neg	Neg	Neg	Neg	Pos	Pos	106
93	Neg	Neg	Neg	Neg	Neg	Pos	1113
94	Pos	Neg	Neg	Neg	Pos	Neg	

95	Neg	Neg	Pos	Pos	Neg	Pos	767
96	Neg	Neg	Neg	Pos	Pos	Neg	88
97	pos	Neg	Neg	Neg	Pos	Neg	509
98	Pos	Neg	Neg	Neg	Pos	Neg	103
99	Pos	Neg	Neg	Pos	Pos	Neg	193
100	Neg	Neg	Neg	Neg	Neg	Neg	167
101	Neg	Neg	Neg	Neg	Pos	Pos	23
102	Neg	Pos	Pos	Pos	Neg	Pos	450
103	Pos	Neg	Neg	Pos	Pos	Pos	245
104	Pos	Neg	Pos	Pos	Pos	Pos	493
105	Pos	Neg	Neg	Pos	Neg	Pos	475
106	Neg	Neg	Pos	Neg	Neg	Pos	568
107	Neg	Pos	Neg	Neg	Neg	Neg	50
108	Pos	Neg	Pos	Neg	Neg	Pos	443
109	Pos	Pos	Neg	Neg	Pos	Pos	547
110	Pos	Neg	Pos	Neg	Neg	Pos	915
111	Neg	Neg	Pos	Neg	Pos	Pos	398
112	Neg	Pos	Pos	Neg	Neg	Pos	822
113	Neg	Neg	Neg	Neg	Pos	Neg	9
114	Neg	Neg	Pos	Neg	Pos	Pos	155
115	Pos	Neg	Pos	Pos	Neg	Pos	323
116	Pos	Neg	Pos	Neg	Pos	Pos	260
117	Pos	Neg	Neg	Pos	Pos	Pos	257
118	Neg	Neg	Neg	Neg	Neg	Pos	225
119	Pos	Neg	Pos	Pos	Neg	Pos	205
120	Pos	Pos	Pos	Pos	Neg	Pos	162
121	Pos	Neg	Pos	Pos	Pos	Neg	265
122	Neg	Neg	Neg	Pos	Pos	Pos	357
123	Neg	Neg	Pos	Pos	Neg	Neg	138

124	Neg	Neg	Neg	Neg	Pos	Neg	912
125	Pos	Neg	Neg	Neg	Neg	Neg	489
126	Neg	Neg	Neg	Pos	Pos	pos	24
127	Pos	Pos	Pos	Pos	Neg	Neg	569
128	Neg	Neg	Neg	Neg	Neg	Pos	540
129	Neg	Neg	Neg	neg	Pos	Neg	229
130	Neg	Neg	Neg	Neg	Neg	Neg	353
131	Neg	Neg	Neg	Neg	Pos	Pos	393
132	Neg	Neg	Neg	Neg	Pos	Neg	259
133	Pos	Neg	Neg	Pos	Neg	Neg	171
134	Neg	Neg	Neg	Neg	Pos	Pos	132
135	Neg	Neg	Pos	Pos	Pos	Neg	78
136	Neg	Neg	Neg	Neg	Neg	Pos	314
137	Neg	Neg	Neg	Neg	Pos	Pos	235
138	Pos	Neg	Neg	Neg	Pos	Neg	598
139	Neg	Neg	Neg	Neg	Neg	Neg	236
140	Pos	Neg	Pos	neg	neg	Pos	16
141	Pos	Pos	Neg	Pos	Neg	Neg	1366
142	Pos	Neg	Pos	Pos	Pos	Pos	1465
143	Pos	Neg	Neg	Pos	Pos	Neg	114
144	Neg	Pos	Neg	Pos	Pos	Pos	798
145	Pos	Pos	Pos	Pos	Pos	Pos	212
146	Neg	Neg	Neg	Pos	Pos	Pos	722
147	Pos	Neg	Pos	Neg	Pos	Pos	180
148	Pos	Neg	Neg	Pos	Neg	Pos	33
149	Neg	Neg	Neg	Neg	Pos	Pos	249
150	Neg	Pos	Neg	Neg	Neg	Pos	241
151	Pos	Neg	Neg	Neg	Pos	Pos	191
152	Neg	Neg	Neg	Neg	Neg	Neg	1055

153	Neg	Neg	Neg	Pos	Pos	Pos	187
154	Pos	Pos	Neg	Neg	Pos	Neg	167
155	Pos	Neg	Neg	Neg	Pos	Pos	229
156	Pos	Neg	Pos	Pos	Pos	Pos	249
157	Pos	Neg	Pos	Pos	Pos	Pos	267

5.4 Tissue Specimens

Histological examination was performed by two pathologists (Professor NR Lemoine and Dr F Campbell) on haematoxylin and eosin (H&E) stained sections of all the blocks which were previously fixed in buffered formaldehyde and then paraffin embedded. Neither the pathologist nor the author were aware of either the previous histology or the clinical outcome of the patient. Only resected ductal adenocarcinoma of the pancreas cases were selected. Appropriate blocks for study were then selected and 5 μm sections cut and mounted on poly-Lysine coated glass slides (Merck Ltd, Lutterwoth, Leics). These were then dried for 12-24 hours at 37°C. They were then labelled with the pathology number of the tumour sample. Tumour grading was evaluated as previously described and staging was according to the UICC (Kloppel et al, 1996). Specimens of normal pancreatic tissue were obtained from healthy organ transplant donors following informed consent from the relatives. Positive control specimens were obtained from the same pathology departments. These were selected if staining with the appropriate antibodies was strong and homogenous.

5.7 Antibodies

The primary antibodies used were all monoclonal and obtained within the United Kingdom. They were refrigerated immediately upon arrival in the laboratory. The c-erbB-3 monoclonal antibody was a generous gift from Professor W. Gullick at the Hammersmith Hospital, London, UK.

TABLE 5.5 Function and dilution of antibodies

antibody	function	dilution
p53 (NCL-p53-DO7)	recognises wild type and mutant p53 protein.	1:300
c-erbB-2 (NCL-CB11)	recognises the internal and external domains of c-erbB-2	1:40
c-erbB-3 (NCL c-erbB-3) Clone RTJ1	recognises an epitope in the cytoplasmic domain of the human c-erbB-3 oncoprotein	1:10
p21	detects p21 protein	1:100
p16	detects human p16 protein	1:100
cyclin D1	detects cyclin D1 protein	1:100

Table 5.6 Positive controls

P53	Gastric cancer
P21	Normal colon
P16	Breast cancer
c-erbB-2	Breast cancer
c-erbB-3	Colon cancer
Cyclin D1	Breast cancer

5.6 Immunohistochemical method

5 μm sections on glass slides (Merck Ltd, Leics) were loaded onto a metal rack and then immersed in two changes of xylene of ten minutes each to remove the wax. The sections were then rehydrated through graded solutions of ethanol (University of Liverpool stores) in concentrations of (100, 100, 90, 90, and 70%) for 5 minutes each. Endogenous peroxidase (Sigma Chemical Co, Poole, and Dorset) was blocked by immersing the sections in methanol and hydrogen peroxide (3% v/v) for 15 minutes. After washing in running tap water for five minutes the sections were subjected to an antigen retrieval step by being immersed in 10mM EDTA (Sigma, Chemical Co, Poole, Dorset) (pH 7.0) and then placed in a microwave oven (Creda Ltd, Blythe Bridge, Stoke on Trent) at 850watts for 15 minutes. The sections were then allowed to cool to room temperature for 15 minutes and then washed in running tap water for 5 minutes to avoid precipitation of the citrate salts. Following this, the slides were transferred in R.O (reverse osmosis) water for a few minutes and then mounted onto plastic cover plates and secured into a sequenza rack. The sequenza racks were filled with Tris buffered saline [TBS (BDH/Merck Chemicals Ltd, Lutterwoth, Leics)] to confirm correct positioning of slides and then 5% bovine serum albumin [1gBSA in 20cc TBS (Sigma, University of Liverpool stores)] added to each well. The primary antibodies (p53 at 1:300; p21 and cyclin D1 at 1:100; c-erbB-2 at 1:40 and c-erbB-3 at 1:10) were diluted in 5% Bovine Serum Albumin (BSA) and then added into each of the wells, excluding the negative control slides on which 5%BSA only was added. Slides were incubated for one hour at room temperature. The sections were then washed with TBS for 5 minutes three times and were then incubated with the 100μL of biotinylated rabbit anti-mouse secondary antibody (Amersham Life Sciences, Bucks, UK) for 45 minutes at room temperature. The slides were again rinsed in TBS as before and 100μL of streptavidin-biotin complex (DAKO Ltd, Bucks, UK) containing 10mL/mL of streptavidin and 9mL/mL of biotinylated horseradish peroxidas

(ABC) made up at least 30 minute before use, was then added onto the slides. After 20 minutes the slides were again rinsed with TBS. The peroxidase staining was visualised using 0.5% (w/v) 3,3′ diaminobenzidine HCL (DAB), (Dako Ltd, Bucks, UK) containing 0.03% (w/v) hydrogen peroxide for 7 minutes and then rinsed in running tap water for three minutes. The slides were then lightly stained with Meyer′s haematoxylin (BDH/Merck chemicals Ltd) for 15 seconds followed by a brief wash and dunking in acid alcohol to remove the excess haematoxylin. A further wash followed by soaking in Scott's tap water (20.0g magnesium sulphate, 3.5g sodium hydrogen carbonate in 1 litre tap water then one crystal of thymol preservative; store at 4°C) which stains the sections blue followed by a final wash. The slides were then dehydrated through graded alcohol solutions followed by immersion in two changes of xylene for 30 seconds before mounting them onto coverslips with DPX mountant BDH/Merck chemicals Ltd, Lutterworth, Leics). Specificity of immunostaining was established using a negative control for each section, missing out the primary antibody and using 100μL of the diluent alone. Also included in each batch of sections was a known positive control as shown.

5.7 Haematoxylin and eosin staining of tissue sections

Prior to use the haematoxylin was filtered. The paraffin embedded slides were then dewaxed in two changes of xylene (University of Liverpool stores) for 10 minutes followed by dehydration in absolute alcohol (University of Liverpool stores) for 3 minutes and 1 minute in 70% alcohol. The slides were then washed in running tap water and then immersed in haematoxylin (BDH/Merck chemicals Ltd, Leics) for 5 minutes followed by washing in tap water for two minutes. Following 10 dips in 0.5% acid alcohol (University of Liverpool stores) the slides were immediately plunged into tap water and washed well. They were then put in Scott's tap water for 30 seconds, washed and then immersed in eosin for 1-2 minutes

and washed in tap water. Finally the sections were dehydrated in absolute alcohol and put through 3 changes of xylene. The slides were then mounted on slides with DPX.

5.8 Scoring of slides

The stained sections were examined using a standard light microscope. Staining results were interpreted independently by the author, two gastrointestinal pathologists (Professor NR Lemoine, Hammersmith, London and Dr F Campbell, Royal Liverpool Hospital, Liverpool**)** and another independent observer unaware of the clinical outcome of the patients under study. The aim was to arrive at reproducible interpretation of staining. There was agreement on the significance of nuclear, diffuse cytoplasmic or membrane staining. Most antibodies either localise (functionally) in the nucleus for example p53, p16, p21 and cyclin D1. Others localise in the cell membrane or in the cytoplasm for example c-erbB-2 and c-erb-B-3. Staining patterns not typical of the antibody were ignored and in some cases experiments were repeated. The staining was assessed in tumour areas and in all cases this was with the agreement of the pathologist. A percentage expression was then estimated, again with the full agreement of the pathologist. Following this a quantitative assessment of the staining was made. Areas of necrosis, giant cell infiltration and neural invasion were noted because this would influence future microdissection for gene sequencing. The background of the slides were then assessed looking for mucinous metaplasia, dysplasia and acini cytoplasmic staining. Nuclear expression was scored by arbitrary division into widespread nuclear staining if present in >75% of cells within the section, focal if there were <25% of cells displaying nuclear staining and absent if there was no nuclear staining. Tumours displaying membranous staining were scored as positive and similarly with cytoplasmic staining. When samples from more than one site of the tumour were available, the mean percentage of positive staining was considered as representative of the tumour. Agreement in scoring was at least 95% in all cases. Specimens were considered to be positive when more than 5% of the

tissue components were unequivocally immunoreactive in the appropriate cellular compartment. Attempts to grade scoring was not easy. The problems ranged from the use of different antibodies, different controls subject to availability and some slides were very old and fixed differently at the time. The actual number of stained cells was not done. With respect to the p16 gene, there was no work to compare with at the time and therefore not easy to determine the quality of staining required. P21 posed similar problems, with very few reliable publications. Inspite of all these problems, it was still important to bear in mind that negative staining did not necessarily mean absence of a mutation. This is because for example a large deletion within a gene may not show a positive result. Additionally, some protein products are involved in coupling thus not exposing the epitopes needed for an antigen –antibody reaction.

5.9 Statistical analysis

The Chi-squared or Fisher's exact test were used for categorical data. Overall survival was calculated by the Kaplan-Meier method and for differences in survival curves the log rank test was used. The log rank test is a non-parametric means of testing the null hypothesis that no difference exists in the survival experience of the groups. Median survival times were calculated for negative and positive staining groups. In the Kaplan Meier method, censored observations are patients who were alive at last follow up or who were lost to follow-up but who did not die during the follow-up period. Variables were first subjected to univariate analysis and after confirmation that the log-plots did not deviate from the proportional hazards assumption, the significant variables were then entered into multivariate analysis using the Cox regression model. Cox regression is used to study the relationship between time to an event and a set of variables**.** All deaths were due to pancreatic cancer. A p value of <0.05 as was accepted as statistically significant. Stat view (version 4.53) for windows was the statistical package used.

6.0 Results of Immunohistochemistry

6.1 Clinicopathological parameters and survival

A total of 157 patients were studied with a median (range) follow up of 1.8 (0.3-7) years. The overall median survival was 33.9 months (95% confidence interval: 28.48 – 39.32) (Table 6.1).

6.2. Overall analysis of clinocopathological factors

Significant prognostic markers were TNM stage of disease and lymph node involvement (Table 6.2). There were no significant correlations between survival and gender, differentiation of tumour, size of tumour, resection margin status and the use of adjuvant therapy. Multivariate analysis showed no significant difference in survival between patients from the UK and Sweden once lymph node status had been taken into account.

6.3 Immunohistochemical Results

6.3.1 p53 results

The slides were scored as positive if there was brown nuclear staining in tumour areas. The level of immunoreactivity was then expressed as a percentage of areas of tumour. Staining outside tumour areas was disregarded but noted. There was predominantly nuclear immunoreactivity of p53 in the tumour specimens graded as positive (although intensity varied between cases) and there was no nuclear reactivity with normal pancreas. Faint cytoplasmic staining without nuclear staining was considered negative. 41 % (n=64) of cases showed immunoreactivity. 18 (90%) out of 20 tumours that were more than 5cm in diameter were negative for p53 immunostaining. There was no correlation with patient survival (Table 6.5).

6.3.2 p21 results

p21 ^{WAF1} nuclear immunostaining in tumours was found in 25% (n=39) cases. Almost 100% positive nuclear activity was seen in normal pancreas. There was no correlation with survival between those cases that were positive for p21 staining and those that were negative.

6.3.3. p16 results

The loss of p16 expression was found in 87% (n=136) of cases. No correlation was found between p16 expression or loss with survival.

6.3.4 c-erbB-2 results

Membranous and some cytoplasmic staining were seen with the c-erbB-2 antibody in pancreatic tumour samples. 33% (n=33) of pancreatic cancer cases showed membranous or cytoplasmic staining. Normal pancreas demonstrated non-specific background immunoreactivity.

6.3.5 c-erbB-3 results

Cytoplasmic staining was seen in pancreatic tumour samples. Normal pancreas demonstrated non-specific background immunoreactivity. c-erbB-3 immunoreactivity was in 56% (n=89) of pancreatic cancer cases.

6.3.6 cyclin D1 results

Nuclear immunoreactivity was found in 72% of pancreatic cancer cases (n=113). Normal pancreas did not show any staining (0%). There was no correlation between cyclin D1 staining or absence of staining with survival.

6.3.7. multivariate analysis

Further analyses of particular patient subsets were performed. This was done to see if different combinations of various markers would give any prognostic information. There was no significant in survival between patients with p53 positive, p21 negative and cyclin D1 positive tumours and the others. Patients with combined c-erbB-2 and c-erbB-3 positive tumours also demonstrated no difference in survival compared with other patients. Patients with five marker abnormalities in any combination did not demonstrate a significant decrease with survival (Table 6.6).

Table 6.1. Clinical parameters of 157 patients who underwent pancreatectomy from the UK and Sweden

Clinical parameter			Number	Not Known	%
Country	UK		88		56
	Sweden		69		44
Median age years (range)	60	(30-77)			
Gender	Male		100		64
	Female		57		36
Histology	Ductal adenocarcinoma		157		
Grade	Well		21		13
	Moderate		77		49
	Poor		59		38
Stage	I		45	4	30
	II		32		20
	III		76		50
Lymph node	Positive		71	2	45
	Negative		84		55
Resection margins	Involved		12	13	8
	Not involved		122		92
Tumour size	<2cm		38	15	27
	2-5cm		84		59
	>5cm		20		14
Adjuvant therapy	Yes		13		8
	No		144		92

Table 6.2. Univariate analysis of clinical parameters and survival in 157 patients who have undergone pancreatoduodenectomy

Parameter		Median survival (months)	95% CI	$\chi 2$	*P* Value
All patients		33.90	28.48- 39.32		
UK		39.14	30.91 – 47.37	4.66	0.03*
Sweden		28.29	21.63 - 34.95		
Male		33.72	27.18 – 40.26	0.24	0.87
Female		33.97	24.43 – 43.51		
Differentiation	well	45.95	30.24 – 61.66	4.24	0.11
	moderate	32.91	24.94 – 40.88		
	poor	29.67	21.60 – 37.74		
Stage I		44.31	33.32 –55.30	16.04	0.0003*
II		41.19	26.77 – 55.61		
III		23.70	18.61 - 28.79		
Lymph node positive		24.23	16.38 – 32.08	14.3	0.008*
Lymph node negative		37.34	29.21 – 45.52		
Resection margins positive		37.60	17.91 – 57.29	0.01	0.92
Resection margins negative		33.61	27.38 – 39.84		
Tumour size	<2cm	8.9	22.51 – 41.13	0.03	0.98
	2-5cm	8.6	26.74 – 42.44		
	>5cm	8.6	19.19 – 30.47		

*Statistically significant value

CI - confidence interval

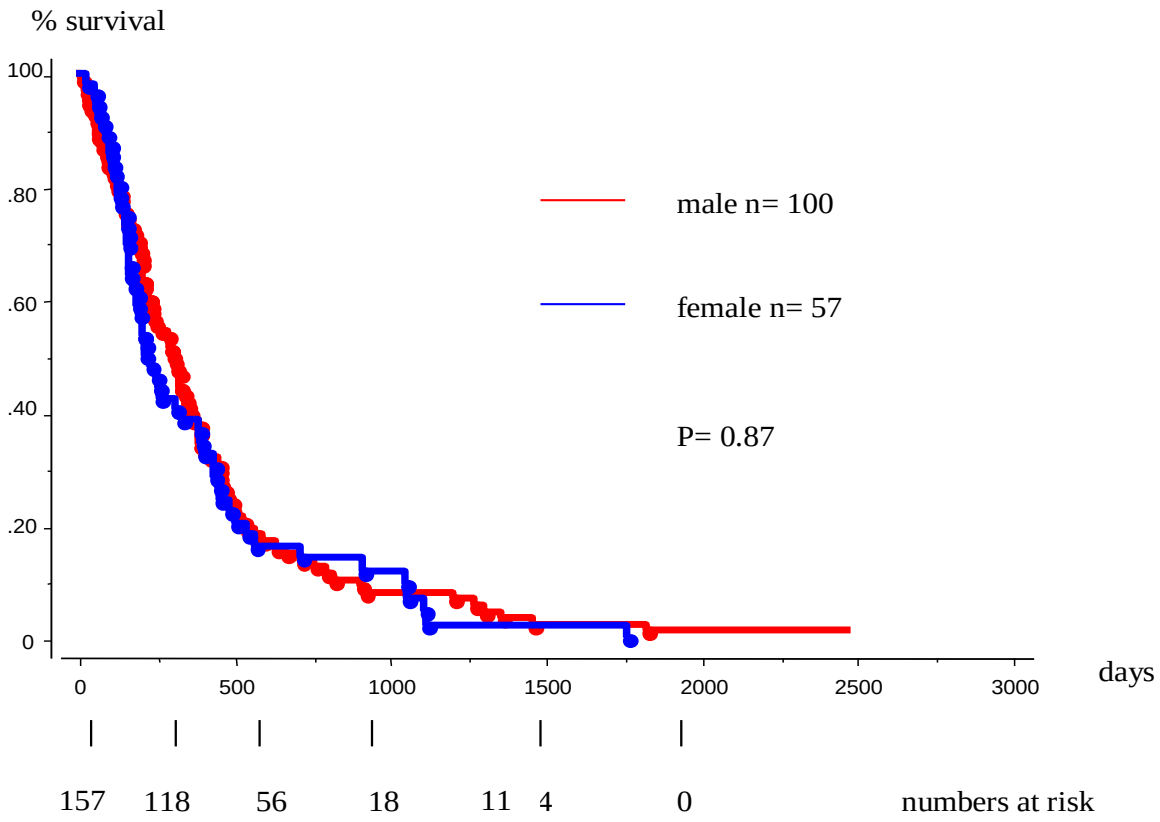


Figure 6.1: Kaplan Meier plot showing gender vs. survival.

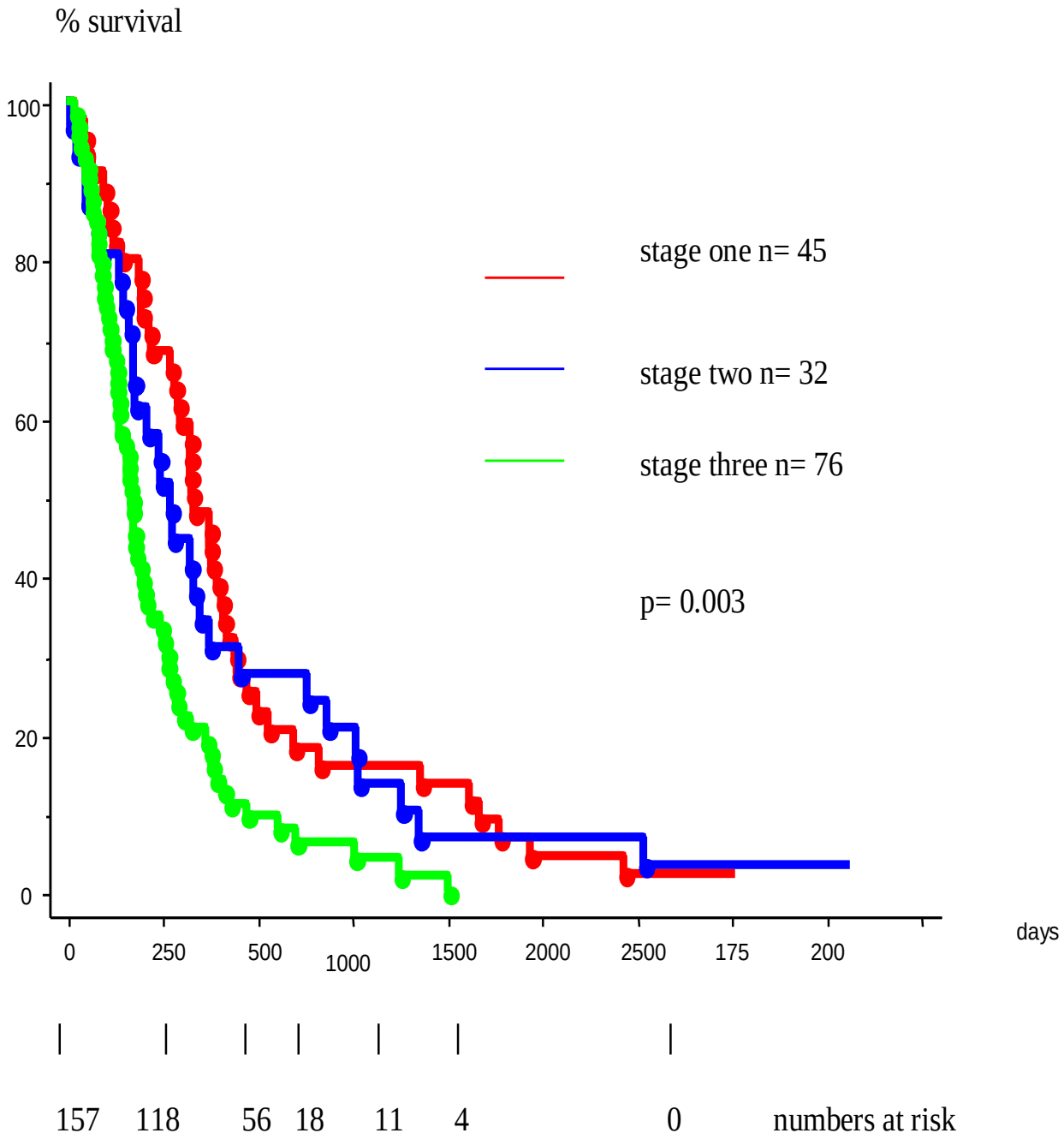


Figure 6.2: Kaplan Meier plot showing stage vs. survival – this was statistically significant.

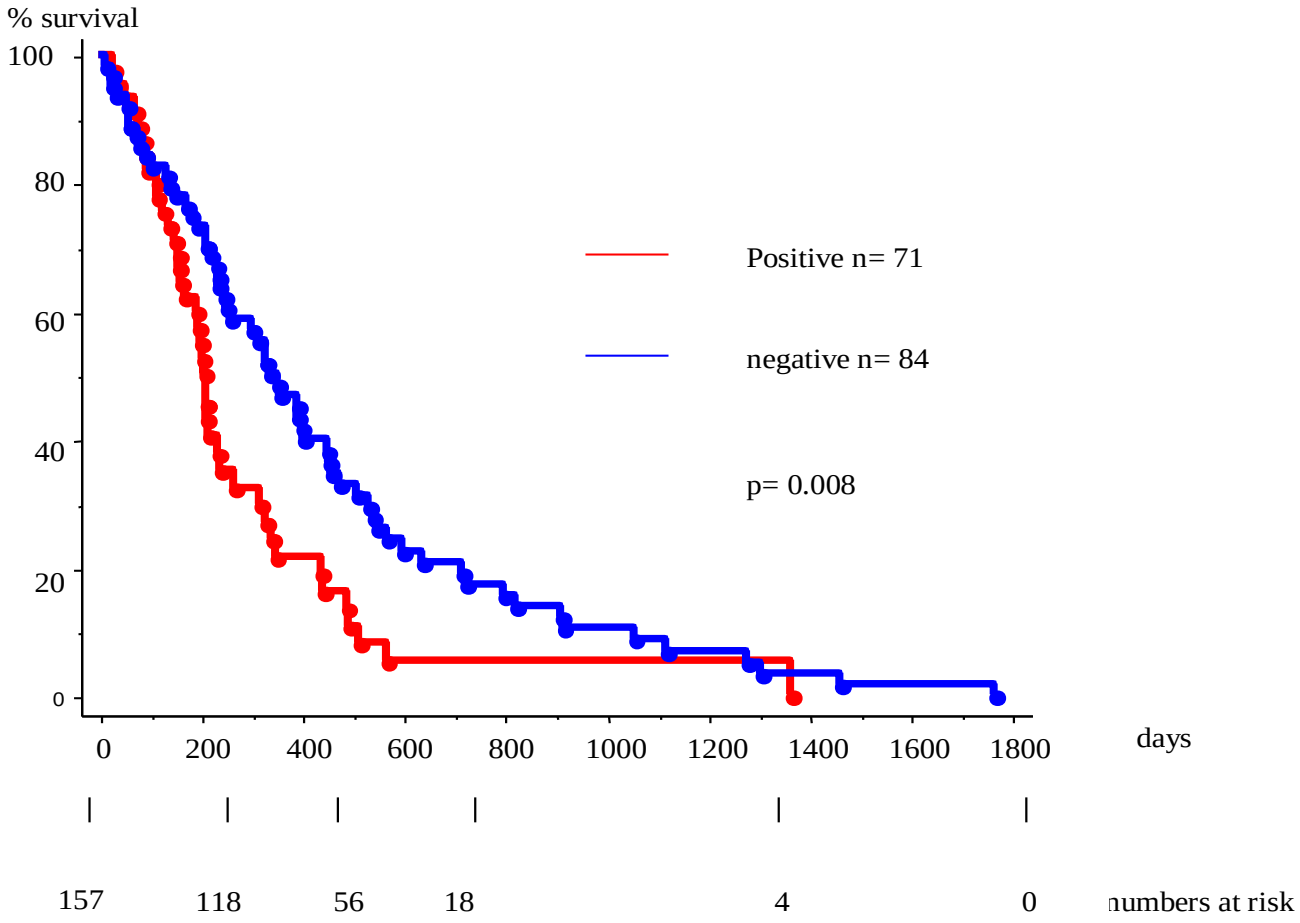


Figure 6.3: Kaplan Meier plot showing lymph node status vs. survival.

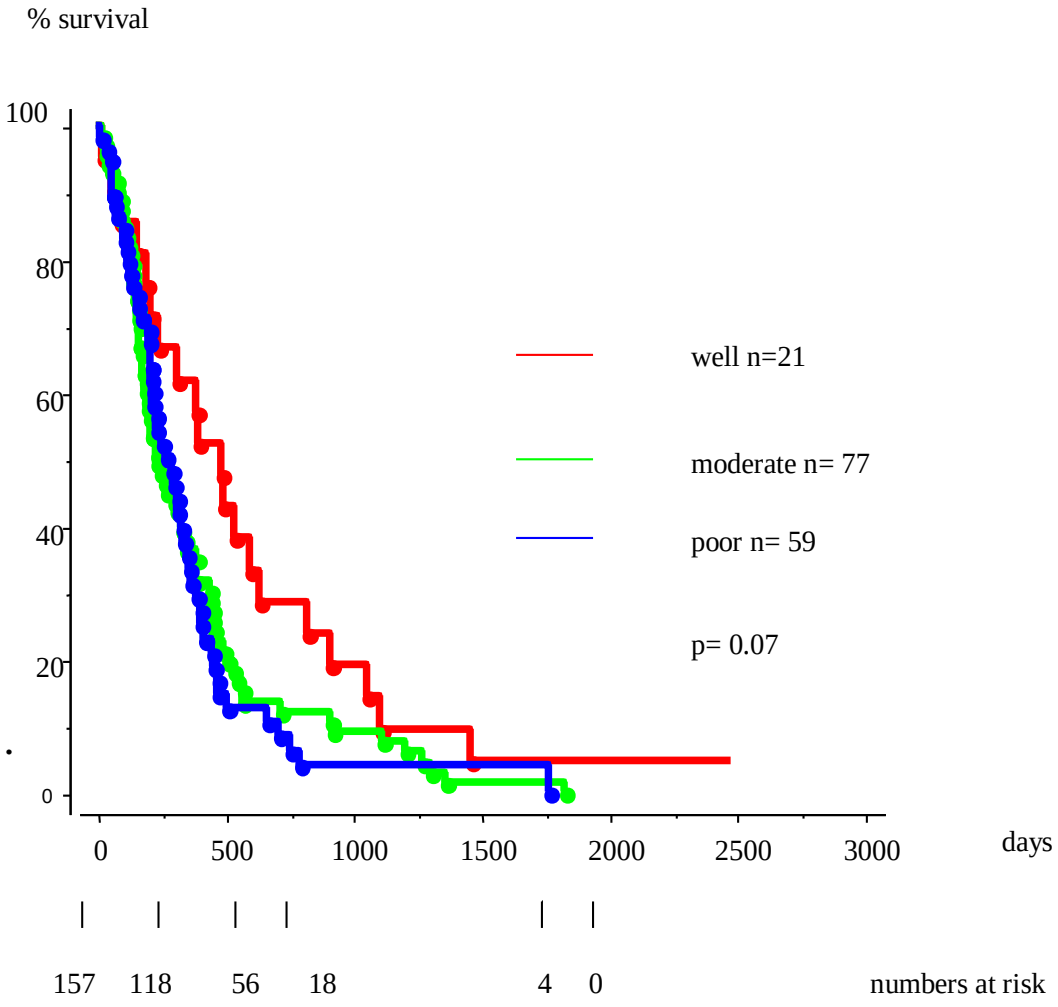


Figure 6.4: Kaplan Meier plot showing differentiation vs. survival.

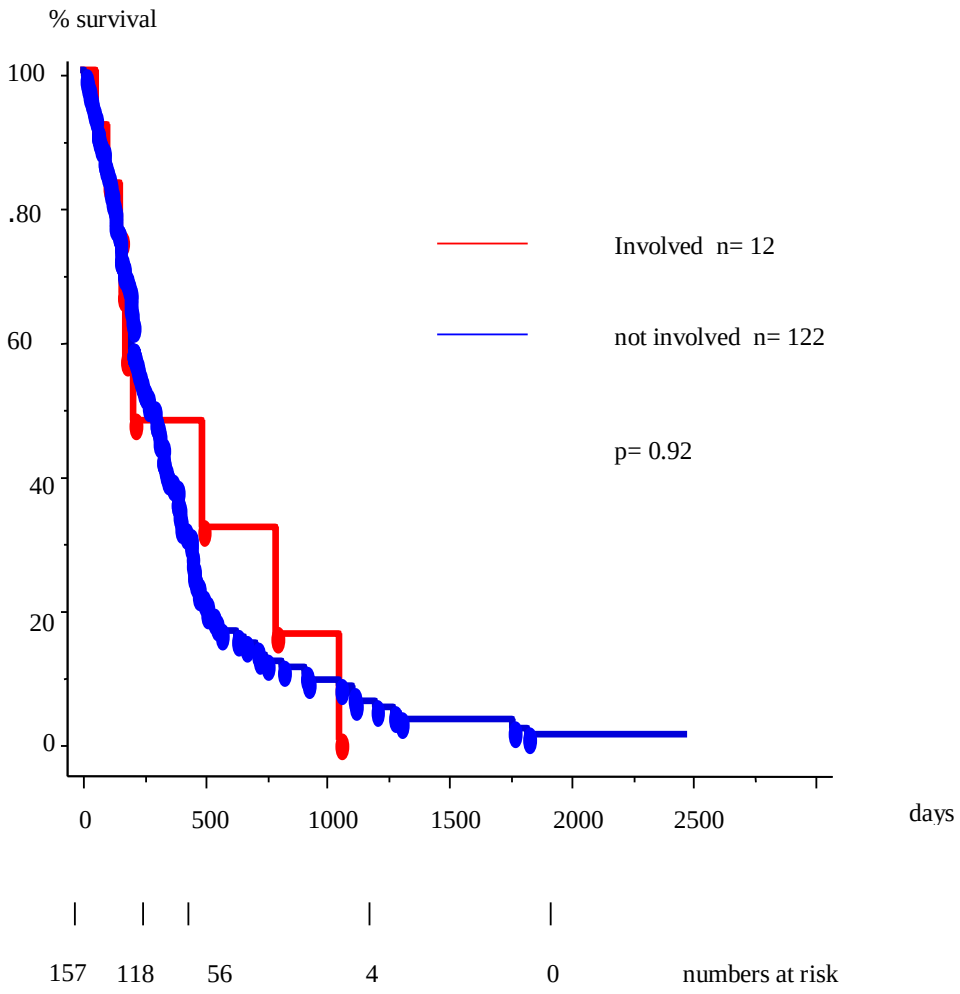


Figure 6.5: Kaplan Meier plot showing resection margins vs. survival.

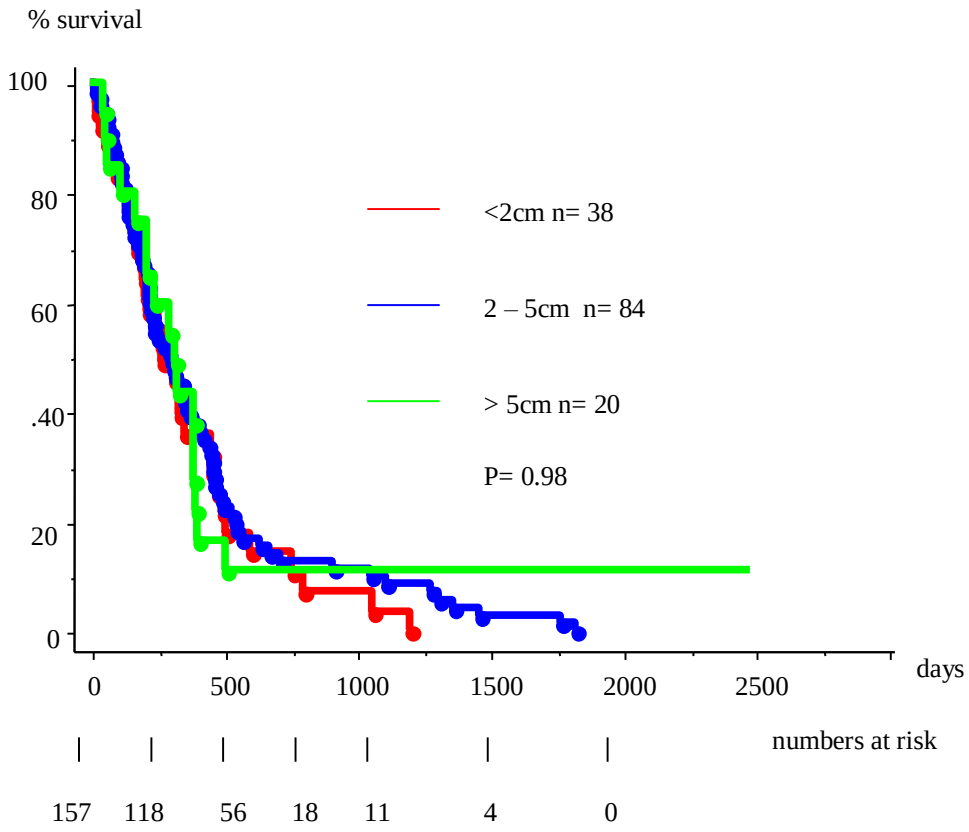


Figure 6.6: **Kaplan Meier plot showing tumour size vs. survival. Tumour size was divided into three groups; less than and equal to 2cm, between 2-5cm and more than 5cm.**

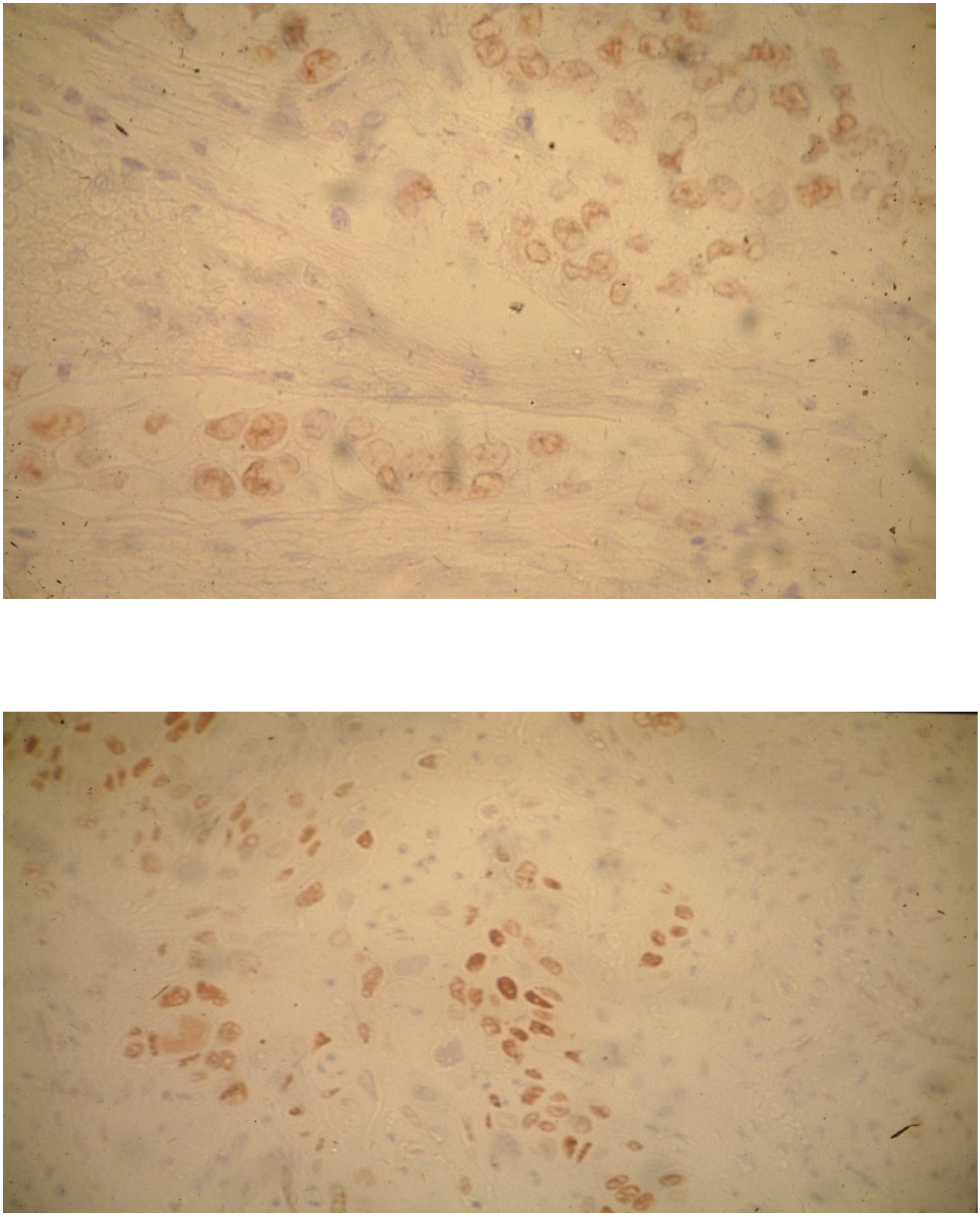


Figure 6.7: P53 nuclear immunostaining in ductal pancreatic cancer with DO7 antibody (low power)

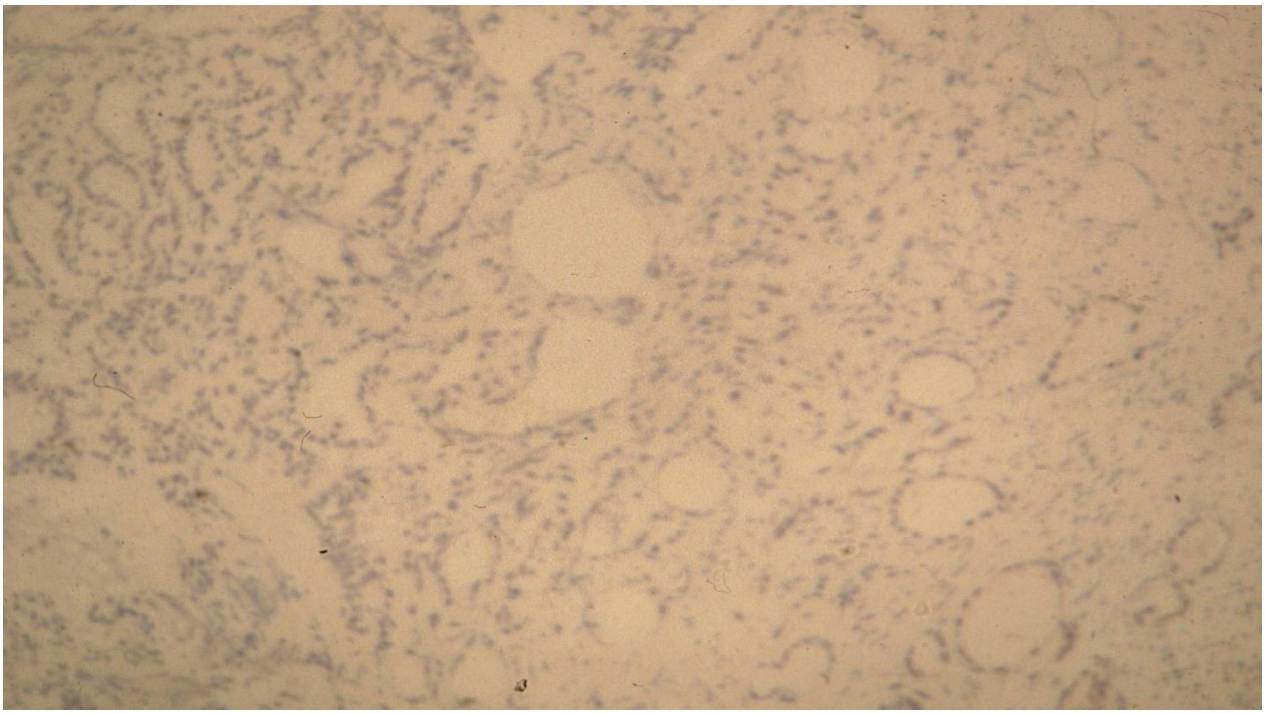


Figure 6.8: P53 negative nuclear staining (control pancreatic cancer slide)

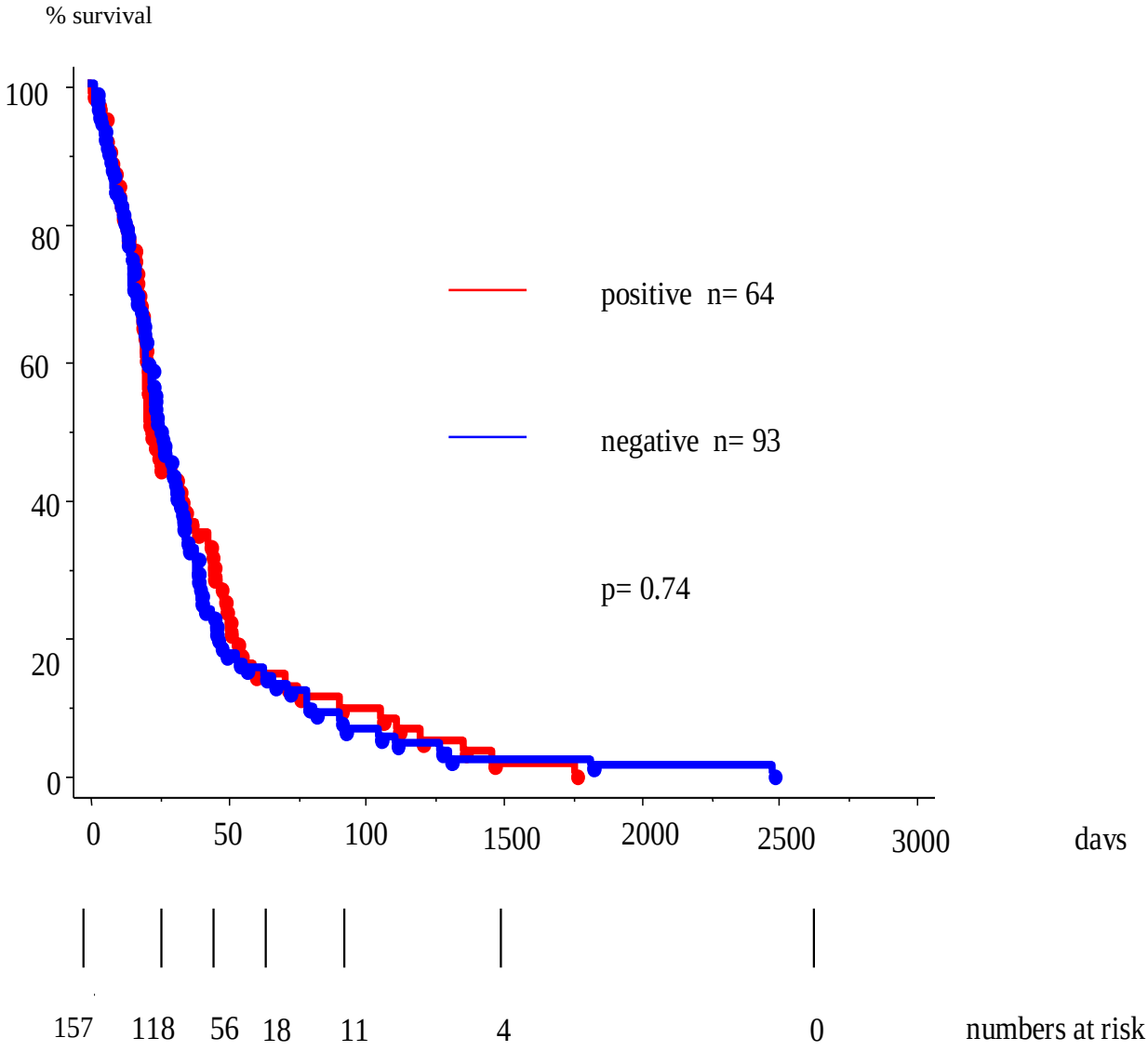


Figure 6.9: Kaplan Meier plot showing p53 immunostaining versus survival.

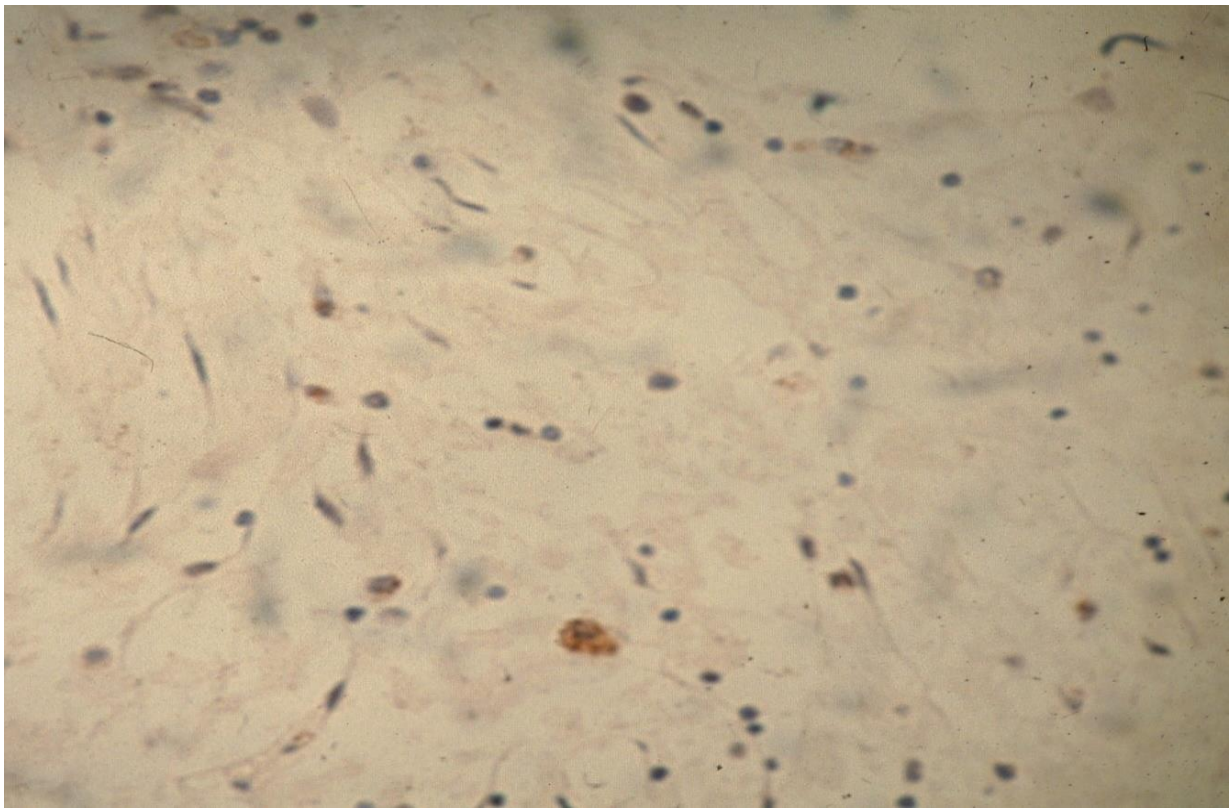


Figure 6.10: p21 nuclear immunoreactivity in ductal pancreatic cancer.

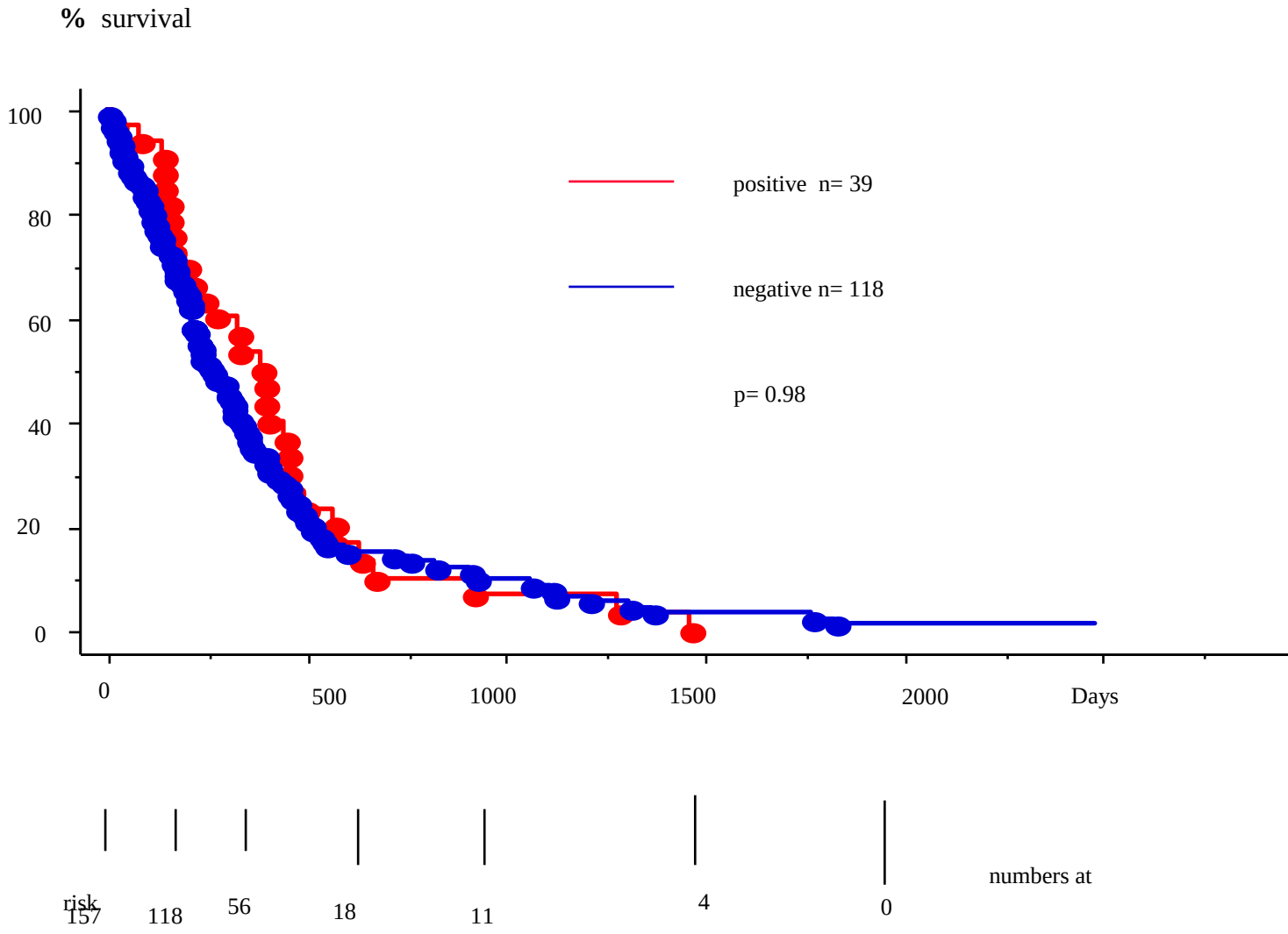


Figure 6.11: p21 Kaplan Meier plot - no statistical significance with positive staining with survival.

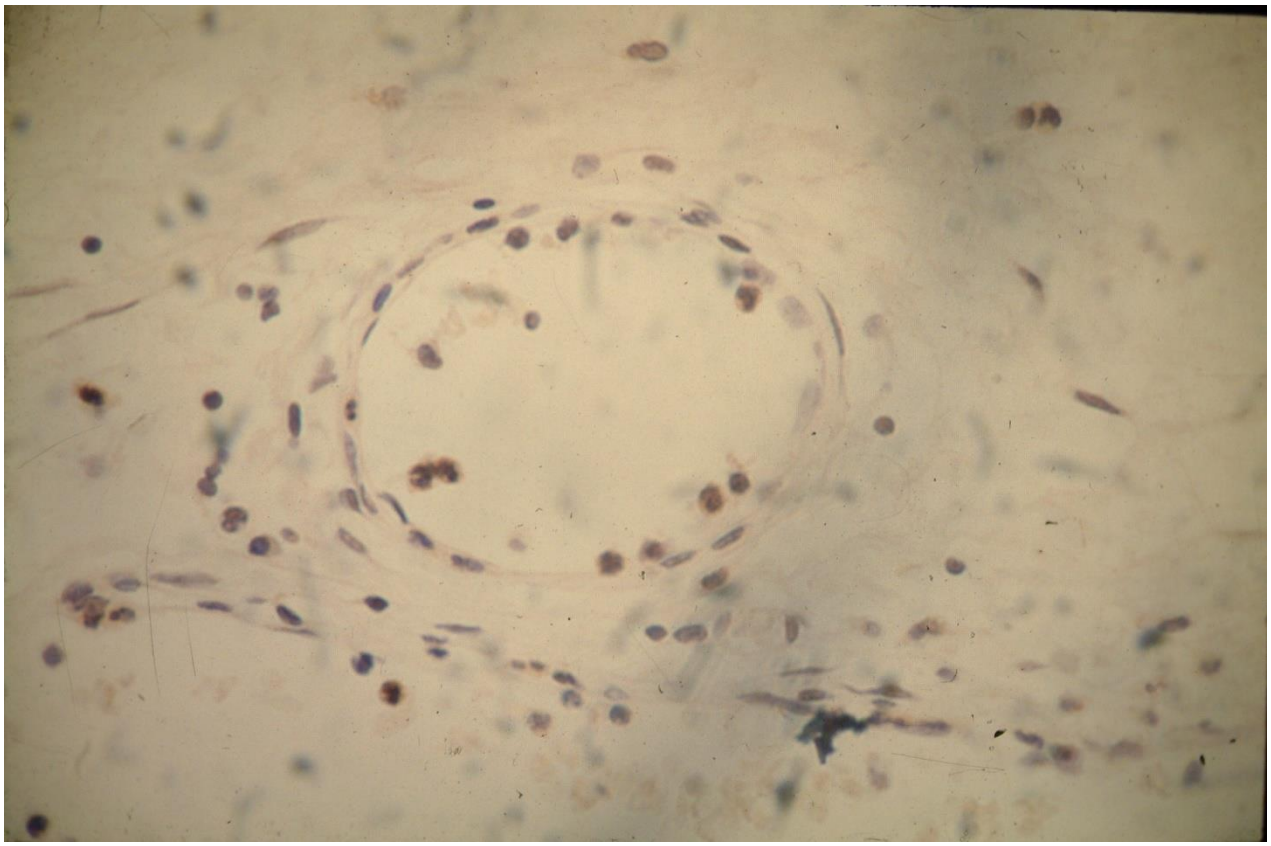


Figure 6.12: P16 nuclear immunoreactivity in ductal pancreatic cancer.

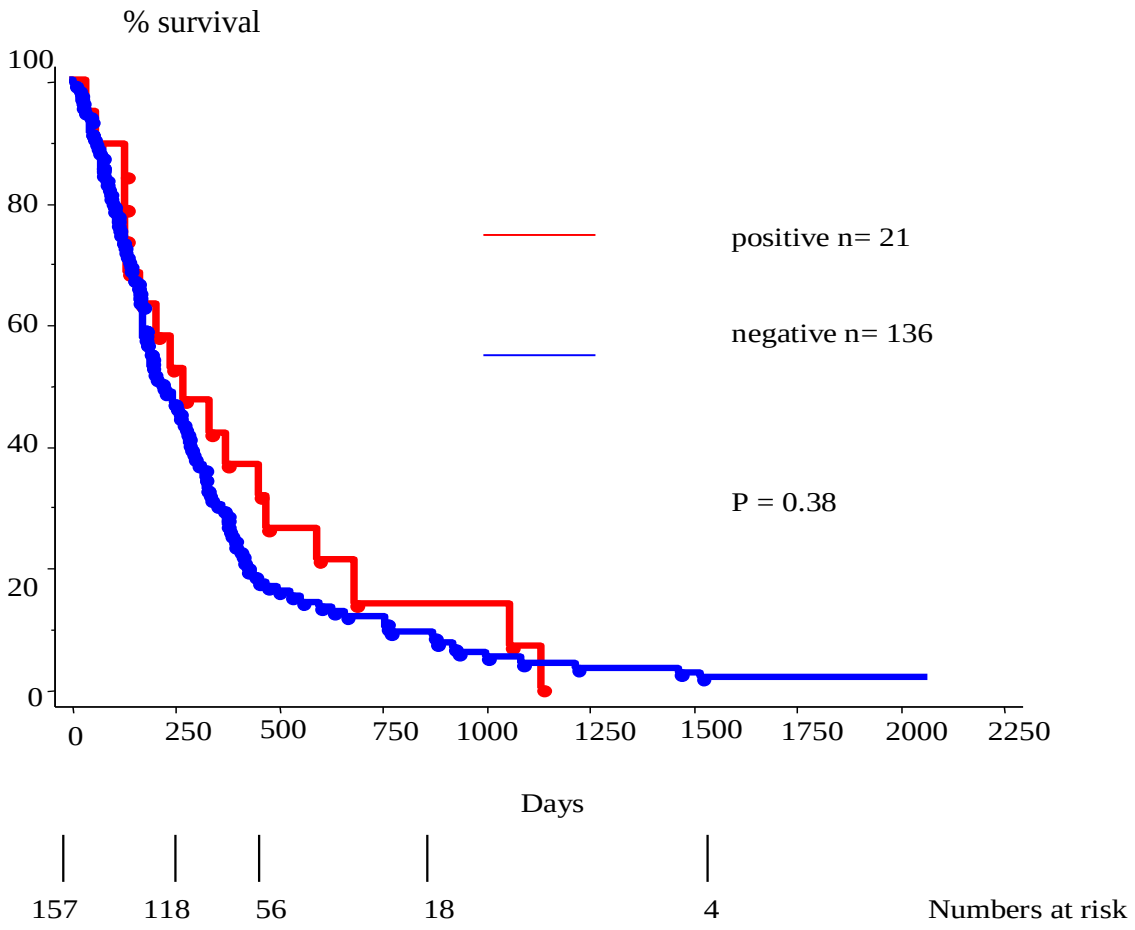


Figure 6.13: **Kaplan Meier plot showing p16 staining Vs survival.**

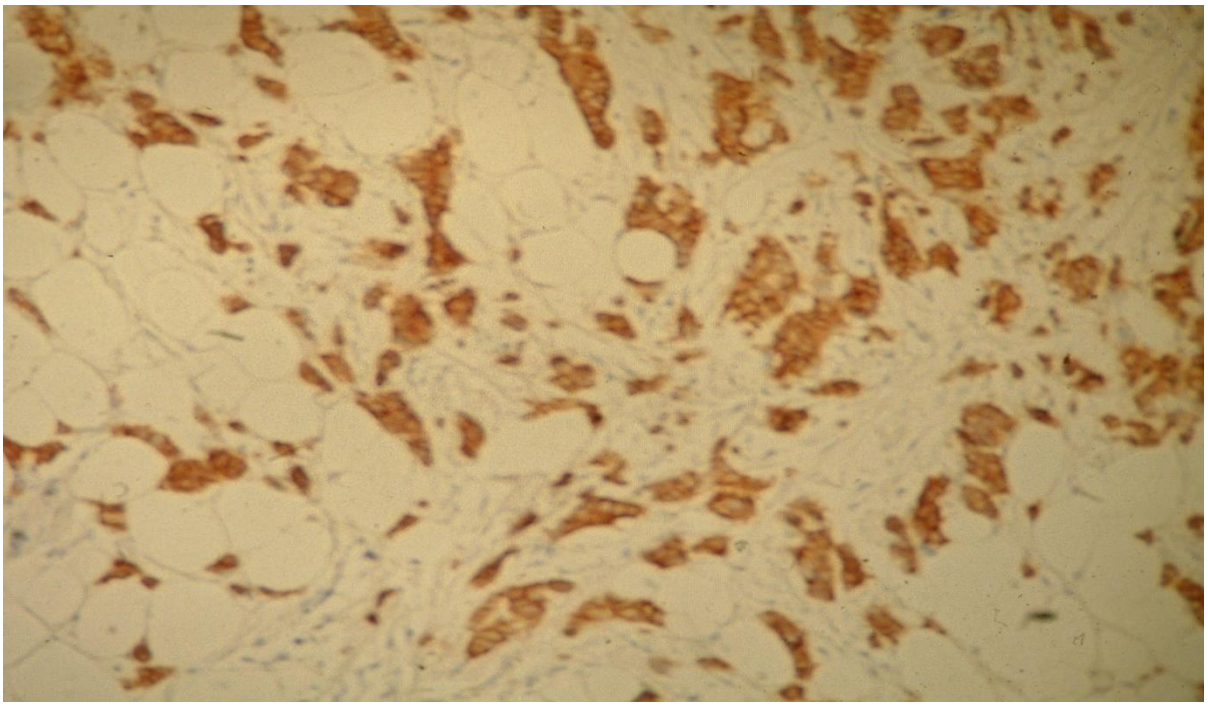


Figure 6.14: c-erbB-2 membranous staining.

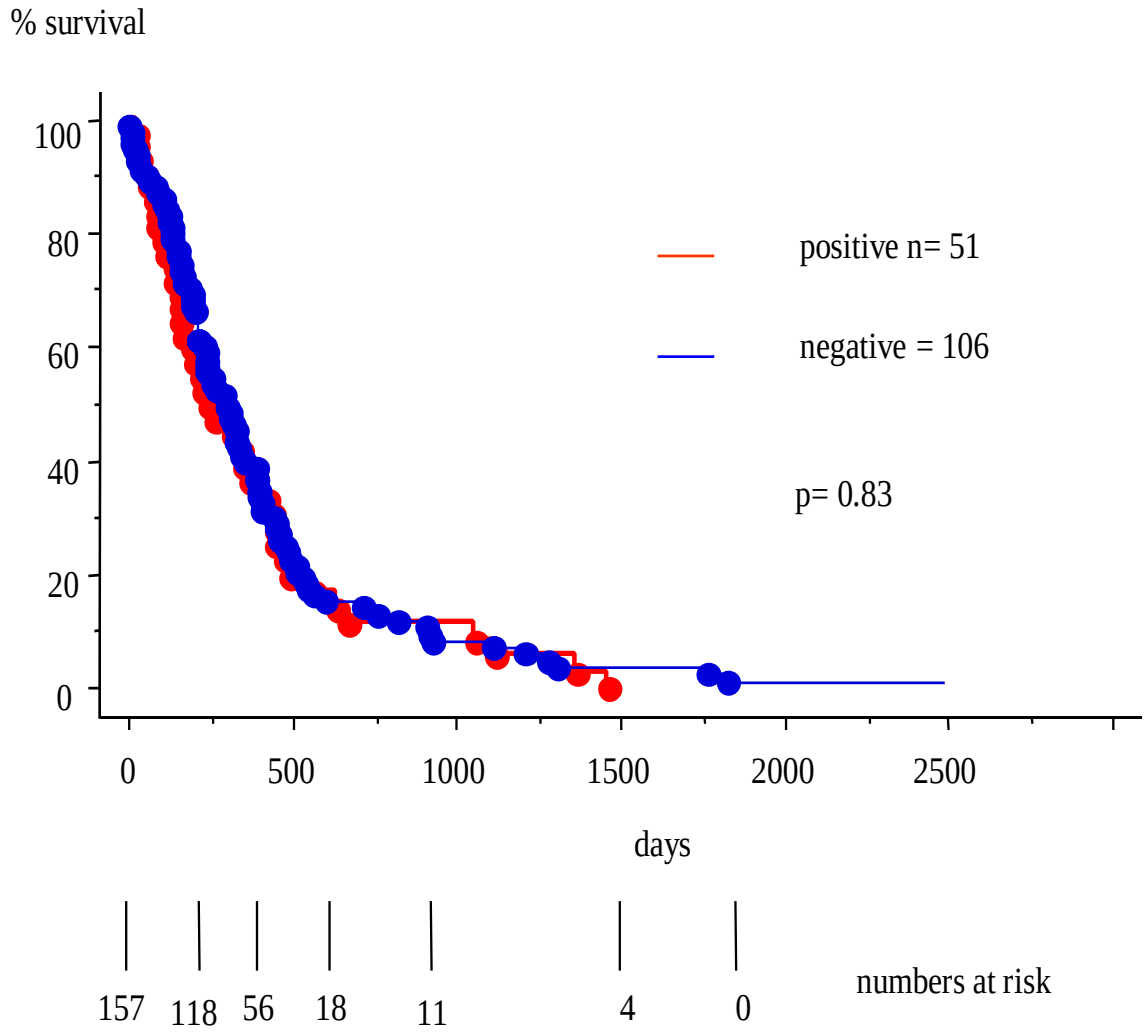


Figure 6.15: Kaplan Meier plot showing c-erbB-2 and survival.

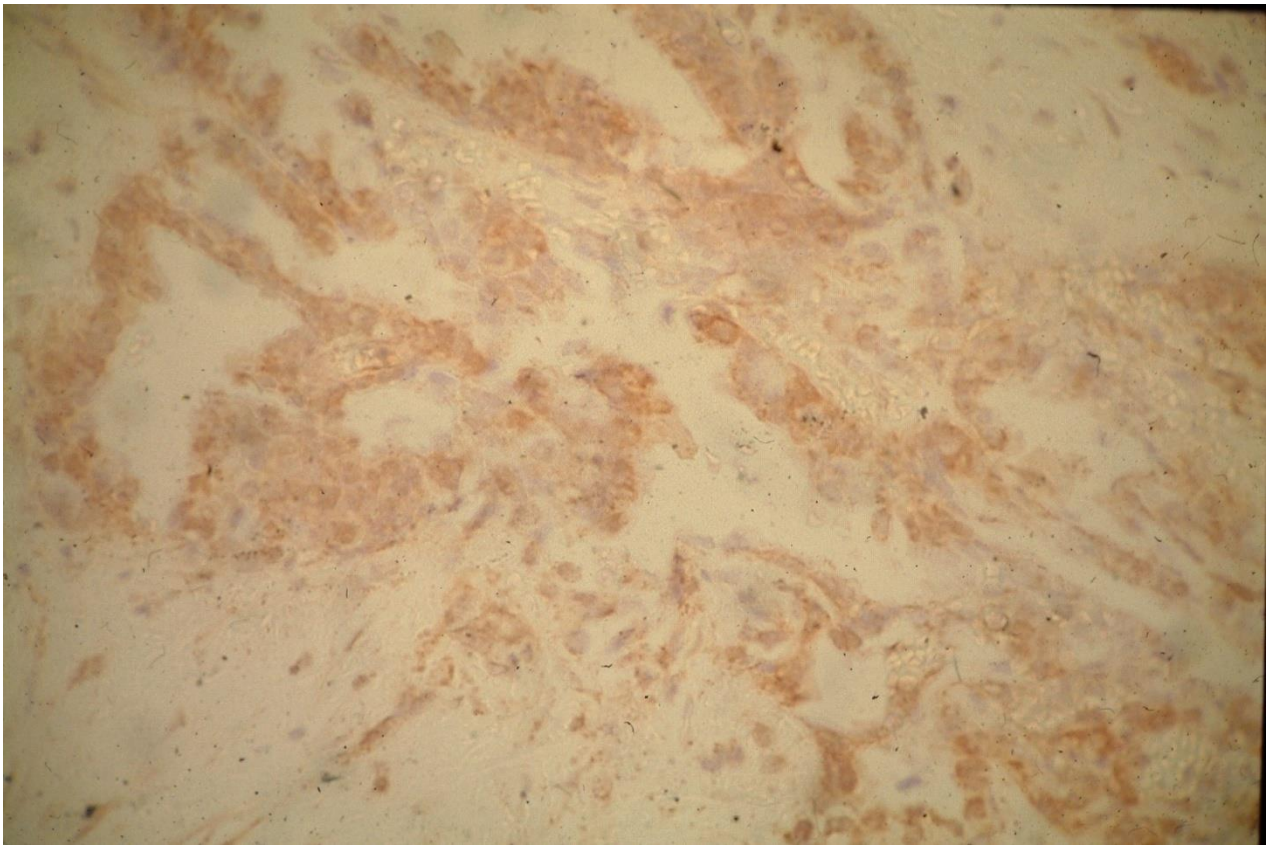


Figure 6.16: c-erbB-3 cytoplasmic staining in ductal pancreatic cancer.

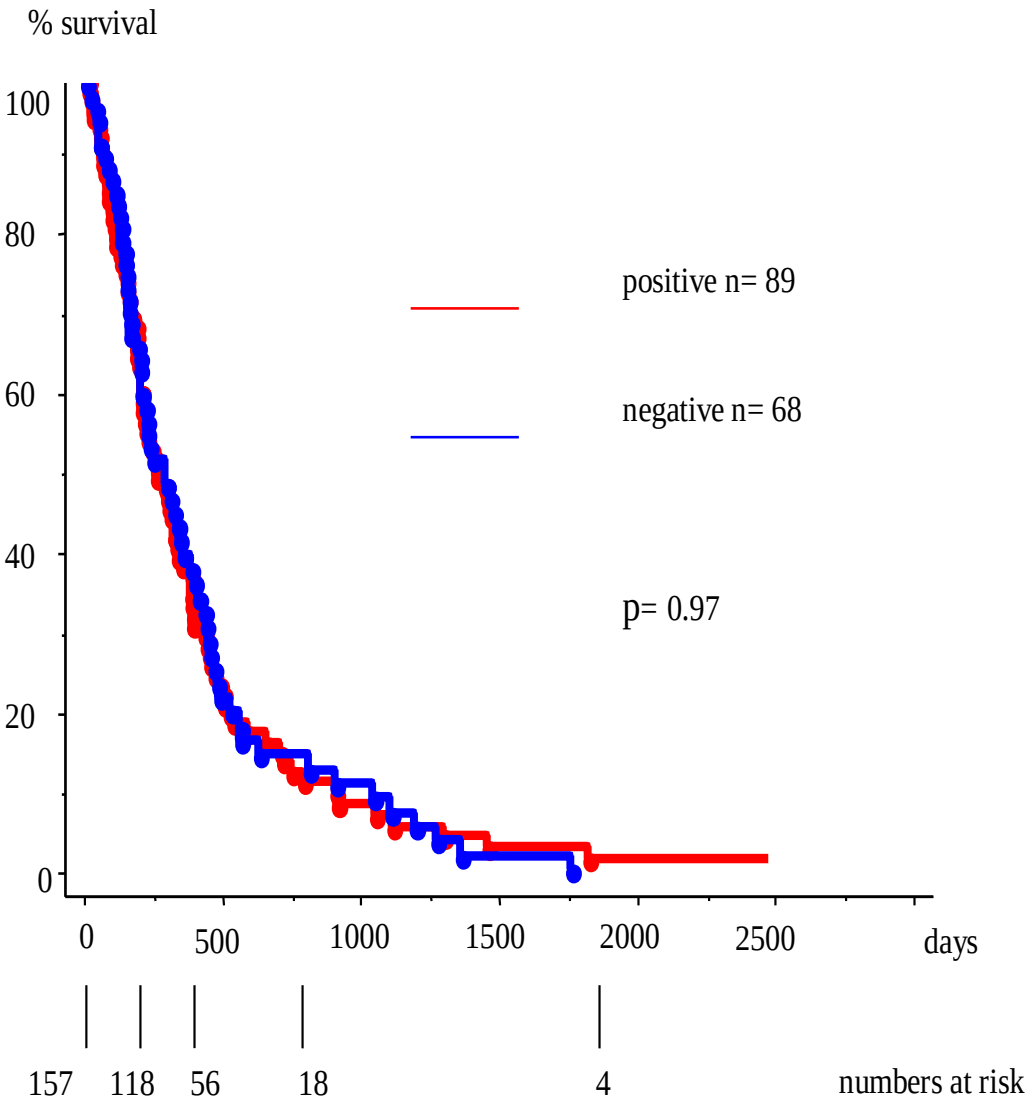


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Figure 6.17: Kaplan Meier plot showing c-erbB-3 and survival.

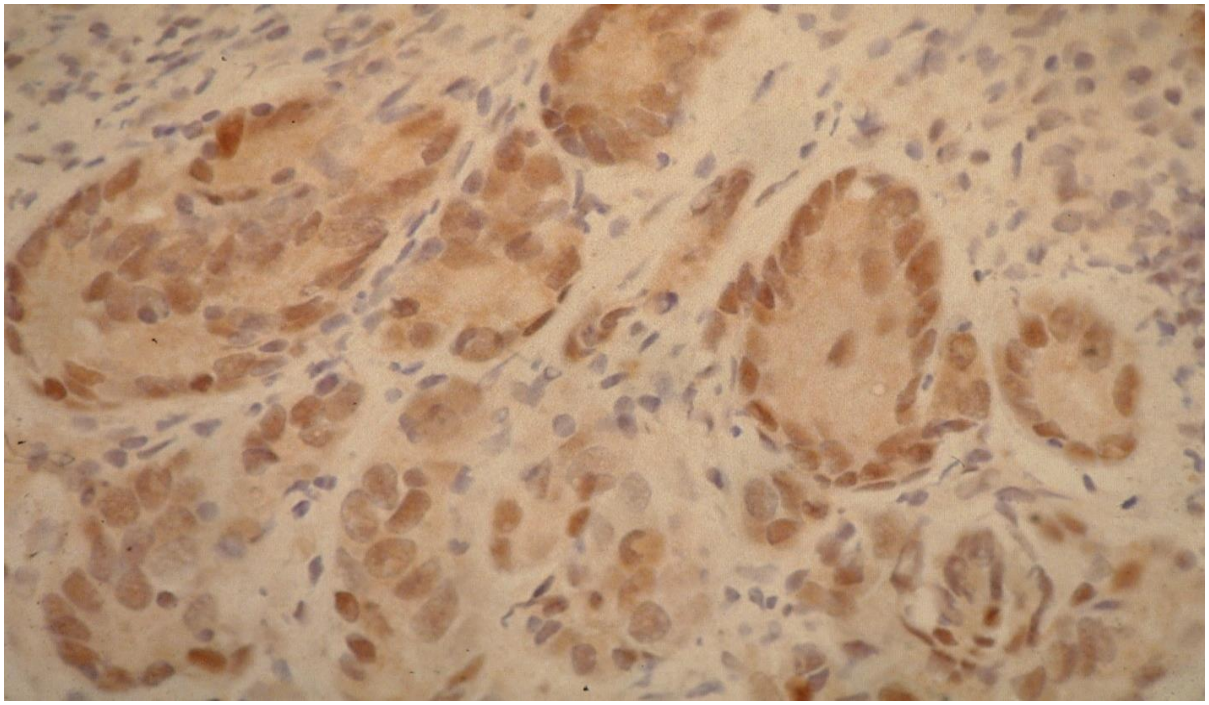


Figure 6.18: cyclin D1 nuclear staining in ductal pancreatic cancer.

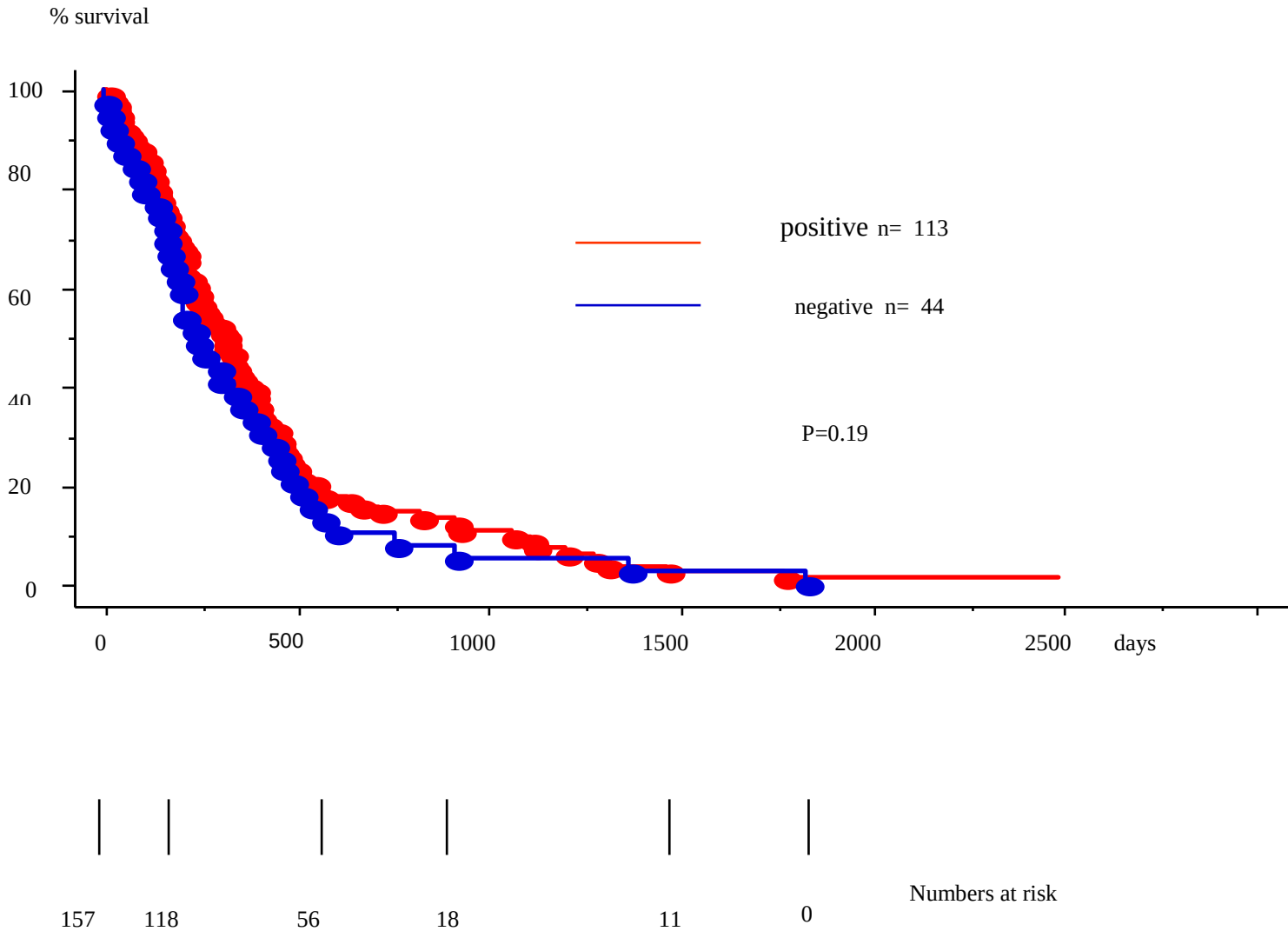


Figure 6.19: Survival of patients in relation to cyclin D1 immunostaining.

Table 6.3. Overall immuno-staining rates for 157 cases of pancreatic adenocarcinoma

Antibody	Positive	
	N	%
p53	64/157	41
$p21^{WAFI}$	39/157	25
$p16^{INK4}$	21/157	13
cyclin D1	113/157	72
c-erbB-2	51/157	33
c-erbB-3	89/157	57

N = number

Table 6.4. A correlation matrix for each antibody and clinical parameters

Clinical parameter	p53	p21	p16	cyclin D1	c-erbB-2	c-erbB-3
gender	P=0.44	P=0.91	P=0.56	P=0.24	P=0.65	P=0.27
stage	P=0.54	P=0.64	P=0.44	P=0.28	P=0.61	P=0.74
differentiation	P=0.30	P=0.77	P=0.42	P=0.47	P=0.42	P=0.57
tumour size	P=0.01*	P=0.70	P=0.58	P=0.51	P=0.19	P=0.99
lymph node status	P=0.30	P=0.34	P=0.64	P=0.05	P=0.16	P=0.81
resection margins	P=0.21	P=0.44	P=0.68	P=0.55	P=0.05	P=0.20

* Statistically significant

Table 6.5. Univariate analysis of the relationship of immunohistochemical staining to patient survival

Molecular marker	N	Median survival (months)	95% CI	χ	*p* value
p53+	64	34.72	26.30 – 43.14	0.29	0.86
p53-	93	33.38	26.21 - 40.39		
p21WAFI+	39	32.83	23.76 – 41.90	3.26	0.98
p21WAFI-	118	21.58	13.35 – 29.81		
p16 INK4I+	21	40.16	24.50 – 55.81	0.75	0.38
p16INK4-	136	32.86	27.10 – 38.62		
cyclin D1+	113	35.79	29.13 – 42.45	1.67	0.19
cyclinD1-	44	29.03	20.06 – 38.00		
c-erbB-2+	51	30.73	22.46 – 39.00	0.05	0.83
c-erbB-2-	106	35.36	28.37 – 42.35		
c-erbB-3+	89	32. 89	25.92 – 39.86	0.13	0.71
c-erbB-3-	68	35.22	26.56 – 43.88		

+ = positive immunostaining, - = negative immunostaining, N = number

Table 6.6. Correlation of patient subgroup immunostaining and survival

Immunostaining subgroup	N	Immunostaining subgroup	N	χ	p value
p53+p21-	45	p53-p21+	18	0.03	0.85
p53+p21-	45	rest	112	0.12	0.72
p53+p21-cyclinD1+	28	p53-p21+cyclinD1-	5	2.13	0.14
p53+p21-cyclin D1+	28	rest	129	0.49	0.48
ErbB2+erbB3+	29	ErbB2-erbB3-	45	0.02	0.89
ErbB2+ebB3+	29	rest	128	0.07	0.79
p53+p21-cyclin D1+ erbB+erbB-3+	7	rest	150	0.13	0.72

+ = positive immunostaining, - = negative immunostaining, N= number

6.4 Summary of immunohistochemical staining results

1) The overall median survival survival was 33.9 months (CI – 28.48 – 39.32) in this study population
2) Significant prognostic factors were TNM stage and lymph node involvement
3) There were no significant correlations between survival and gender, differentiation, size of tumour, resection margin status and the use of adjuvant therapy
4) Multivariate analysis showed no significant difference in survival between patients from the UK and Sweden once lymph node status was taken into account.
5) The pattern of expression of p53, p21WAF1, p16INK4, cyclin D1, c-erbB-2 and c-erbB-3 did not significantly correlate with patient survival
6) Correlation between immunohistochemistry and clinical parameters showed a significant association between size of tumour and p53 immunoreactivity ($p < 0.01$)
7) There were no significant differences in survival between patients with combined p53 positive, p21 negative and cyclin D1 positive tumours with the other markers
8) Patients with combined c-erbB-2 and c-erbB-3 positive tumours also demonstrated no difference in survival when compared with other markers
9) Patients with abnormalities in all the markers did not demonstrate a significant decrease in survival when compared with the rest

6.5 Conclusions

The molecular markers studied occur with significant frequencies in pancreatic cancer but do not correlate with survival in resected ductal pancreatic cancer either independently or in combination.

7.0 p53 mutation analysis

7.1 Introduction

Studies of p53 alterations at the genomic and protein level show that overexpression of the p53 protein correlates significantly with allelic loss (El Diery WS et al, 1997) and missense mutation in exons 5-9 (Kupryjanczykk J et al, 1993; McManus D et al, 1994). But nonsense mutations, splice site mutations and most deletions do not result in protein accumulation (Kupryjanczykk J et al, 1993; Righetti SC et al, 1996). At least 50% of pancreatic cancers show allelic loss (deletion of one copy of the gene) at the p53 locus (Berrozpe G et al, 1994; Redston MS et al 1994; Rozenblum E et al, 1997). Most studies of mutations in the p53 tumour suppressor gene in tumours have examined mainly exons 5-8, the so called mutational "hot-spot" region. In one study, up to 30% of p53 mutated pancreatic cancers were negative for p53 immunohistochemical staining (Scarpa A et al, 1993) since intragenic deletions associated with the loss of the normal allele prevented protein synthesis. Also, the few large studies that have examined the entire coding region of the p53 gene suggest that 10% to 25% of all mutations occur outside exons 5-8 (Hartmann A et al, 1995). It is important therefore that mutational analysis of the p53 gene is performed in conjunction with immunohistochemical analysis so that the relationship between mutation, expression, treatment and prognosis can be more fully understood. The aim of this part of the work was to confirm the findings of p53 positive and negative staining immunohistochemistry by mutation analysis in a selected group of the cases that were either p53 positive or negative. Microdissected pancreatic cancer specimens were processed using a new multiplex PCR system and then sequenced.

7.2. Mutation detection methods

The number of methods that are available for mutation detection vary in their complexity, costs, labour-intensiveness and type of mutations detected. There are two main approaches for mutation detection; scanning and diagnostic. Scanning methods detect both known and unknown mutations and are more frequently used when large numbers of samples and/or large genes are being investigated. By contrast diagnostic methods are generally applied to the detection of a single base pair changes within single genes. A summary of the main techniques used for mutation detection is shown in Table 7.1.

Microdissection of paraffin-embedded formalin-fixed samples used for routine diagnosis allows analysis of DNA from normal and diseased microscopic lesions. The problem with archival formalin-fixed paraffin-embedded tissue is that extracted DNA is often fragmented because of fixation; this restricts the size of the product that can be amplified. Therefore to cover the entire 1.8-kb of p53 coding region and splice sites, multiple products need to be generated. Unfortunately the limited amount of material generated from microdissection makes it difficult to perform multiple amplification reactions. Multiplex polymerase chain reaction (PCR) is one method that can be used to amplify multiple loci in one reaction (Henegariu O et al, 1997; Angelopoulou K et al, 1998; Thompson-Hehir J et al, 2000). Used in conjunction with nested PCR, multiple specific products can then be generated on which mutational analysis can be performed.

Previous work by Thompson-Hehir J et al, (2000) has suggested that multiplex PCR followed by a second PCR to amplify individual products, could potentially reduce the amount of clinical material used by up to 95%. The rationale behind this was that single exon amplification

requires 2μL for each exon in the first round, whereas only 2μL of the same micro dissected material would be required for multiplex PCR to amplify all exons.

Table 7.1. Summary of current methods used for mutation detection.

Technique	Scanning/Diagnostic	Known/Unknown mutation detection	Sensitivity %	Fragment length
SSCP	Scanning	Both	80-90%	300bp
HA	Scanning	Both	80%	200-600bp
DGGE	Scanning	Both	99%	<1000bp
NIRCA	Diagnostic	Both	95%	1000bp
CCM	Diagnostic	Both	90-95%	1400bp
EMC	Diagnostic	Both	>98%	1300bp
ASO	Diagnostic	Known	N/a	N/a
PASA/ARM	Diagnostic	Known	N/a	N/a
DNA chips	Diagnostic	Known	99%	N/a
Sequencing	Both	Both	95%	<700bp

Single Strand Conformational Polymorphism (SSCP), Heteroduplex Analysis (HA), Denaturing Gradient Gel Electrophoresis (DGGE), Non-Isotopic Rnase Cleavage Assay (NIRCA), Chemical Cleavage Mismatch (CCM), Enzyme Mismatch Cleavage (EMC), Allele Specific Oligonucleotides (ASO), Amplification of Specific Alleles (PASA/ARMS PCR).

7.3. DNA sequencing

DNA sequencing provides information about the position and type of mutation whether the mutation is known or unknown. The most commonly used method is chain termination dideoxysequencing (Sanger F et al, 1977). Dideoxynucleotides which, when incorporated into the growing DNA strand by a DNA polymerase cause termination of elongation. By using appropriate concentrations of both deoxynucleotides and dideoxynucleotides in a sequencing reaction, a chain termination event is generated for each base position in the piece of DNA being sequenced. Originally DNA sequencing used the Klenow fragment from DNA polymerase to generate the sequencing reaction products and radionucleotides for product detection. Generally DNA sequencing is now carried out by cycle sequencing using thermostable DNA polymerases and fluorescent nucleotides or primers for product analysis and automated data collection and analysis.

The other methods used for mutation detection have their advantages and disadvantages and these will be discussed briefly. SSCP enables a large number of samples to be processed, especially if the method is multiplexed. CCM has a detection rate nearing 100% but has the disadvantage of using toxic chemicals. DGGE has one of the highest reported mutation detection rates but requires customized primers and specialised equipment. Although DNA sequencing can be time consuming and expensive it does provide both the position and type of nucleotide change for a given fragment of DNA.

7.4 Materials and methods

7.4.1 Source of clinical samples

Formalin-fixed, paraffin-embedded pancreatic cancer samples were obtained from the pathology archives of the department of pathology at the Royal Liverpool University hospital, Liverpool, United Kingdom. Human Placental Genomic DNA (Sigma, UK) was used throughout as controls.

7.4.2 Preparation of slides for microdissection

For cases chosen for mutation analysis, formalin fixed paraffin-embedded tissue blocks were retrieved from archives. A 5μm section was cut followed by six serial 10μm sections and a 5μm section, all of which were then prepared on labelled slides. To avoid cross contamination between cases a disposable microtome blade was used and cleaned with xylene after each tissue block. Before cutting from each tissue block a few sections were cut from a blank paraffin block. Slides for microdissection were stored in a plastic container until ready for use. The first 5μm section was stained with haematoxylin and eosin and reviewed by a pathologist to select tissue regions for microdissection. Appropriate areas were marked on these slides with a marker pen. The slides were dewaxed by soaking in xylene (heated to 37°C), for 20 min followed respectively by a 2x10min, soak in xylene (at room temperature), 5 min, soak in absolute ethanol, 5min, soak in 75% ethanol, 5 min soak in 25% ethanol and a final 5 min soak in sterile distilled water. The slides were stained using 0.1% toluidine blue (30second soak) and washed 3 times using distilled water (Liverpool University Stores).

7.5 Microdissection

After staining with 0.1% toluidine blue the slides were washed twice in fresh distilled water and held in water prior to microdissection. Slides were then mounted on the microscope stage and a pool of microdissecting buffer (10mM EDTA, 1mM Tris-HCl, 10mM Tris base) was placed over the slide with a disposable pipette, a deep pool of microdissecting buffer was dropped on to the slide to cover the entire section. Microdissection of the ducts with tumour was carried out using a stereodissecting microscope and an electrolytically polished tungsten needle replaced between slides and controlled by a micromanipulator (Leitz mechanical manipulator) as described previously (Going et al, 1996). Siliconised tips with ends cut off at an angle to give a wide flat mouth were held with the cut edge parallel to the slide to facilitate removal of the tissue specimens. Tissue fragments of tumour cells were microdissected avoiding were possible blood vessels and regions of necrosis. These fragments within the same low power field (x50) were collected in 12.5μl buffer (10mM Tris HCL pH 8.0, 1mM EDTA) and stored in sterile 0.5mL polypropylene micro centrifuge tubes. Control samples from normal pancreas from each case were preserved. Digitised computer images were archived before and after microdissection as shown in Figure7.1. These images and haematoxylin and eosin stains of the microdissected sections and serial sections were used for subsequent pathological review.

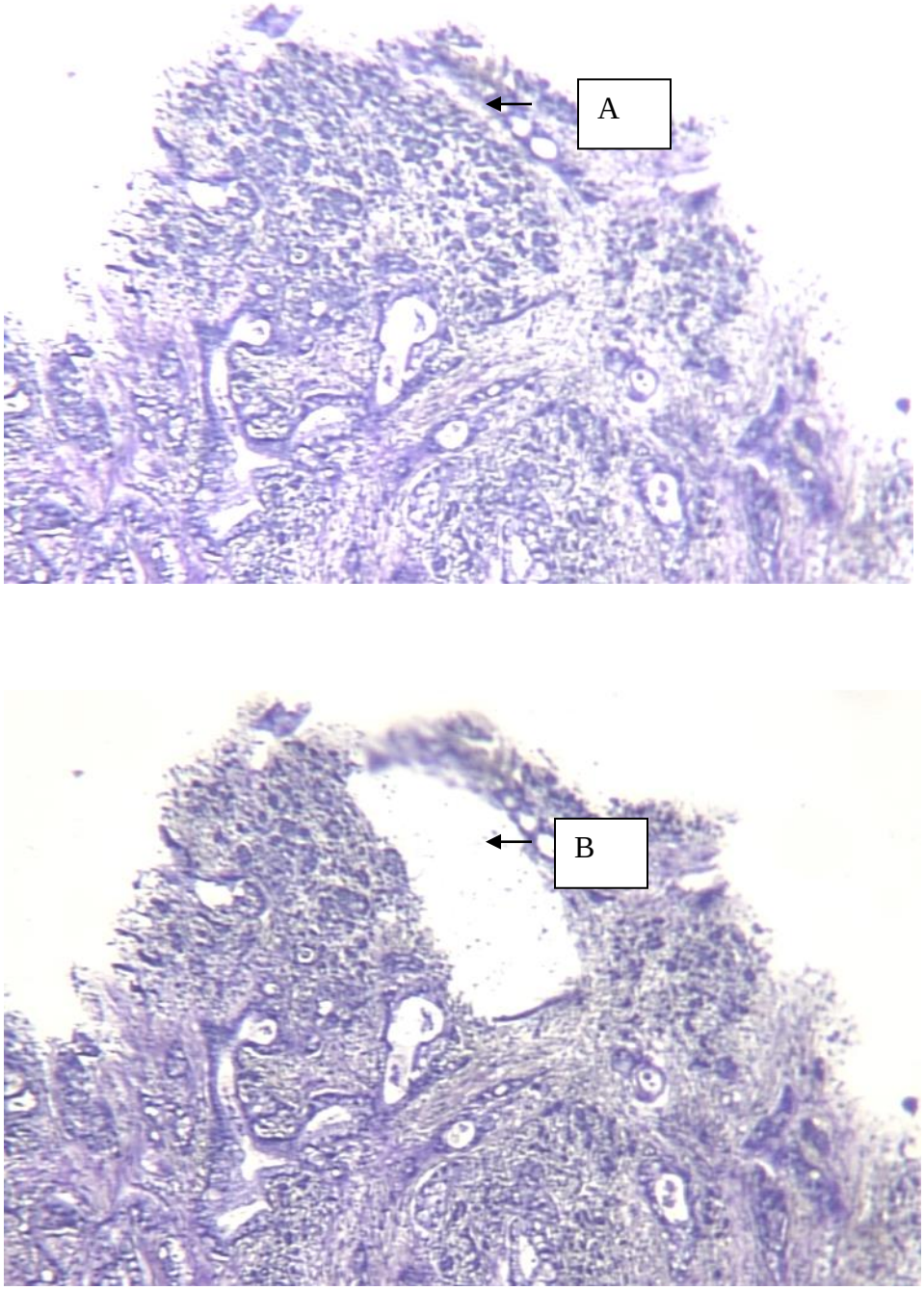


Figure 7.1: Video capture images of pre and post microdissection, top and bottom respectively. A malignant duct (A) and microdissected malignant duct (B) are shown by arrows.

7.6 DNA extraction from microdissected tissues

All subsequent manipulations of microdissected tissue were carried out in a class 11 laminar flow cabinet. An equal volume of Proteinase K digestion buffer [(20mg/mL proteinase K (Sigma), 0.2% Tween 20 (Sigma)] and microdissection buffer was added to the microdissected tissue and the sample incubated at 37ºC for 48 hours. The Proteinase K was inactivated by incubation in a boiling water bath for 15 minutes. The proteinase K digestion buffer stock used was made up as follows:

880ml microdissection buffer

20µl Tween-20 (polyoexyethylene Sorbitan Monolaurate)

100ųl proteinase k (@20mg/ml)

7.7. Oligonucleotide primers

Primer sequences were selected from p53 genomic sequence (GenBank accession number U94788) using Oligo software version 5. Oligonucleotides were purchased from Oswel DNA services (Southamptom, UK) and from Gnosys (Cambridge, UK) and arrived as a freeze-dried pellet. To each primer, 100µL of sterile water was added and they were incubated at 4°C either for 4 hours or overnight to allow primer resuspension. To prevent contamination and repeated freeze thawing of the oligonucleotides a system was designed for primer storage. The concentration of each oligonucleotides in 100µL of water was determined. The appropriate volume of stock oligonucleotide to prepare a 500µL, 50µM aliquot was then dispersed into a series of sterile, labelled micro centrifuge tubes. The tubes were labelled with the primer name and the amount of water required to be added to make the oligonucleotide to 50ğM e.g. 1NF1/247µL, 3R1/329µL. These stocks were stored at minus 20° C until required.

7.8 Polymerase Chain Reaction

Polymerase chain reactions (PCR) were either multiplex or single product. Amplification of p53 products using the multiplex method employed two rounds of PCR, the first being multiplex and the second being hemi-nested amplification of specific p53 products from the first round mix. Single product amplification, employed one or two rounds of amplification, with second the round being hemi-nested amplification of specific products. First round reactions contained 1xPCR buffer Π (Perkin Elmer), 1.5-3.0mM $MgCl_2$, 200ųM dNTPs, 0.025μl^{-1} Amplitaq Gold and 1x Multiplex primer mix. Second round or single exon reactions were prepared as for first-round reactions except both forward and reverse primers were included at 1μM. When first round amplified product was used as a target in second round PCR the first round reaction was diluted to 1:16 prior to the addition to second round reactions. First round PCR cycling conditions were 94^{o}C for 10 min, then 94^{o}C 30 sec, 30^{o}C for 60sec, 72^{o}C for 60sec for 8 cycles, followed by 36 cycles of 94^{o}C, 60^{o}C for 30sec and 72^{o}C for 30 sec, followed by 72^{o}C for 10min and finally 4°C hold. Second round and single exon PCR cycling conditions were 94^{o}C for 10min, and then 42 cycles at 94^{o}C for 30sec, 72^{o}C for 30sec followed by 72^{o}C for 10min and finally 4^{o}C hold. All reactions were carried out in foil sealed 96 well Thermofast plates (Advanced Biotechnologies, UK) and cycled in a Perkin Elmer 9600 thermal cycler (Biosystems).

7.9 Agarose gel electrophoresis

All gels were an equal volume of 3% Seakem LE (FMC Bioproducts) and 3% Nusieve (Flowgen, Staffordshire) agarose in 1xTAE (Tris-acetate-EDTA buffer) containing ethidium bromide (0.5ug.ml-1). Sample volume loaded was variable but contained loading dye (40% sucrose, 0.025%(v/v) bromophenol blue, and 0.025% xylene cyanol) at $1/6^{th}$ to $1/10^{th}$ of the sample. Following electrophoresis the gels were scanned using a Fluorimager SI (Molecular Dynamics, Chesham, Bucks) with Image quant software (Molecular Dynamics).

7.10 Drying of PCR products

Where there was judged to be low sample/product yield, the samples were dried down using a standard Gyrovap vacuum drier and resuspended in an appropriate volume of sterile water.

7.11 Clean up of PCR Products

Prior to sequencing the reaction components e.g. dNTPs and primers which would interfere with the sequencing were removed using Qiaquick tm 96 well PCR purification kit (Quiagen, West Sussex, UK) according to the manufacturer's instruction. Samples were eluted in 60μL of sterile water and 4μL of product was anaylsed by electrophoresis. Eluates were dried down by overnight incubation at 37°C and samples were resuspended in the appropriate amount of sterile water to give a concentration within the range recommended for cycling sequencing.

7.12 DNA sequencing

ABPI PRISM dRhodamine Dye Terminator Ready Reaction Cycle Sequencing kits (Perkin Elmer) with AmpliTaq DNA Polymerase FS (Perkin Elmer) were used for direct sequencing of PCR products. The sequencing kits were used as instructed except volumes of ready mix added were reduced by 50%. The reaction mixtures consisted of 4μL of DNA template (15ng-45ng), 2μL of forward or reverse primer at 0.8pmoles μl^{-1} and 4μL of ready reaction mix. Reactions were set up using 96 well Thermofast plates (Advanced Biotechnologies). The cycling parameters were 96°C for 2 min, and then 25 cycles at 96° C for 10sec, 50°C for 5sec and 60°C for 4min and finally 4°C hold.

7.13 Ethanol precipitation in micro centrifuge tubes

Each sequencing reaction was transferred to a labelled, sterile micro centrifuge 0.5mL tube and 25uL of a 24:1 96% (v/v) ethanol/3M sodium acetate (pH5.2) stock was added to each tube, vortexed briefly and incubated at room temperature for 20min. Samples were then centrifuged in a micro centrifuge at full speed for 30 min and the supernatant discarded. 125μL of 70%(v/v) ethanol was added to each tube, the samples were vortexed briefly and centrifuged as before for 15 min. The supernatant was discarded and the tubes left to dry at 37ºC for 10-15min. Samples were either stored at minus 20ºC or re-suspended in 2μL sequencing loading dye (de-ionsed formamide and 25mM EDTA-containing 50mg/mL blue dextran, in a ratio of 1:5) and used immediately. 1.5μl of the sample was loaded on a 55 polyacrylamide gel (Long Ranger) and electrophoresed on a 377 automated flourescent sequencer (PE Biosystems) for 3-6 hours.

7.14 Analysis of sequencing data.

Analysis was carried out blind without knowledge of the p53 status of the specimens. Sequence data was analysed using Factura and sequence Navigator software (PE Biosystems). Sequence variations detected manually in both forward and reverse directions were confirmed by further analysis of a second independent PCR amplified product from the same tissue extract. Both forward and reverse directions were confirmed by further analysis of a second independent PCR amplified product from the same tissue extract.

Chapter 8 Results of p53 mutation analysis

8.1 DNA extraction from microdissected samples

17 cases were chosen for mutation detection and of these 12 (70%) yielded detectable DNA (See Table5). Of these 12 cases, seven were negative for p53 nuclear staining while the remaining five were p53 positive. The author was blinded as to the p53 status of the samples until after sequencing results were verified with an independent observer. A pathologist (Dr F Campbell, Royal Liverpool Hospital) had previously selected tumour areas for microdissection. In the p53 positive staining group at least 60% of the tumour areas showed widespread p53 nuclear staining.

Table 8.1. Clinical information on micro dissected sample

CASE	BACKGROUND	P53 STAINING	% STAINING	DIFFERENTIATION
3	mucinous metaplasia	positive	70%	well to moderate
4	normal	negative	absent	mod.focal poor
5	normal	negative	absent	poor to moderate
6	mucinous metaplasia	positive	90%	poor
10	mucinous metaplasia	positive	60%	well to moderate
11	normal	positive	75%	moderate
12	normal	negative	absent	poor. perineural
13	normal	negative	absent	mod. Giant cells
14	mucinous metaplasia	positive	80%	mod. To poor
15	mucinous metaplasia	negative	absent	moderate
16	normal	negative	absent	poor.giant cells
17	Mucinous metaplasia	positive	100%	Poor. Huge nerve

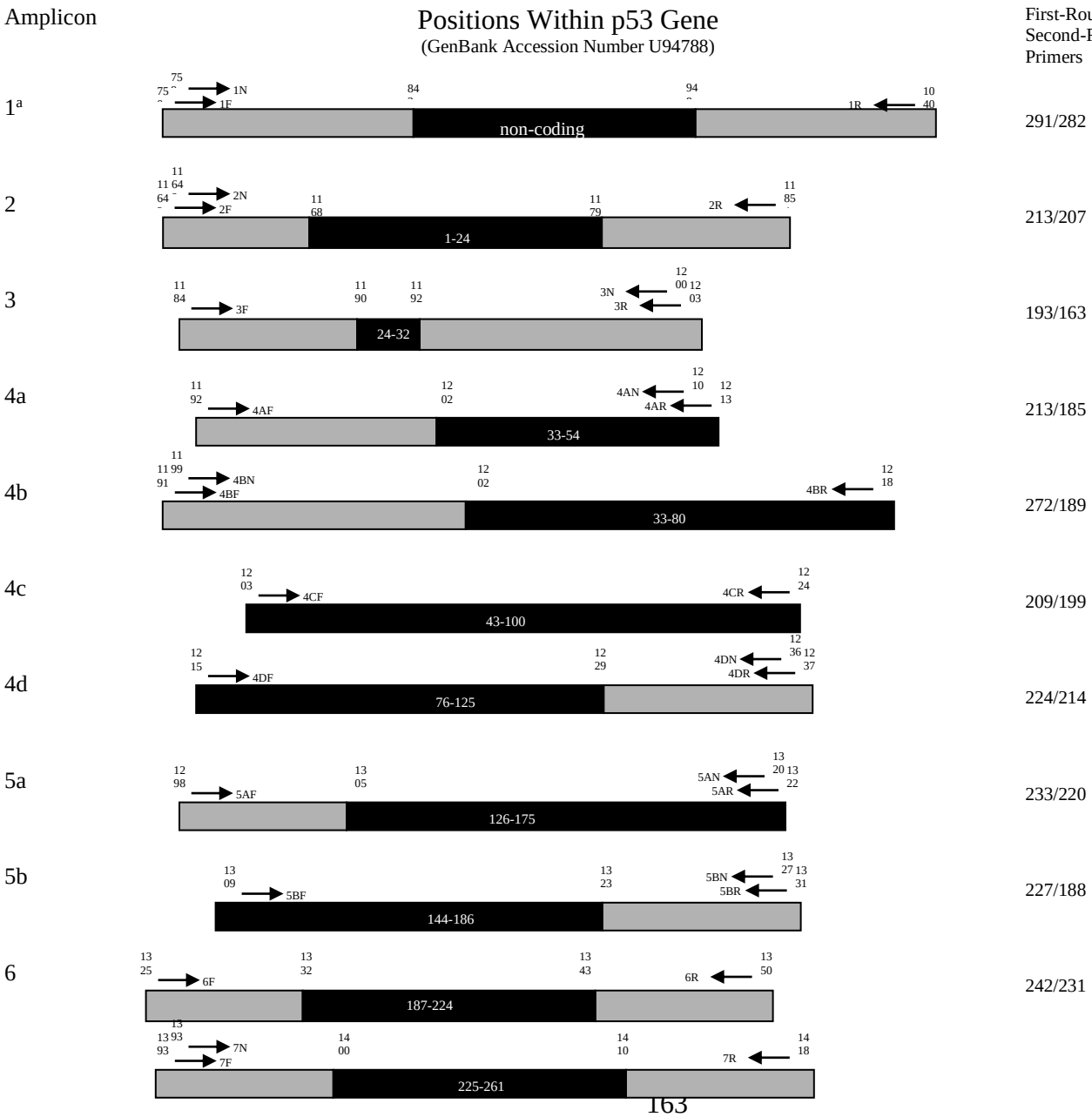
Figure 8.1: Agarose gel electrophoresis of duplicate second round polymerase chain reaction products from micro dissected pancreatic cancer showing amplification of exons 1 – 11.

TABLE 8.2. p53 primers nested, second-round primers are highlighted in bold

PRODUCTS	FORWARD PRIMER	SEQUENCE (5'-3')	REVERSE PRIMER	SEQUENCE (5'-3')
First-Round	1F	TTTGTGCCAGGAGCCTCGCA	1R	CCCAGCCCCAGCGATTTTCCCGAGCTGAAA
Second-Round	1N	GGAGCCTCGCAGGGGTTGATG	1R	as above
First-Round	2F	CAGGGTTGGAAGCGTCTCAT	2R	TCTCTGCTAGGGGGCTGGGGT
Second-Round	2N	TGGAAGCGTCTCATGCTGGA	2R	as above
First Round	3F	CCCTAGCAGAGACCTGTGGG	3R	CGGCAAGGGGGACTGTAGAT
Second-Round	3F	as above	3N	AGCAGTCAGAGGACCAGGTCCTCA
First-Round	4AF	TCTGGTAAGGACAAGGGTTG	4AR	GGAGCAGCCTCTGGCATTCTGGGAGC
Second-Round	4AF	as above	4AN	CATCTGGACCTGGGTCTTCA
First-Round	4BF	CTGAAAACAACGTTCTGGTAAGG	4BR	TGCAGGGGCCGCCGGTGTA
Second-Round	4BN	CTGACTGCTCTTTTCACCCATC	4BR	as above
First-Round	4CF	GCAATGGATGATTTGATGCTGT	4CR	CCGTAGCTGCCCTGGTAGGT
Second-Round	4CN	ATTTGATGCTGTCCCCGGAC	4CR	as above
First-Round	4DF	GCACCAGCAGCTCCTACA	4DR	CAAACAAAAGAAATGCAGGGGGAT
Second-Round	4DF	as above	4DN	AAATGCAGGGGGATACGG
First-Round	5AF	TTCCAGTTGCTTTATCTGTTCA	5AR	CGCTCATGGTGGGGGCAG
Second-Round	5AF	as above	5AN	GGCAGCGCCTCACAACCT
First-Round	5BF	GCCAAGACCTGCCCTGTG	5BR	AAGAGCAATCAGTGAGGAATCAGA
Second-Round	5BF	as above	5BN	GGCAACCAGCCCTGTCGTC
First-Round	6F	AGACGACAGGGCTGGTTG	6R	CTCACCCGGAGGGCCACTGA
Second-Round	6N	CTGGTTGCCCAGGGTCCCCA	6R	as above
First-Round	7F	GGCCTCCCCTTGCTTGCCACA	7R	GGCCCAGGGGTCAGCGGCAA
Second-Round	7N	CCCCTGCTTGCCACAGGTCT	7R	as above
First-Round	8F	GTTTTTTAAATGGGACAGGTAGGA	8R	GGTGATAAAAGTGAATCTGAGGCATAAC
Second-Round	8N	GGGACAGGTAGGACCTGATTTCCT	8R	as above
First-Round	9F	CAAGAAGCGGTGGAGGAGACCAAGGGT	9R	CCCCCTGATGGCAAATGCCCCAA
Second-Round	9F	as above	9N	GCCCCAATTGCAGGTAAAACAGTCA
First-Round	10F	CAATTGTAACTTGAACCATCTTTTAACT	10R	TGAGAATGGAATCCTATGGCTTTC
Second-Round	10F	as above	10N	GGAATCCTATGGCTTTCCAACCTA

First-Round	11F	GGGCACAGACCCTCTCAC	11R	GTTCAAAGACCCAAAACCCA
Second-Round	11F	as above	11N	CCCAAAACCCAAAATGGC

[1]with reference to Genbank accession number U94788



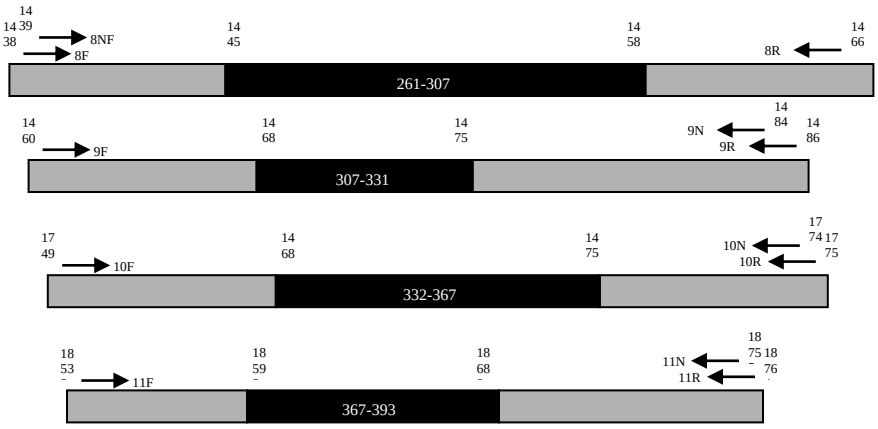


Table 8.3: **Primers used to generate each amplicon including localisation of 5'ends. Nucleotide positions in brackets represent the position of the 5'end of the nested second-round primer. Coding sequence is shown in black and intron sequence in grey. [a]Exon 1 is non-coding; the region shown in black forms part of the primary transcript**
.

8.2 Analysis of sequence variations in archival tumour samples

Most of the cases studied produced good sequence data but not in all exons exons (See Table 8.4). Human placental genomic DNA (Sigma, Dorset, UK) were used as controls for sequence analysis and mutation detection. Sequence data was considered complete when there was bi-directional read for the full coding sequence transcribed region for exon 1 and 10 bases upstream and downstream of the coding region for each product.

Table 8.4. Summary of sequencing yield from exons

Exon	Case3	4	5	6	10	11	12	13	14	15	16	17	Total/12	%
1	p	p	p	g	p	g	g	g	g	g	p	g	7	58
2	g	g	g	g	g	g	g	g	g	g	g	g	12	100
3	p	p	p	p	p	g	g	g	g	g	g	g	7	58
4a	p	p	g	p	g	g	p	g	g	g	g	g	9	75
4b	p	g	g	g	g	g	p	g	g	g	g	g	10	83
4c	p	g	p	p	p	g	p	p	g	p	g	g	7	58
4d	g	g	g	g	g	g	p	g	g	g	g	g	11	91
5a	g	g	g	g	g	g	g	g	p	p	g	g	10	83
5b	g	g	p	g	g	p	p	p	g	g	g	g	9	75
6	g	g	g	g	g	g	g	g	g	g	g	g	12	100
7	g	g	g	g	g	g	g	g	g	g	g	g	12	100
8	g	g	p	g	g	g	g	g	p	g	g	g	11	89
9	p	p	g	p	g	g	g	g	p	g	g	g	9	75
10	g	g	g	g	p	p	p	p	g	g	g	g	8	66
11	g	g	g	g	g	g	g	g	g	g	g	g	12	100
total/15	9	11	11	12	11	13	11	12	12	13	14	15	146/180	81
% yield	60	73	73	80	73	86	73	80	80	86	93	100		

g = good sequence

p = poor sequence

Table 8.5. Summary of sequence variations detected in pancreatic cancer samples.

Sample	Region	Variation	Type	p53	Grade
3	exon 5b	mutation	Base change G-A@113	positive	well/mod
10	exon5b	mutation	Base change G-T@103	positive	well/mod
10	exon 7	polymorphism	G-T base change	positive	well/mod
11	exon2	polymorphism	C-insertion@173,174,177	positive	moderate
12	exon2	polymorphism	C-insertion@173,174,177	negative	poor
13	exon2	polymorphism	C-insertion@173,174,177	negative	mod
14	exon3	polymorphism	C-insertion @32	positive	poor
15	exon3	polymorphism	C-insertion@32	negative	mod
16	exon3	polymorphism	C-insertion@32	negative	poor
17	exon4a	polymorphism	G-T@ 208	positive	poor

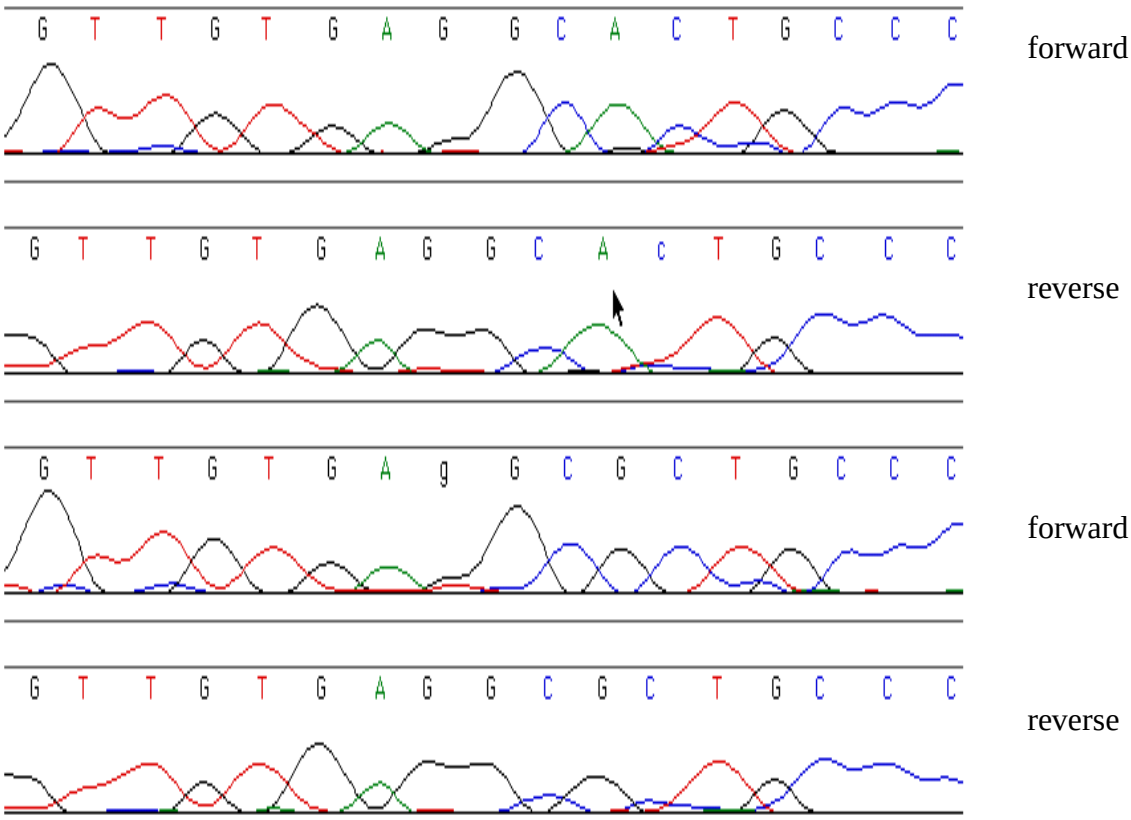


Figure 8.2: **Electrophoregram showing mutation as a base change between G-A@133base Exon 5b in case 3 (p53 positive). Sequence variants detected in both forward and reverse directions in duplicate. The experiments were repeated by analysis of independent PCR products amplified from the same tissue extract. When PCR products from the same sample shared the base change, it was defined as a "confirmed variant".**

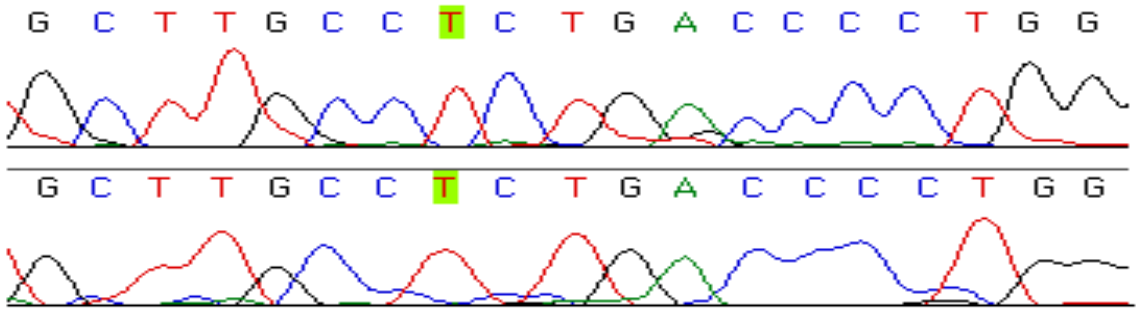
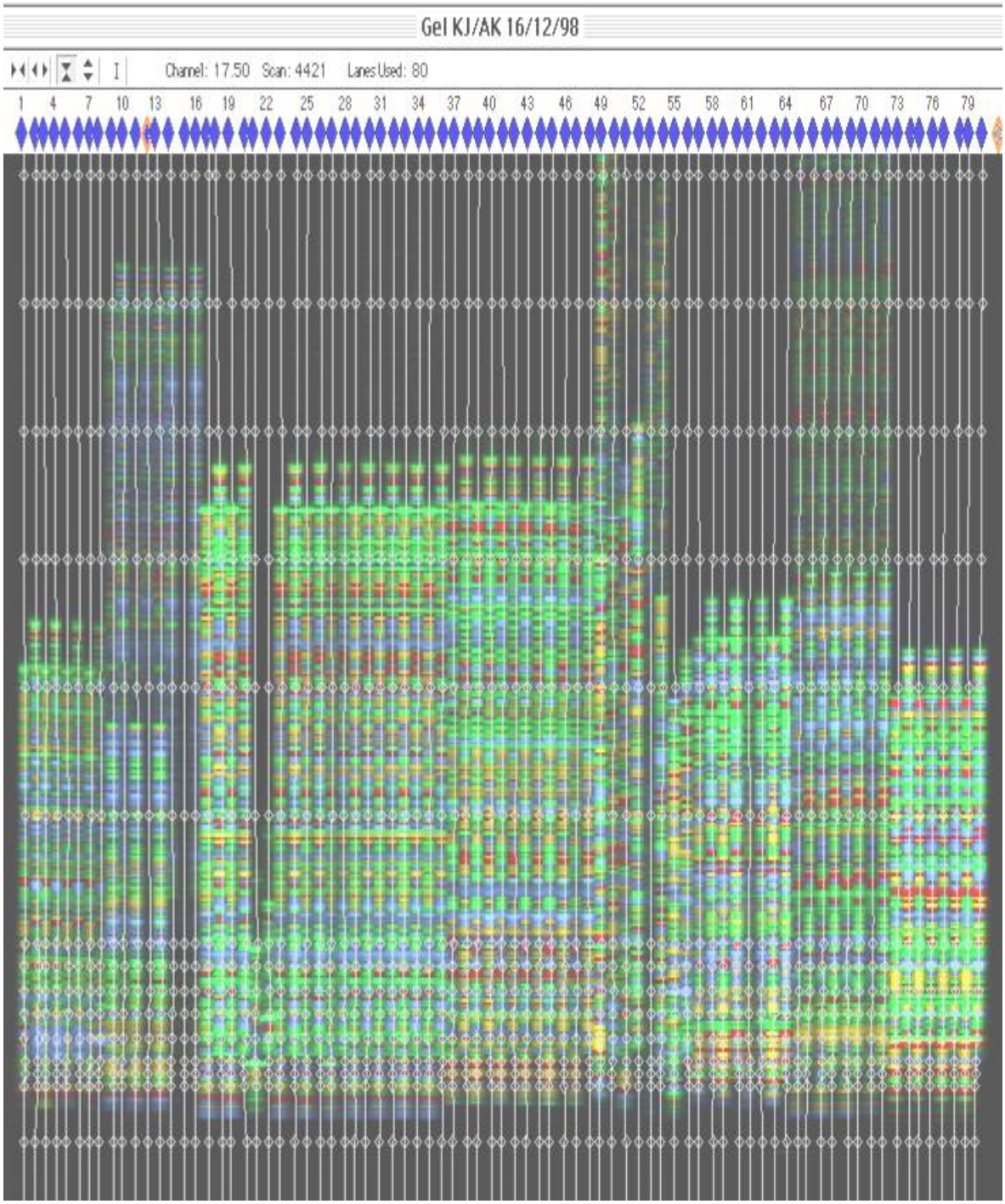


Figure 8.3: **Electrophoregram showing polymorphisms in Cases 11, 12, 13 in Exon 2 @173,174,177 in codon 21. Case 11 was p53 positive while cases 12 and 13 were p53 negative.**

Figure 8.4: Gel image from an ABI 377 DNA sequencer 96 gel lane format.



8.3. Summary of results

1) Confirmed p53 mutations were found in cases 3 and 10 in Exon 5b (See Figure 8.3). These samples were previously p53 positive on immunostaining

2) We detected a C-insertion at base positions 173, 174 and 177(Figure 8.4) in intron 2, and a G-T change at position 14168 in intron 7 (See Figure. 8.4). These variations had previously been deposited in Genbank (U94788).

3) There were 10 taq polymerase errors (Case 14; Ex3 bases 90, 96, 108; case 15 and 16, exon 3 base 90, 96, 108; case 17, exon 4a at base 52 found in sequencing. These were genetic variants found from PCR-induced errors. Distinction was made between true sequence variants (genetic variants or mutations) and sequence variants resulting from PCR-induced errors, with the former being defined as being present in multiple independent PCR products.

8.4. Conclusion from this study

The entire p53 gene was sequenced successfully using a multiplex PCR system. Mutations were confirmed in two of six cases that were previously positive for p53 on immunostaining. There were no mutations found in the p53 negative staining cases. A number of polymorphisms previously unreported to Genebank were found but more work is needed to correlate this with clinical parameters.

Chapter 9 K-ras mutation studies

9.1 Introduction and aims

In ductal adenocarcinoma of the pancreas point mutations in codon 12 of the K-ras gene are amongst the most common genetic abnormality found (Hruban RH et al, 1993). In previous studies the presence or the type of K-ras mutations in pancreatic tumours has not been shown to significantly correlate with patient survival (Dergham ST et al, 1997; Hruban RH et al, 1999). The aim of this part of the study was to analyse K-ras mutations using microdissected samples and correlate the findings with survival. This work was carried out in collaboration with the department of Molecular Medicine at Haukeland University, Norway.

9.2 Single Strand conformation polymorphism (SSCP)

This is a rapid and simple method of studying mutations. The electrophoretic mobility of a particle in a gel is sensitive to both its shape and size. In the absence of denaturing, single stranded DNA has a three folded structure that is determined by intramolecular interactions and therefore by its sequence. In SSCP analysis, a mutated sequence is detected as a change of mobility in polyacrylamide gel electrophoresis caused by its altered folded structure. The target sequence is first labelled and amplified simultaneously by the PCR of the genomic DNA or cDNA using labelled substrates. The PCR product is then denatured and resolved by polyacrylamide gel electrophoresis and mutations are detected as altered mobility of separated single strands in the autoradiogram. Further characterisation of the mutation can be done by eluting the mutated allele from the gel used for autoradiography and amplifying again for sequence determination.

9.3 Methods

9.3.1 Patients

143 clinical samples from patients in the immunohistochemical study were used. The clinical details are shown in Table 9.1. The samples were selected by myself and the work was carried out with collaborators in Norway who had no knowledge of clinical details of patients until completion of study.

9.3.2 Extraction of DNA

Tissue blocks or tumour specimens which had been formalin-fixed, paraffin- embedded and slide mounted were used to prepare the DNA for analysis. Two methods were then used to extract DNA. Tissue sections were mixed with 100μL of buffer [(10mM Tris- HCL ph 8.0 and 5% Chelex (Biorad)] and the tubes were heated to 95°C for 5 minutes. After centrifugation for 30seconds, 5μL of the supernatant was immediately added to separate PCR reactions mixes. Alternatively 5μL tumour areas were scraped off microscopic slides and paraffin was removed with 1ml xylene incubation at 50°C for 30 minutes. The samples were then washed twice with 96% ethanol, centrifuged and allowed to dry at room temperature. DNA was extracted using the Puregene kit. 500μL of lysis buffer was added to the sample with 10uL of 10mg/ml proteinase K, incubated overnight at 50°C and then cooled. A 100μL aliquot of protein precipitation solution was added followed by a short centrifugation. The supernatant was removed and DNA precipited using isopropanol. Pellets were centrifuged for ten minutes at 12,000 revolutions then washed with 70% ethanol. Once the pellets were dry the DNA was dissolved in 50μL of TE (10mM Tris-HCl, 1mM EDTA, pH 8.0).

9.4 PCR Amplification

9.4.1 primers

Oligonucleotide primers 5"- AACCTTATGTGTGACATGTTAAT-3" and 5"-AATGGTCCTGCACCAGTAAT-3" were designed to target K-ras exon 1 to produce a 221 base pair (bp) product**.**

9.4.2 PCR Amplification

A 2-5 µ1 of sample DNA was added to a PCR reaction mix consisting of 10mM Tris-HCl ph 9.0, 50mM KCl, 1.5mM MgCl2, 0.01% (w/v) gelatin, 0.1% Triton**,** 125 mM dNTP, 0.5M of each primer and 0.2 units of Taq polymerase (Supertaq, HT Biotechnology Ltd, UK) in a total volume of 100µL. PCR conditions were 94^{o}C for 2 minutes, then 40 cycles at 94^{o}C for 15 seconds and then at 60^{o}C for 30 seconds, followed by 72^{o}C for 2 minutes using a Perkin Elmer TC 1 thermocycler.

9.5 Single Strand conformation polymorphism (SSCP) analysis

A 4µ1 aliquot of PCR product was denatured by mixing with an equal volume of deionised formamide and incubated briefly at 80^{o}C. The samples were then applied to a 20% homogeneous polyacrylamide gel (Phast gel, Pharmacia**,** Sweden) with buffer strips containing 0.88 M L-Alanine, 0.25M Tris pH 8.8 and run on the Pharmacia Phast system automatic electrophoresis and development unit. The programme used was pre run: 400V, 10mAA, 2.5W, 100Vh, loading: 400V, 1mA, 2.5W, 3 Vh, separation: 400V, 10mA, 2.5W, 300Vh. After electrophoresis the gels were silver stained. This method has the ability to identify all the mutations so far reported in exon 1 of K-ras (Ulvik A et al, 1995). A

minimum of three separate analyses was performed for each sample to eliminate the possibility of PCR artefact.

9.6 Sequence analysis

PCR products were directly sequenced (standard Sequenanse kit, Amersham Life Science, Little Chalfont, Buckinghamshire, UK) to confirm the above mutations. Standard reagents other than those specified were purchased from BDH Laboratory Supplies Ltd, Poole, Dorset, UK.

9.7 Statistical analysis

Chi-squared or Fisher's exact test were used for categorical data. Survival data were analysed using the Kaplan-Meier method and statistical significance of the difference in survival between the two groups was calculated using the log rank test. Multivariate analysis was used to test for independent significance. Statistical significance was set at $P<0.05$.

9.8 Results of K-Ras mutation analysis

The clinical data on all patients studied is shown in tables 9.1 and 9.2. Case numbers that were used in immunohistochemistry were retained. From 143 cases DNA was obtained in 93 cases and of these 73 (75%) were found to have mutations

Table 9.1. Clinical data for K-ras analysis in Swedish cases.

Case	TISS	PCR	SSCP	SSCP1	Survival (Days)
1	N-	+	wild type (GGT GGC)	GGTGGC	7
2	¥	¥		¥	135
3	N++	-----	wild type (GGT GGC)	GGTGGC	420
4	N-	+	GGT GGC > GaT GGC	GaTGGC	161
5	N	+	wild type (GGT GGC)	GGTGGC	269
6	N-	+	GGT GGC > GcT GGC	GcTGGC	**0**
7	N-	-----	wild type (GGT GGC)	GGTGGC	104
8	N-	-----	wild type (GGT GGC)	GGTGGC	154
9	N++	-----	GGT GGC > GaT GGC	GaTGGC	327
10	N-	-----	GGT GGC > GtT GGC	GtTGGC	473
11	N-	+	wild type (GGT GGC)	GGTGGC	461
12	N++	+	GGT GGC > aGT GGC	aGTGGC	670
13	N++	+	GGT GGC > GaT GGC	GaTGGC	402
14	N++	(+)	wild type (GGT GGC)	GGTGGC	389
15	N-	OK	wild type (GGT GGC)	GGTGGC	498
16	N	OK	GGT GGC > GGT GaC	GGTGaC	390
17	N-	-----	wild type (GGT GGC)	GGTGGC	53
18	N-	(+)	wild type (GGT GGC)	GGTGGC	131
19	N	OK	GGT GGC > GaT GGC	GaTGGC	120
20	N-	+	wild type (GGT GGC)	GGTGGC	252
21	N-	+	GGT GGC > GtT GGC	GtTGGC	206
22	N++	+	GGT GGC > aGT GGC	aGTGGC	511
23	N	OK	GGT GGC > GaT GGC	GaTGGC	637
24	N+	(-)	GGT GGC > GtT GGC	GtTGGC	210
25	N	-	GGT GGC > GtT GGC	GtTGGC	210
26	N-	(+)	wild type (GGT GGC)	GGTGGC	390
27	N	-----	-	¥	210
28	N-	+	GGT GGC > GtT GCC	GtTGGC	533
29	N-	+	wild type (GGT GGC)	GGTGGC	13
30	N	(+)	wild type (GGT GGC)	GGTGGC	342
31	N+	+	GGT GGC > GaT GGC	GaTGGC	1120

32	N-	+	wild type (GGT GGC)	GGTGGC	716
33	N+	+	wild type (GGT GGC)	GGTGGC	403
34	N	-----	GGT GGC > GaT GGC	GaTGGC	210
35	N-	(+)	GGT GGC > GaT GGC	GaTGGC	137
36	N+	+	GGT GGC > cGT GGC	cGTGGC	60
37	N-	+	GGT GGC > GaT GGC	GaTGGC	29
38	N-	(+)	wild type (GGT GGC)	GGTGGC	204
39	N-	+	wild type (GGT GGC)	GGTGGC	210
40	N	(+)	wild type (GGT GGC)	GGTGGC	112
41	N-	(+)	wild type (GGT GGC)	GGTGGC	30
42	N+	-----	wild type (GGT GGC)	GGTGGC	92
43	N--	+	wild type (GGT GGC)	GGTGGC	347
44	N	+	GGT GGC > aGT GGC	aGTGGC	1308
45	N	+	GGT GGC > GtT GGC	GtTGGC	454
46	N	+	GGT GGC > GtT GGC	GtTGGC	219
47	N+	-----	GGT GGC > GtT GGC	GtTGGC	440
48	N-	+	GGT GGC > cGT GGC	cGTGGC	181
49	N	+	GGT GGC > GaT GGC	GaTGGC	337
50	N	OK	GGT GGC > GtT GGC	GtTGGC	150
51	-	(+)	GGT GGC > GtT GGC	GtTGGC	1769
52	N-	-----	-	¥	234
53	N	+	GGT GGC > GaT GGC	GaTGGC	125
54	N-	-----	GGT GGC > GtT GGC	GtTGGC	798
55	-	-----	-	¥	458
56	-	-----	GGT GGC > GaT GGC	GaTGGC	157
57	N+	(+)	GGT GGC > GaT GGC	GaTGGC	316
58	N+	-----	wild type GGT GGC	GGTGGC	157
59	N	+	wild type GGT GGC	GGTGGC	150
60	N-	+	GGT GGC > GcT GGC	GcTGGC	195
61	N	+	GGT GGC > GaT GGC	GaTGGC	37
62	N-	+	wild type GGT GGC	GGTGGC	1278
63	-	-----	GGT GGC > GtT GGC	GtTGGC	330
64	N-	OK	GGT GGC > GaT GGC	GaTGGC	267
65	N+	-----	GGT GGC > GtT GGC	GtTGGC	90
66	N--	+	wild type GGT GGC	GGTGGC	900
67	N	+	wild type GGT GGC	GGTGGC	240
68	N	-----	wild type GGT GGC	GGTGGC	77
69	N	-----	wild type GGT GGC	GGTGGC	330

¥ absent

Table 9.2. Clinical data for K-ras analysis in United Kingdom cases.

Case	TISS	PCR	SSCP	SSCP1	Survival (days)
70	N	+	wild type GGT GGC	GGTGGC	367
71	¥	¥		¥	63
72	N	-----	-	¥	305
73	N++	-----	-	¥	451
74	N-	-----	-	¥	450
75	N	OK	GGT GGC > GaT GGC	GaTGGC	34
76	N++	+	GGT GGC > GaT GGC	GaTGGC	197
77	N-	-----	GGT GGC > GtT GGC	GtTGGC	393
78	N-	+	GGT GGC > GtT GGC	GtTGGC	293
79	N+	OK	GGT GGC > GaT GGC	GaTGGC	118
80	-	-----	-	¥	313
81	¥	¥		¥	1827
82	N+	(+)	GGT GGC > GAT GGC	GaTGGC	190
83	N++	+	GGT GGC > GaT GGC	GaTGGC	760
84	N++	-----	wild type GGT GGC	GGTGGC	229
85	N	OK	GGT GGC > cGT GGC	cGTGGC	300
86	N	+	GGT GGC > GaT GGC	GaTGGC	1060
87	N+	+	GGT GGC > GaT GGC	GaTGGC	2485
88	¥	¥		¥	1206
89	N-	-----	wild type GGT GGC	GGTGGC	170
90	N	OK	GGT GGC > cGT GGC	cGTGGC	353
91	¥	¥		¥	924
92	N-	-----	wild type GGT GGC	GGTGGC	106
93	N	-----	wild type GGT GGC	GGTGGC	1113
94	N+	-----	wild type GGT GGC	GGTGGC	

95	N-	+	GGT GGC > GaT GGC	GaTGGC	767
96	N-	+	GGT GGC > GaT GGC	GaTGGC	88
97	N+	+	wild type GGT GGC	GGTGGC	509
98	N	(+)	wild type GGT GGC	GGTGGC	103
99	N-	+	GGT GGC > GaT GGC	GaTGGC	193
100	N	+	wild type GGT GGC	GGTGGC	167
101	N-	+	wild type GGT GGC	GGTGGC	23
102	N	+	GGT GGC > cGT GGC	¥	450
103	N-	+	GGT GGC > GtT GGC	GtTGGC	245
104	N++	+	GGT GGC > GaT GGC	GaTGGC	493
105	N	+	GGT GGC > GtT GGC	GtTGGC	475
106	N+	+	GGT GGC > GGT GaC	GGTGaC	568
107	N++	+	wild type GGT GGC	GGTGGC	50
108	N++	+	GGT GGC > cGT GGC	cGTGGC	443
109	N++	+	GGT GGC > GtT GGC	GtTGGC	547
110	N++	+	GGT GGC > GtT GGC	GtTGGC	915
111	¥	¥		¥	398
112	N	+	GGT GGC > GaT GGT	GaTGGC	822
113	¥	¥		¥	9
114	N++	-----	GGT GGC > GtT GGC	GtTGGC	155
115	N-	+	wild type GGT GGC	GGTGGC	323
116	N+	+	wild type GGT GGC	GGTGGC	260
117	N+	+	GGT GGC > GtT GGC	GtTGGC	257
118	N++	+	GGT GGC > GtT GGC	GtTGGC	225
119	N++	+	GGT GGC > GtT GGC	GtTGGC	205
120	N++	+	GGT GGC > GtT GGC	GtTGGC	162
121	N++	+	GGT GGC > GaT GGC	GaTGGC	

122	N	+	wild type GGT GGC	GGTGGC	357
123	N-	+	wild type GGT GGC	GGTGGC	138
124	¥	¥		¥	912
125	¥	¥		¥	489
126	N-	+	wild type GGT GGC	GGTGGC	24
127	N	+	GGT GGC > GtT GGC	GtTGGC	569
128	N+	(+)	wild type GGT GGC	GGTGGC	540
129	N+	+	wild type GGT GGC	GGTGGC	229
130	N--	(+)	wild type GGT GGC	GGTGGC	353
131	N	+	wild type GGT GGC	GGTGGC	393
132	N++	+	GGT GGC > GaT GGC	GaTGGC	259
133	N+	-----	wild type GGT GGC	GGTGGC	171
134	N	+	GGT GGC > GaT GGC	GaTGGC	132
135	N	+	GGT GGC > GaT GGC	GaTGGC	78
136	N--	-	GGT GGC > GtT GGC	GtTGGC	314
137	N+	-----	wild type GGT GGC	GGTGGC	235
138	N	-	GGT GGC > GtT GGC	GtTGGC	598
139	N-	(+)	wild type GGT GGC	GGTGGC	236
140	N	+	GGT GGC > GtT GGC	GtTGGC	16
141	N++	-----	GGT GGC > GtT GGC	GtTGGC	1366
142	N+	+	wild type GGT GGC	GGTGGC	1465
143	N	+	wild type GGT GGC	GGTGGC	114

9.6.1 SSCP and mutation analysis

DNA extraction, PCR and SSC was successful in 97 specimens of pancreatic adenocarcinoma and 73 (75%) were found to have a K-ras mutation (Table 9.4).

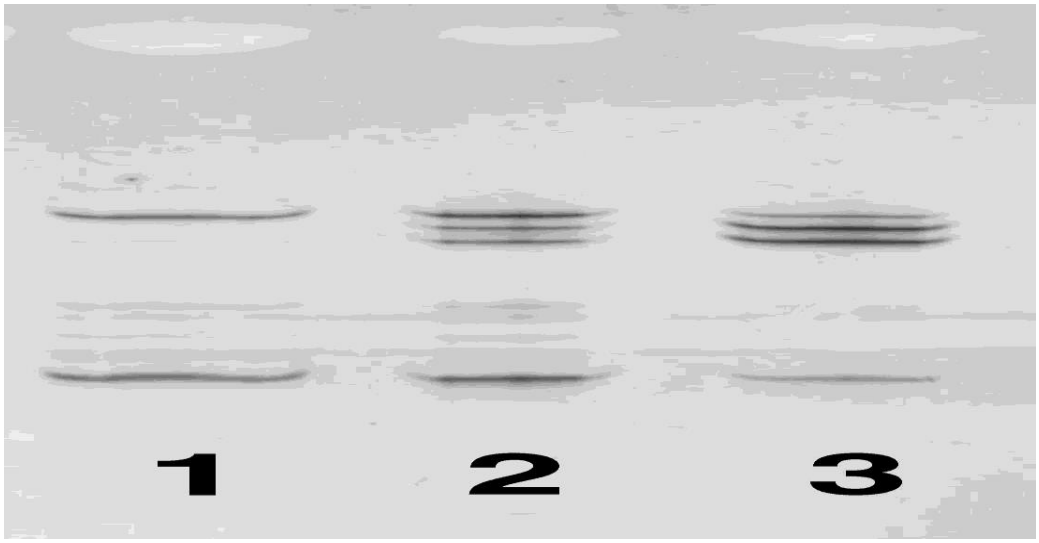


Figure 9.1: **1: Wild type K-ras codon 12 GGT**
2: Samples heterozygous for wild type and GTT
3: Samples heterozygous for wild type and GTT (less wild type tissue than in lane 2)

9.10 K-ras mutation and clinicopathological parameters

There were no significant associations between K-ras mutation status or mutation subtype with gender, stage, grade, tumour size and resection margin status (Table 9.3). There was a significant association between the subtype of mutation and lymph node involvement ($p<0.04$). 75% of GtT tumours were lymph node negative, 100% of aGT and GcT tumours were lymph node positive. Survival analyses revealed no significant correlation between the presence or absence of K-ras mutation and patient survival, but there was improved survival by analysis of mutation subtypes.

Table 9.3. Incidence of K-ras mutations in pancreatic adenocarcinoma

Authors and year	Number of patients	Mutation frequency (%)
Almoguerra et al, 1988	22	95
Smit et al, 1988	30	93
Grunewald et al, 1989	65	75
Mariyama et al, 1989	13	77
Tada et al, 1990	18	100
Shibata et, 1990	27	74
Motojima et al, 1991	18	100
Lemoine et al, 1992	16	75
Hruban et al, 1993	82	83
Pellegata et al, 1994	35	71
Dergham et al 1997	76	84
Kubrusly et al, 1999	11	100
Luttges et al, 1999	35	100

Table 9.4. Correlation between K-ras mutation status and single mutation subtype (GaT GtT cGT aGT Gct or GaC) and clinical parameters and survival

Parameter	wild type vs. mutations		wildtype vs each mutation subtype(Gat GtT cGT Gct or Gac)		mutation subtype only (Gat GtT cGT aGT Gct or GaC)	
	X^2	*p value*	X^2	*P value*	X^2	*P value*
Gender	0.084	0.81	5.07	0.54	5.04	0.41
Grade of tumour	4.87	0.08	8.60	0.74	4.05	0.95
Stage of tumour	1.71	0.43	14.33	0.28	12.69	0.24
Tumour size	0.05	0.98	14.3	0.28	14.46	0.15
Lymph node status	0.29	0.59	12.27	0.06	11.86	0.04∗
Resection margins	0.25	0.62	0.70	0.99	0.53	0.99
Adjuvant therapy	0.7	0.41	1.73	0.94	1.54	0.91
Survival	2.15	0.14	23.4	0.0007∗	17.59	0.0004∗

∗ Statistically significant value.

Table 9.5. Frequency of K-ras codon 12 and 13 mutations detected by SSCP in 97 tumour samples and median survival

K-ras Mutation subtype	Codon	Number	%	Median survival (months)	95% CI
GGT (wild type)	-	24	25	31.15	23.45 – 38.85
GaT (ASP)	12	31	31	29.20	17.93 – 40.47
GtT (VAL)	12	29	29	31.41	20.18 – 42.64
cGT (ARG)	12	6	6	15.81	4.25 – 27.37
aGT (SER)	12	3	3	42.43	27.23 – 57.63
GcT (ALA)	12	2	2	8.75	1.40- 16.10
GaC (ASP)	13	2	2	36.04	29.11 – 42.97

ASP=aspartic acid; VAL= valine; ARG=arginine; SER=serine; ALA=alanine

9.11 Summary

a) DNA extraction, PCR and SSCP was successful in 97 specimens of pancreatic adenocarcinoma

b) 73 cases (75%) were found to have a K-ras mutation

c) The were no significant associations between K-ras mutation status or mutation subtype with gender, stage, grade, tumour size and resection margin status

d) There was no significant association between the subtype of mutation and lymph node involvement ($p<0.04$)

e) 75% of GtT tumours were lymph node negative

f) All (100%) of aGT and GcT tumours were lymph node positive

g) Survival analyses revealed no significant correlation between the presence or absence of mutation subtype

h) There was improved survival by analysis of mutation subtypes ($p =0.0004$)

9.12 Conclusion from this study

Survival analyses revealed no significant correlation between the presence or absence of K-ras mutation and patient survival but there was improved survival by analysis of mutation subtypes.

CHAPTER 10 Discussion

10.1 Introduction

A comprehensive analysis of potentially useful prognostic molecular markers in ductal pancreatic cancer has been carried out in a large series of clinical specimens from a multicentre population to clarify previous conflicting reports. Previous studies on molecular prognostic markers were small, usually involving the study of a single gene and reported conflicting survival outcomes. Our study has addressed these concerns and we have found that these molecular markers occur with high frequency in ductal pancreatic cancer, although no correlation with survival was found except in particular subsets of K-ras mutations. Following an apparently curative resection for ductal pancreatic cancer, most patients develop local recurrence within the resection bed as well as liver metastases (Andren-Sandberg A et al, 1997). Thus present surgical procedures are inadequate for cure except in a few cases and, where adjuvant treatment is used it must be effective not only against systemic spread of the disease but also against local recurrence. Staging of pancreatic cancer has become critical given that there is now a better understanding of its pathogenesis and the obvious improvements in surgical care. Currently there are two main staging systems in use, the UICC (TNM systems) and that of the Japanese Pancreas Society. These two systems are fundamentally different and have wide confidence intervals (Zerbi A et al, 1994) making them unsuitable for clinical decision making. The recent explosion in knowledge of molecular biology has generated an interest in the possible use of altered gene products as possible prognostic markers, particulary in cancer treatment.

10.2 Clinical Characteristics

We were fortunate to have available resected pancreatic cancer specimens from three different centres with very good clinical data.. A significant difference in survival between British and Swedish patients (p=0.01) on univariate analysis was found but this disappeared following multivariate analysis, the reasons for this was unclear. The only significant prognostic factors were TNM stage for disease (p=0.002) and lymph node involvement (p=0.0002). We found lymph node involvement in 70 (45%) of cases and this is in agreement with other reports of up to 70% resected pancreatic cancers (Cameron JL et al, 1991; Bassi C et al, 1995; Yeo CJ et al, 1995; Delcore R et al, 1996). There is however an uncertain relationship between lymph node involvement and survival in pancreatic cancer. Therefore lymph node status does not influence adjuvant treatment as it does in other malignancies. Those who favour extended lymphadenectomy during pancreatic resections argue that potential foci are removed and also that there is interruption of lymphatic tumour spread. Some have even suggested removing the retroperitoneal tissue, including the coeliac plexus (Nagakawa T et al, 1993; Beger HG et al, 2002) but this has not gained wide acceptance (Osaki H et al, 1998). In a prospective multicentre randomised trial, 83 patients were randomised to traditional or extended lymphadenectomy (Pedrazzoli S et al, 1998). The overall survival was the same in the two groups ($p<0.05$). Although there was a correlation between stage and survival, there is still a problem of "stage migration" given the differences in the available staging systems. There also remains the problem of identifying patients whose disease is likely to progress rapidly or those with a better prognosis. In our study there were no significant correlations between survival and gender (p=0.87), differentiation (p=0.07), tumour size (p=0.98), resection margin status (p=0.92) and the use of adjuvant treatment (p=0.67). Only 13 patients (8%) had adjuvant

treatment via the UKPACA and ESPAC trial. Some studies have reported tumour size to be a significant prognostic factor by both univariate (Allison DC et al, 1991; Geer RJ and Brennan MF, 1993) and multivariate analyses (Allison DC et al, 1991; Geer RJ and Brennan MF, 1993: Yeo CJ et al, 1995). Tumour size also correlates with peripancreatic (e.g. retro perineal or portal vein) invasion, which has also been found to be a strong prognostic factor (Kobari M et al, 1996). Our results do not agree with this, and this could possibly be due to the subjective nature of the interpretation of size of a tumour and in 15 of our cases there was no comment by the reporting pathologist on the size. Recently, Neoptolemos et al (2001) have reported on the largest randomised adjuvant study to date where they examined the influence of resection margins on survival in 541 patients. About 20% of patients had positive resection margins (R1). The patients had either chemo radiation or chemotherapy and the median follow up was 10 months. Resection margin status was found to be a significant prognostic factor with a median survival of 10.9 months for R1 as compared to 16.9 months for R0 margins. There was a survival benefit for chemotherapy (19.7 months) but not for chemo radiation (14.0 months) irrespective of resection margin status. Those patients with RO margins who received chemotherapy had a longer survival compared with those who did not.

10.3 Results of immunostaining

Reports of p53 overexpression in pancreatic cancer have been small and with conflicting results. Previous studies of p53 abnormalities in paraffin sections, frozen material and in pancreatic cancer cell lines have revealed similar p53 nuclear expression (van den Berg FM et al, 1993). The immunohistochemical data in this study revealed p53 immunoreactivity in 41% of the cases. These findings are in general agreement with previous studies reporting overexpression

rates of between 30 – 75% (Barton CM et al, 1991b; Zhang et al, 1994; Lundin et al, 1996; Rozenblum et al, 1997). Similar results have been reported in metastatic pancreatic lesions both regional and distant (Ruggeri BA et al, 1997). The presence of p53 overexpression and or/mutations has been shown to correlate with survival in a number of human malignancies (Lane DP et al, 1991; Hollstein M et al, 1994). In agreement with three other studies, p53 expression, in this study did not correlate with survival (Lundin et al, 1996; Dergham et al, 1997a; Ruggeri et al, 1997). In contrast some smaller studies have reported reduced survival (Yokoham et al, 1994; Weyrer et al, 1996; Linder et al, 1997). In addition there was no significant association between p53 and the pathological parameters studied, although a trend toward a higher histologic grade (more poorly differentiated tumour) was observed. Two previous studies have investigated expression of p53 and p21 as predictors of survival associated with resectional adjuvant chemotherapy. One study found that patients with tumours overexpressing p53 and given adjuvant treatment had significantly longer survival (Nio et al, 1998) but in another study improved survival of patients was found only in those cases in which tumours overexpressed p53 and did not express p21 (Nio et al, 1999). Also there was no correlation on multivariate analysis with any of the other prognostic markers studied. Even if an association with survival was found in other studies it is obvious that the correlation is not sufficiently strong enough on which to base decision making. Given the central role of p53 in the cell cycle the lack of prognostic significance is unclear. It has been suggested that p53 mutation is a relatively late event in the progression of pancreatic cancer (Hruban RH et al, 2000) and therefore would not influence survival. Another explanation is that immunohistochemical staining does not always correlate with mutation status. In the p53 negative staining cases it is possible that the p53 had a very large deletion or had a nonsense

mutation. p53 protein stability depends not only on mutation but on binding to mdm-2 (Lane DP and Hall PA, 1997). Mdm2 overexpression may affect p53 function as previously discussed. The stability of the p53 protein is affected by different fixation methods and this particularly applies to weak staining. Using frozen tissue and a panel of anti-p53 antibodies, Barton et al (1991) found an overexpression of mutant p53 protein in 60% of cases of pancreatic cancer but only 20-25% nuclear immunoreactivity in paraffin embedded tissue. This difference in p53 immunoreactivity was probably because the epitope recognised by the antiserum is only partially resistant to formalin fixation Barton CM et al (1991). Negative staining does not necessarily mean that there is no abnormality in the p53 gene and conversely positive staining does not always indicate mutant protein. Fisher et al (1994) showed that some fixatives appear to preserve antigenicity of the p53 protein better than others particularly to tumours showing only weak nuclear staining. They concluded that formal saline is a good preservative for antigenicity. Tissue fixation in this way has a good morphology and fixation is rapid and gives excellent preservation of DNA for use in flow cytometry and in analyses using the polymerase chain reaction technique (Fisher CJ et al, 1994). The fact that p53 is commonly altered in cancer does not necessarily imply that it is a good prognostic marker, on the contrary a genetic change present in a majority of the specimens of a particular tumour type is unlikely to be predictive of diverse outcomes. A second assumption often made about p53 alteration is that it is a binary event, with p53 alteration being either normal or altered in a tumour. It is important when considering p53 in tumours to remember that different mutations are variable in their phenotype. Some mutations are recessive “loss of function” whereas others are dominant “gain-of function” mutations more typical of dominant oncogenes (Steele RJ et al 1998). Further various assays used to study p53 have differing levels of sensitivity for the various forms of p53 alteration. The

recently suggested progression model for pancreatic cancer shows that p53 mutation is a relatively late event in the progression of pancreatic cancer. It is possible that mutations of other oncogenes or tumour suppressor genes are more critical in promoting progression of pancreatic tumours. Inspite of the correlation with survival, p53 occurs at significant frequency in pancreatic cancer and it may still be important to study the different types of p53 mutations and correlate this with survival.

The overall rates of p21 positivity conform with previous reports (Ruggeri B et al, 1997; Coppla D et al, 1998). p21 overexpression was not found to correlate with any of the clinicopathological variables studied and is in keeping with the few reports in the literature (Okada N et al, 1995; Coppola D et al, 1998; Ruggeri BA et al, 1997). Mutation analysis studies of p21 in some malignancies has revealed few somatic mutations (Ruggeri BA et al, 1997) leaving its tumour suppressor role unresolved. Further, $p21^{WAF1}$ null mice do not exhibit a higher frequency of spontaneous tumours as do p53 null mice (Ruggeri et al, 1997). Most recent studies have failed to show a correlation between p21 expression and p53 suggesting that in addition to the p53 dependent pathway for p21 induction other mechanisms exist. Correlation between p21 immunoreactivity and the clinical parameters studied was not statistically significant. Multivariate analysis with other prognostic markers did not show any correlation. In the normal colon positive control p21 immunoreactivity was found in the nuclei of crypts as previously reported (Yasui W et al, 1997). There was no correlation with patient survival, which contradicts previous smaller studies, which have postulated a link between p16 inactivation and survival (Naka et al, 1998). A multivariate analysis of p16 overexpression with other prognostic markers did not show any statistical significance. The overall rates of positivity of c-erbB-2 were similar

to those published, but unlike the smaller studies by Friess et al (1995) and Lei et al (1995) there was no relationship with prognosis

C-erbB-2 overexpression was not associated with survival in this study. This is in contrast with two previous smaller studies (Friess H et al, 1995; Lei SZ et al, 1995). Lei et al (1995) studied 21 cases of pancreatic cancer using the monoclonal antibody (Mab) CB11 and found overexpression in 10 cases (47.6%). Overexpression of c-erbB-2 was closely and inversely related to survival; 19.1 +/- 11.7 months for those that were negative for c-erbB-2 and 7+/- 3.8 months for those that were positive ($p<0.01$). In a recent study by Jacobs et al (2000) looking at interlaboratory agreement in assessing overexpression of c-erbB-2 in breast cancer, they found the agreement in interpreting cases as positive or negative was 97%. This was remarkable given that although the same polyclonal antibody was used they employed different automated immunostainers, detection systems, and methods of scoring were used. The success of clinical trials using Herceptin (trastuzumab, a humanised anti-erbB-2 antibody) as therapy for patients with advanced breast cancer (Pegram MD et al, 1998) has generated further interest in this oncoprotein. The minority of patients who had significant improvement expressed very high levels of c-erbB-2. Currently the Hercep Test kit (Dako, Carpinteria, CA) has become popular. It has the advantage of being easy to use and promotes reproducibility because it contains comprehensive standardised reagents. (Roche PC et al, 1999). An ideal scoring system should report the percentage or proportion of positive or negative cells and their intensity as merely reporting positive or negative can be misleading because there are many assays in use. There are a limited number of studies with the c-erbB-3 oncoprotein but our findings agree with those by Friess et al (1995) and Lei et al (1995). Multivariate analysis with other clinical parameters as well as with other prognostic factors did not show any correlation.

The levels of immunoreactivity of cyclin D1 (72%) was similar to previous reports and overexpression did not correlate with survival in this study in agreement with other studies (Ruggeri D et al, 1997; Coppola D et al, 1998). The high expression rate suggests that cyclin D1 is involved in the pathogenesis of pancreatic cancer. In a study of 75 patients with transitional cell carcinoma of the bladder, Shin et al (1997) found that overexpression was not associated with reduced survival. The loss of p16 expression in 87% cases was consistent with the high frequency of p16 mutation in pancreatic cancer found in other studies (Caldas C et al, 1994; Naumann M et al, 1996). No correlation was found with survival and this is in conflict with recent small studies (Naka T et al, 1998). In a study of 32 patients with pancreatic cancer, nineteen cases (59%) were positive for p16 and were associated with poor survival (Naka T et al, 1998).

Multivariate analysis was carried out between all the molecular markers. This was done because it is becoming clear that pancreatic cancer formation is associated with multiple cancer related genes, including K-ras, p53, p16 and DPC4 genes that occur at high frequencies (Rozenblum E et al, 1997). The change from a benign to a malignant condition is not regulated by a single gene but by multiple mutations in a single cell. There was no correlation with survival however between the different combination markers. This was not surprising given that those individual markers did not independently show any correlation

10.4 Immunohistochemistry

Immunohistochemistry is a useful laboratory method; however it has limitations with respect to quantitation and reproducibility. Problems include threshold for detection of antigen (varying factors such as antibody concentration will alter staining), interpretation of staining (what is

positive and negative) and specificity of antibodies. Most laboratories routinely fix material in some variant of formaldehyde which gives good morphology. However this introduces problems of sensitivity in the detection of a significant number of antigens due to a phenomenon known as masking of epitopes in fixed tissues. Immunoreactivity for many antigens is lost or reduced and this appears to be proportionate to the length of fixation. The exact mechanism by which formaldehyde acts in fixing tissues is not fully understood, but it involves cross-linking of reactive sites within the same protein and different proteins via methylene bridges (Fox CH et al, 1985; Mason JT et al, 1991). To optimise staining results several techniques exist for unmasking or retrieving antigens based firstly on treating sections with proteolytic enzymes and more recently on heating sections at high temperature in water or a variety of solutions prior to immunostaining (Taylor CR et al, 1996). Recent evidence suggests that release of calcium ions from tissues may play a role in unmasking epitopes and for this reason chelating agents e.g. EDTA were used in the antigen retrieval step in my experiments. For example, there is an increase in positive staining for the p53 protein in some tumours with antigen retrieval and different results with different antibodies (Lambkin HA et al, 1994).

10.5 p53 mutation analysis

This part of the study was done to confirm the results of immunohistochemistry. The limitations of immunohistochemistry have been discussed previously in this thesis. We sort to study mutations in all exons (1 –11) of the p53 gene. By limiting analysis to the DNA binding domain of the p53 protein most studies that only look at the so called “hot spot’ regions, up to 25% of mutations can be overlooked (Hartmann A et al, 1995). Successful microdissection of pancreatic

tumour samples and subsequent multiplex PCR of the DNA obtained, followed by sequencing was undertaken in 12 cases. Microdissection was performed by using a polished tungsten needle with no contamination. This shows that it is possible to carry out complex genetic analysis with inexpensive equipment. Multiplex PCR is an economical method of utilising scarce DNA specimens (e.g. pancreatic cancer). We were able to analyse the full coding sequence, splice sites, and polymorphisms from one 2µL aliquot of 5 micron tissue section clinical sample. All the exons in all 12 cases chosen for study were successfully sequenced. p53 mutations were found in 2 (33%) of the 6 cases that showed p53 positive nuclear staining. There were no mutations in the other six cases that were p53 negative. Previous work has reported mutation rates of up to 75% in pancreatic cancer (Rozenblum et al, 1997). The mutational yield was lower than expected and the most likely explanation for this was the small numbers studied. In a number of cases where poor sequence was obtained we cannot be certain that mutations were missed. It is possible that fixation of tumours from old specimens could contribute. For example, microdissection could only be done if tumour cells actually seen clearly on slides. The other problem was possibly the difficulties we encountered with alcohol and gel electrophoresis. This made it difficult to confirm presence of tumour products.

For each sequence variation detected, validation was made by further sequencing of additional independent PCR products. This allowed us to determine whether the changes seen were PCR-induced errors or were genuine sequence variations. The G-T change within intron 7 seen in cases 10 and 17 was not identified in a search of the GenBank database. Analysis of these products with an alternative sequencing chemistry may help to validate these findings, although the presence of a T at this nucleotide in those samples would imply that the p53 sequence U94788 either contained a sequencing artifact at that position or that the source represented a

polymorphic variant. The PCR method described was designed to amplify low-copy number, fragmented DNA from microdissected archival tumour samples. This mutational analysis study was carried out at a time when the multiplex PCR system was being optimised in the laboratory where I carried out the experiments. It was unknown at the time whether a multiplex system would work for microdissected pancreatic tissue although this had been shown in ovarian and brain tissue in the same laboratory (Clatterbridge, Wirral, Liverpool, UK). It was found during the course of the experiments that minute amounts of alcohol carried over from the DNA purification stage interfered with sequencing reactions. This led to experiments being repeated under different conditions several times until the cause of the poor sequencing was found. This of course cannot be an explanation for the lower mutation frequency but is worth noting given that alcohol is routinely used in these experiments. A further drying out process was subsequently included in the purification stage and this resulted in good sequencing yield. In spite of the fact that the risk of PCR induced errors is increased when amplifying from the small amounts of amplifiable DNA available when working with microdissected tissue, we were able to satisfactorily eradicate false mutations by repeat PCR from the same sample. For the amplicons shown, each aliquot from the pancreatic cancer samples contained amplifiable material but amplification from duplicate samples did not always produce detectable PCR product. The fact that not all genomic DNA products amplified with equal intensity in the second-round amplification may reflect this.

10.6 K-ras mutation analysis

Our results of the frequency and types of K-ras mutations were very similar to previous large series (Hruban et al, 1993; Dergham et al, 1997b). There was no correlation between survival

and the presence or absence of K-ras mutations in agreement with previous reports (Hruban et al, 1993; Dergham et al, 1997b). Hruban et al (1993) studied 82 resected formalin-fixed paraffin-embedded pancreatic adenocarcinomas for the presence of point mutations at codon 12. Mutations were found in sixty-eight (83%) of cases and of these 33(49%) were guanine to cytosine transversions, 27 (39%) were guanine to thymine transversions and eight (12%) were guanine to cytosine transversions. The prevalence of mutations was higher in ex-smokers (89%) and current smokers (86%) when compared to those who had never smoked (68% p=0.046). There was no correlation however with survival. In a more recent study, Dergham et al (1997) extracted DNA from 81 patients with pancreatic cancer and studied K-ras muations in them by SSCP. The incidence of K-ras mutation was 83% but there was no correlation with survival (Dergham ST et al, 1997). The association of K-ras mutations and survival with adjuvant chemo radiation was demonstrated in one recent study. Patients with pancreatic cancer and K-ras positive nodal micro metastases (pathologically classified as stage 1 disease) were shown to demonstrate better survival with adjuvant therapy compared to patients with nodal micro metastases who underwent surgery alone (Demeure et al, 1998). Analysis of the K-ras mutation subtype revealed significant associations with patient survival. All of the mutations identified have been shown to contribute to malignant transformation but whether there are differences in K-ras downstream signalling by certain mutations is unclear. Previous studies in colorectal cancer have demonstrated that patients with G to A transitions and G to C transversions tend to have a worse prognosis than those with G (T transversions (Span et al, 1996). In addition codon 13 mutated tumours were less likely to progress to local or distant metastases (Finkelstein et al, 1993). Our results agree with this, as patients with K-ras mutations GaT, cGT and GcT demonstrated shorter median survival times when compared with other mutations. There have

been reports of differences in the pattern of K-ras mutations between Europeans (Scarpa A et al, 1994). Differences in the site of the mutation and in the ratio of transitions to transversions were observed between an Italian and Spanish population. Dong et al (1998) has reported differences in the substitution of K-ras mutations between the western population compared to the Chinese and Japanese. The implied ethnic or environmental differences may be important because the pathogenesis of the tumour may implicate geographical genotoxic substances. If a causal relationship were to be found , this could go a long way in the understanding of this aggressive tumour.

10.7 Conclusions from present study

This study represents the largest bank of pancreatic ductal adenocarcinoma that has been examined for molecular prognostic markers at the same time and with identical methods. The molecular markers (p53, $p16^{INK4}$, $p21^{WAF1}$, cyclin D1, c-erbB-2 and c-erbB-3) studied by immunohistochemistry occur in significant proportions in pancreatic cancer but do not correlate with survival. Mutations were confirmed in a small number of p53 positively staining cases using an economical method of DNA amplification. The presence of K-ras mutation alone did not correlate with survival but there were significant differences in survival according to the type of K-ras mutation ($p=0.0004$).

10.8 Future studies

Further studies of specific p53 and K-ras mutations as prognostic and treatment response markers are needed. It is possible that different types of these mutations may have important epidemiological, prognostic or therapeutic implications in pancreatic cancer. Recently, p53 mutations have been reported to be helpful in predicting response of adjuvant treatment in some tumours (Berns EM et al, 2000). With regard to K-ras mutations, future studies looking at adjuvant treatment data for mutation subtype will be important. This would possibly help to explain whether mutation subtype could predict response to adjuvant treatment or whether patients have a poor outcome because they did not respond to chemotherapy. This study could not address this problem because of the limited data on adjuvant treatment. Most recently, there has been more work on complete sequencing of the pancreatic cancer genome (Niki, A et al, 2011). This breakthrough will no doubtful provide an opportunity to exploit this knowledge for developing treatment methods such as gene therapy.

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APPENDICES

Appendix 1

Suppliers of chemicals and reagents (Immunohisochemistry - IHC)

Chemicals and Reagents	Supplier
Avidin biotin complex	DAKO Ltd, High Wycombe, Bucks
Biotinylated sheep secondary antibody	Amersham LIFE SCIENCE, Buck
Citric acid	Sigma Chemical Co, Poole, Dorset
DAB (3-diaminobenzidinetetra-Hydrochloride)	Sigma Chemical Co, Poole, Dorset
DPX mountant	BDH/Merck chemicals Ltd, Leics.
EDTA	Sigma Chemical Co, Poole, Dorset
Eosin	Sigma Chemical Co, Poole, Dorset
Ethanol	University of Liverpool stores
Foetal calf serum	ICN Biomedicals Ltd, Irvine, Scotland
Harris′ Haematoxylin	BDH/Merck chemicals Ltd, Leics
Horseradish peroxidase labelled anti-mouse secondary IgG (Goat)	Sigma Chemical Co, Poole
Hydrochloric acid	BDH/Merck chemicals Ltd Lutterworth, Leics.
Hydrogen peroxide solution (30%)	Sigma Chemical Co, Poole, Dorset
Poly-L-lysine	Sigma Chemical Co, Poole, Dorset
R.O (Reverse osmosis) water 15 mega ohms	University of Liverpool stores
Sodium chloride	BDH/Merck chemicals Ltd Lutterworth, Leics.
Sodium hydroxide pellets	BDH/Merck chemicals Ltd Lutterworth, Leics.
Tris (Tris (hydroxymethyl) methalamine/	

HCl)	BDH/Merck chemicals Ltd, Lutterworth, Leics.
Xylene	University of Liverpool stores
Methanol	Diagnostic Developments, Lancs., UK

Appendix 2 (IHC)

Suppliers of equipment

Equipment	**Supplier**
Centrifuge	Hereaus Equipment Ltd, Brentwood, Essex
Eppendorf tubes (0.5&1.0)	Sarstedt Ltd, Beaument Leys, Leics
Filter paper	BioRad Laboratories
Gilson pipette tips	Sarstedt Ltd, Beaument Leys, Leics
Microscope slides	Merck Ltd, Lutterworth, Leics.
Microwave oven	Creda Ltd, Blythe Bridge, Stoke-on- Trent
Shandon Sequenza	Life Sciences International (UK) Ltd, Hants
Universal tubes	Sterilin Ltd, Hounslow, Middlesex
Silicon tips	Sigma

Appendix 3 (IHC)

Preparation of solutions

Solution	**Method**
Acid Alcohol	Make up a 70% methanol solution Using distilled water. Add 1% Hydrochloric acid to this solution
and	
	store at room temperature
Citrate buffer (0.01)	Dissolve 10.5g citric acid powder in 5 L distilled water. Adjust pH to 6.0 with HCL
DAB (0.05%) (3- diaminobenzidinetetra-Hydrochloride)	Dissolve 10 g DAB in 100mL TBS. Measure out 2 ml aliquots and store at –20°C. When required, take 2ml aliquot and dissolve in 400ml TBS
Ethanol solutions (300ml)	100% = 300ml ethanol 90%=270 ml ethanol + 30ml water 70%= 210ml ethanol + 90ml water
Hydrogen peroxide solution (3%)	30ml of 30% hydrogen peroxide were added to 300ml of distilled water.
Scotts' Tap Water	Dissolve 20.0 g magnesium sulphate, and 3.5 g sodium hydrogen carbonate in 1 litre of tap water. Add one crystal of thymol (preservative) and store at 4ºC
Bovine serum albumin	1g BSA in 20mLs TBS
Tris- Hcl 50mM, pH 7.6 (TBS)	Dissolve 40.85 g sodium chloride and 30.3 g Tris in 1 Litre of distilled water. Make up to 5L with distilled water. Adjust pH to 7.6 with HCL.

Appendix 4 Materials (Mutation analysis)

PCR reagents	**Supplier**
10x PCR buffer II	Perkin Elmer
100 mM deoxynucleotides (dNTP's)	Pharmacia
25mM Magnesium Chloride	Perkin Elmer
Sterile filtered distilled water	Sigma
Amplitaq Gold (5µ/ µL)	Perkin Elmer
Taq polymerase	Supertaq, HT
Primers	Oswel
Agarose gel Electrophoresis	
Nusieve 3:1 Agarose	Flowgen
10x TAE buffer:	
1210g Tris base	Sigma
500ml of 0.5M EDTA (ph 8.0)	Clatterbridge (UK)
285.5ml glacial acetic acid	Fisons
214.5 ml distilled water	**Sigma**
Gel loading buffer (w/v) bromophenol blue, 30% (v/v) glycerol	
Vistra green	Amersham
Ethidium Bromide (0.5µg/ml)	Sigma
Marker VI and IX (0.5 – 1.0 µ**g)**	Boehringer
Purification	
QIAquick PCR purification kit	Qiagen
QIAquick 96 purification kit	Qiagen

Appendix 5 Classification of pancreatic intraepithelial neoplasia

The normal duct and ductular epithelium is a cuboidal to low-columnar epithelium with amphophilic cytoplasm. Mucinous cytoplasm, nuclear crowding, and atypia are not seen.

Squamous (transitional) metaplasia
A process in which the normal cuboidal ductal epithelium is replaced by mature stratified squamous or pseudostratified transitional epithelium without atypia.

PanIN-1A (pancreatic intraepithelial neoplasia 1-A)
These are flat epithelial lesions composed of tall columnar cells with basally located nuclei and abundant supranuclear mucin. The nuclei are small and round to oval in shape. When oval, the nuclei are oriented perpendicular to the basement membrane. It is recognised that there may be considerable histologic overlap between non-neoplastic flat hyperplastic lesions and flat neoplastic lesions without atypia. Therefore, some may choose to designate these entities with the modifier term "lesion" (PanIN/L-1A) to acknowledge that the neoplastic nature of many cases of PanIN-1A has not been unambiguously established.

PanIN-1-B (pancreatic intraepithelial neoplasia 1-B)
These epithelial lesions have a papillary, micropapillary, or basally pseudostratified architecture but are otherwise identical to PanIN-1A.

PanIN-2 (pancreatic intraepithelial neoplasia 2)
Architecturally these mucinous epithelial lesions may be flat but are mostly papillary. Cytologically, by definition, these lesions must have some nuclear abnormalities. These abnormalities may include some loss of polarity, nuclear crowding, enlarged nuclei, pseudo-stratification and hyperchromatism. These nuclear abnormalities fall short of those seen in PanIN-3. Mitoses are rare, but when present are nonluminal (not apical) and are not atypical. True cribriform structures with luminal necrosis and marked cytologic abnormalities are generally not seen and, when present, should suggest the diagnosis of PanIN-3.

PanIN-3 (pancreatic intraepithelial neoplasia 3)
Architecturally, these lesions are usually papillary or micropapillary; however, they may rarely be flat. True cribriforming, the appearance of "budding off" of small clusters of epithelial cells into the lumen, and luminal necrosis should all suggest the diagnosis of PanIN-3. Cytologically, these lesions are characterised by a loss of nuclear polarity, dystrophic goblet cells (goblet cells with nuclei oriented toward the lumen and mucinous cytoplasm oriented toward the basement membrane), mitoses that may occasionally be abnormal, nuclear irregularities, and prominent (macro) nucleoli. The lesions resemble carcinoma at the cytonuclear level, but invasion through the basement membrane is absent.

Adapted from R.H Hruban et al (2001) Am J Surg Pathol. 25(5), 2001.

Appendix 6 PTNM pathological classification

The pT, pN and pM categories correspond to the T, N and M categories. pN0 Histological examination of the regional lymphadenectomy specimen will ordinarily include 10 or more lymph nodes

1 = peripancreatic tissues include the surrounding retroperitoneal fat (retro peritoneal soft tissue or retro peritoneal space), including mesentery (mesenteric fat), mesoclon, greater and lesser omentum and peritoneum. Direct invasion to bile ducts and duodenum includes involvement of ampulla of Vater.

2 = adjacent large vessels are the portal vein, celiac artery, and superior mesenteric and common hepatic arteries and veins (not splenic vessels)

The classification system applies only to carcinomas of the exocrine pancreas. There should be histological or cytological confirmation of the disease. The following are the procedures for assessing T, N, and M categories:

T categories	Physical examination, imaging, and/or surgical exploration
N categories	Physical examination, imaging, and/or surgical exploration
M categories	Physical examination, imaging, and/or surgical exploration