Clinical Review and Experimental Evaluation of Tumour M2-Pyruvate Kinase in Pancreatic Cancer

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DECLARATION CONCERNING THESIS PRESENTED FOR THE DEGREE OF DOCTORATE OF MEDICINE

I, **YOGESH KUMAR**, student of University Department of Surgery, Royal Free & University College Medical School, Hampstead Campus, Rowland Hill Street, London NW3 2PF, solemnly and sincerely declare, in relation to the thesis entitled:

Clinical review and experimental evaluation of Tumour M2-pyruvate kinase in pancreatic cancer

That work was done by me personally and the material has not previously been accepted in whole, or in part, for any other degree or diploma.

Signature: **Date:**

Dedication

I dedicate this thesis to my parents whose sacrifices have brought me to this stage. I also dedicate this thesis to my wonderful wife and children who supported my ups and downs during research and provided me their full moral support.

Abstract

The treatment of pancreatic cancer is challenging. Patients are often beyond curative surgical therapy and palliative treatment with chemotherapy provides limited benefit. New markers of cancer activity and therapeutic targets are required.

This thesis has firstly reviewed the available literature on Tumour M2-PK, a dimeric form of M2 isoenzyme of pyruvate kinase, in GI cancer and carried out a meta-analysis of the clinical data on pancreatic cancer. Experimental work evaluated the measurement of M2-pyruvate kinase in human pancreatic cancer cell lines with altered microenvironment (hypoxia, acidic pH or glucose-deprived condition). Tumour M2-PK level was measured using ELISA, total M2-PK by immunoblotting and pyruvate kinase activity by spectrophotometric analysis. Apoptosis or necrosis was detected by measuring active Caspase 3/7 and 8, Bcl-2, Bax and Annexin V staining. Localisation of M2-PK in pancreatic cancer cell was studied by immunocytochemistry.

The clinical review has shown that Tumour M2-PK is not an organ-specific marker of GI cancer but is elevated with positive predictive value of 86–88% in gastro-oesophageal and colorectal cancers. In pancreatic cancer the diagnostic odds ratio (DOR) of an elevated Tumour M2-PK was similar to those of CA19-9 with overall sensitivity of 94% and specificity of 55%. Higher levels of Tumour derived M2-pyruvate kinase were observed in Colo 357 cell lines compared to Panc-1 cells. Exposure of Colo 357 cells to altered culture conditions resulted in decreased cell proliferation accompanied by elevated Tumour M2-PK levels with unchanged total M2-PK levels suggesting tetramer-

dimer switch-over, which was confirmed by the corresponding change in the pyruvate kinase activity. No correlation of Tumour M2-PK level or PK activity with apoptotic or anti-apoptotic markers was observed. Immunocytochemistry suggested M2-PK localisation to intracellular membrane-bound structures with no translocation to nucleus or mitochondria under altered tumour microenvironment.

Conclusion: Tumour M2-PK is a potential marker of pancreatic cancer. Altering the tumour microenvironment causes a switch to measured M2-PK levels. This may allow cells to overcome cell apoptosis and could be a pathway facilitating tumour survival.

Acknowledgment

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Thesis Description

Chapter 1 is an overview on glycolysis, tumour biology and tumour metabolism with reference to Tumour M2-PK. There is also a brief synopsis of the importance of tumour microenvironment in pancreatic cancer in this chapter.

Chapter 2 Tumour M2-PK is discussed as a cancer marker in various GI cancers. A clinical review of all the available studies on Tumour M2-PK in GI cancer is carried out.

Chapter 3 The studies on Tumour M2-PK in pancreatic cancer are reviewed and meta-analysed. A diagnostic odds ratio (DOR) was calculated for each study from the available data and pooled together to give a summary estimate DOR for Tumour M2-PK and CA19-9. An overall sensitivity and specificity is calculated comparing Tumour M2-PK with CA19-9 in pancreatic cancer.

Chapter 4 The Materials and Methods.

Chapter 5 The measurement of Tumour M2-pyruvate kinase levels in human pancreatic cancer cell lines.

Chapter 6 The influence of hypoxia (1% O_2) or acidic pH (6.5) or glucose-free culture condition on Tumour M2-PK level and total M2-PK expression in pancreatic cancer cell lines.

Chapter 7 The relationship between Tumour M2-PK and apoptosis/necrosis in pancreatic cancer.

Chapter 8 The localisation of M2-PK in tumour cells in normal culture conditions versus the altered tumour microenvironment.

Chapter 9 General discussion of the thesis including methodological considerations, results and conclusions drawn from the experiments has been carried out and future directions have been suggested.

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1 Chapter 1: Glycolysis, Tumour metabolism and Tumour Microenvironment

1.1 Glycolysis and Tumour Biology

Glycolysis—literally lyses of glucose—first requires the conversion of glucose to pyruvate and then to lactic acid (Figure 1.1). In most mammalian cells, glycolysis leads to production of pyruvate which gets oxidised to CO_2 and H_2O in mitochondria in the presence of oxygen. Under anaerobic condition mitochondrial respiration is inhibited and pyruvate is converted to lactic acid. This inhibition is called 'Pasteur effect', after Louis Pasteur (1). In cancer cells, conversion of glucose to lactic acid occurs even in the presence of oxygen and is known as aerobic glycolysis or the 'Warburg effect'. This phenomenon was first reported by Warburg in the 1920s (2), leading him to the hypothesis that cancer results from impaired mitochondrial metabolism. Although the 'Warburg hypothesis' has been proven incorrect, the experimental observations of increased glycolysis in tumours even in the presence of oxygen have been repeatedly verified (3). Interest in metabolic property of cancers has varied over time and has been rekindled recently in the form of tumour metabolome.

The term 'tumour metabolome' (in analogy to tumour genome and tumour proteome) was coined by Mazurek and Eigenbrodt in 2001 for the metabolic characteristics of tumour cells (4-6).

Most early structural and functional investigations of glycolytic enzymes have focused on their glycolytic functions. Recent studies have provided evidence that some glycolytic

enzymes have complicated multifaceted roles in mammalian cell biology (7). These roles include:

Transcriptional regulation

The unexpected nuclear localisation of several glycolytic enzymes, including Hexokinase, LDH, GAPD and ENO1, has been reported in yeast and mammalian cells (7) providing convincing evidence that nuclear forms of these glycolytic enzymes participate in transcription and /or DNA replication (7).

Apoptosis

Early studies indicate that in mammalian cells hexokinase and GAPD localise to mitochondria to participate in anti-apoptotic and pro-apoptotic process respectively (8-10). While the mitochondrial hexokinase blocks the voltage-dependent anion channel (VDAC) to block apoptosis (9), the mechanism of pro-apoptotic function of GAPD remains unknown (7).

Proliferation and Metastases

Although no specific enzyme has been identified, it is the aerobic glycolysis that allows the tumour cells to proliferate and metastasise (11). Lactic acid as a by-product of upregulated glycolysis lowers the extracellular pH resulting in apoptosis or necrosis of normal cells (12) while the tumour cells remain resistant by upregulation of certain membrane ionic transporters (13). Acidosis also contributes to breakdown of extracellular matrix by involving the metalloproteinases and/or cathepsins, which promote the

degradation of the ECM and basement membranes, thus facilitating tumour cell mobility and metastases (14;15).
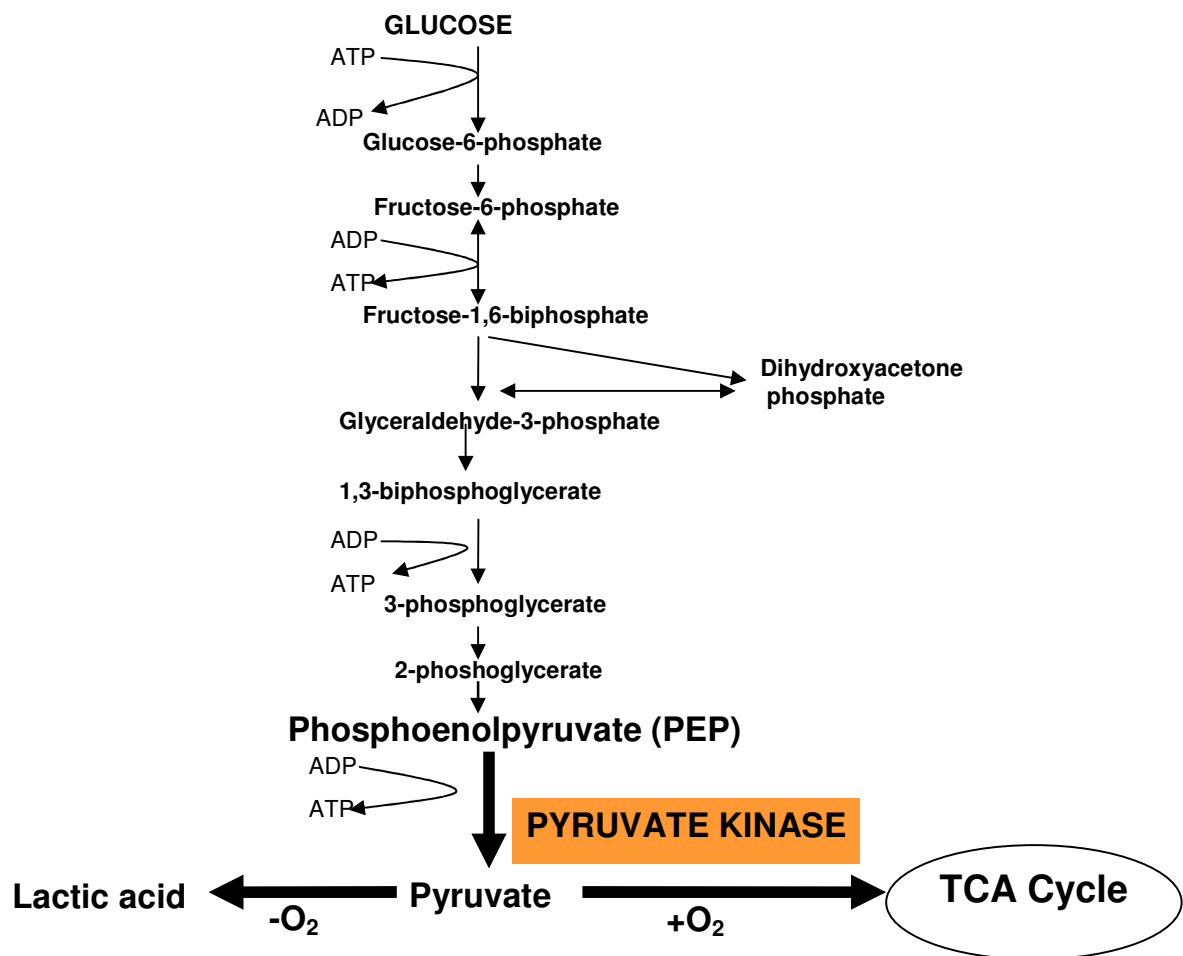
Figure 1.1: Glycolytic pathway in mammalian cells (TCA- Tricarboxylic acid or citric acid or Krebs cycle)

1.2   Pyruvate kinase (PK) and tumour metabolism

Pyruvate kinase mediates the transfer of high-energy phosphate of phosphoenolpyruvate to generate ATP and pyruvate in differentiated cells. Pyruvate kinase (PK) has different isoenzymes; L-PK is present in tissues such as the liver and kidney, R-PK is present in erythrocytes and M1-PK is found in tissues requiring large amounts of energy such as the brain and muscle (16-20) also see Figure 1.2). M2-PK is present in all proliferating cells such as embryonic and adult stem cells but especially in tumour cells. M2-PK can occur in a highly active tetrameric form with high affinity for its substrate phosphoenolpyruvate (PEP) and in an inactive dimeric form with a low affinity to PEP (4-6;16;19-23). The tetrameric form is associated with other glycolytic enzymes within the so-called glycolytic enzyme complex which leads to a very effective conversion of glucose to lactate (6;17-20). In tumour cells the dimeric form is always predominant and has therefore been labelled as Tumour M2-PK (6;19;21;22) .

The dimeric form switches to the tetrameric form with high levels of fructose 1,6 bi-phosphates in tumour cells (6). During tumourogenesis tissues with totally different basic metabolism, e.g. liver and brain, shift to the same metabolic phenotype (6). The common result is increased glycolysis, reduction of PK activity, glutaminolysis, expansion of phosphometabolites and a shift of metabolism to the synthesis of nucleic acids, amino acids and phospholipids (4-6;16;19;20;22-26). Energy production is facilitated by an alternative pathway called glutaminolysis (degradation of the amino acid glutamine to
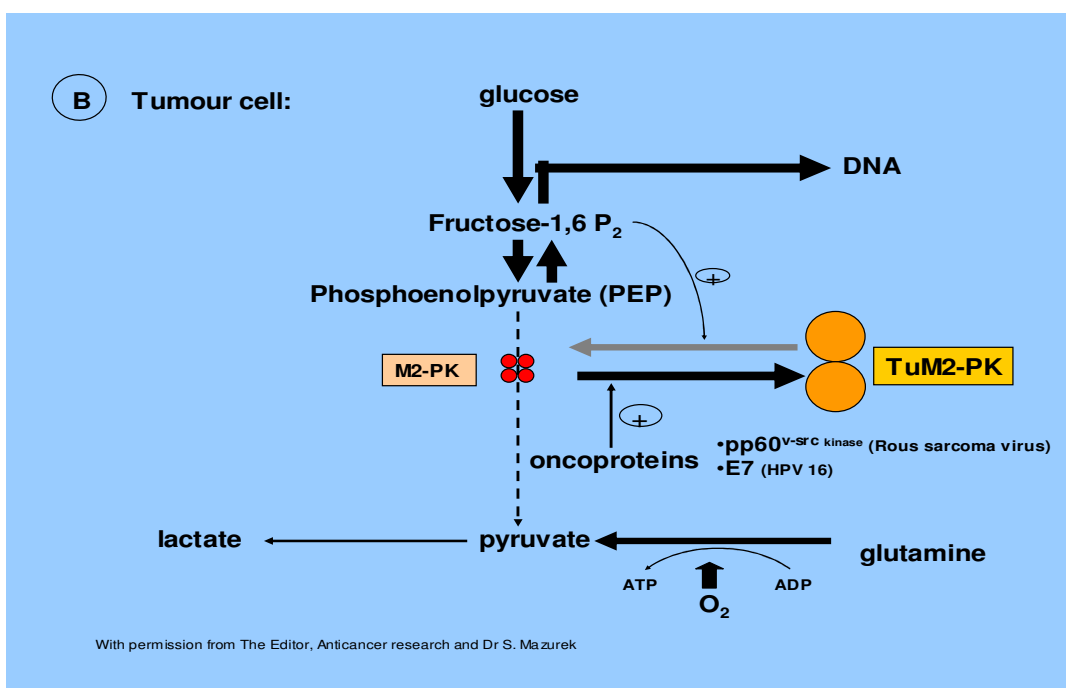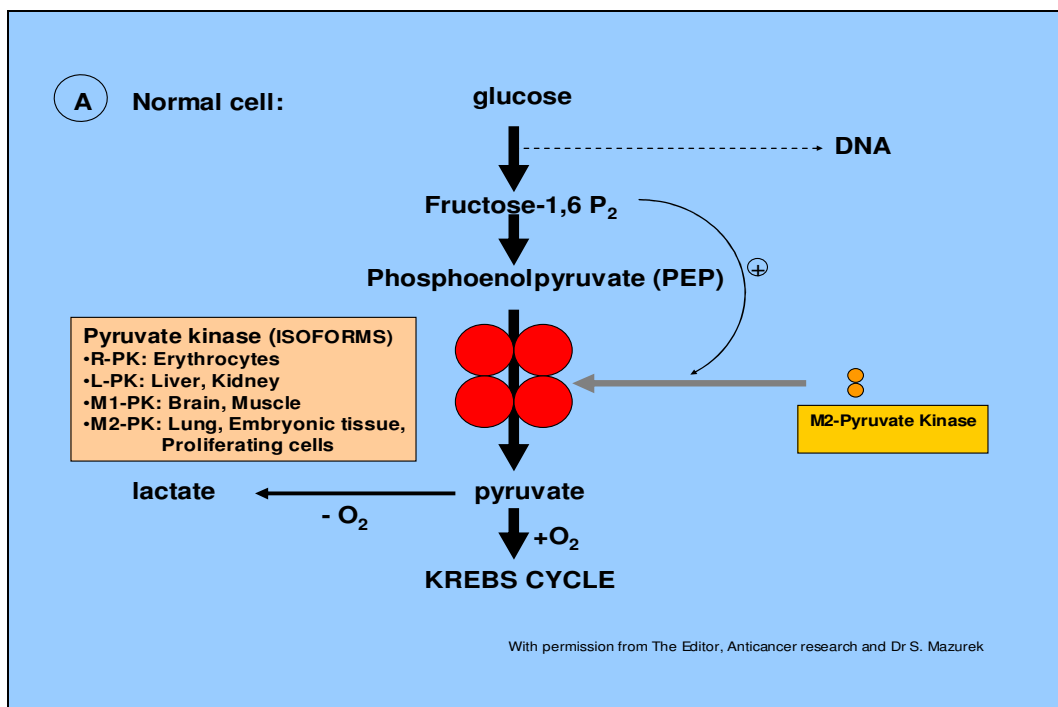
Fig: 1.2: Isoforms of pyruvate kinase in normal and neoplastic cell

lactate) – (27) which depends on an adequate oxygen supply and high NAD(P) levels (6;16;20;28).

In the absence of oxygen, M2-PK is reactivated to the tetrameric form by high fructose 1, 6 biphosphate levels and glutaminolysis is inhibited, thereby switching glucose metabolism to energy production. Thus M2-PK may act as a sensor of tumour metabolome allowing the tumour cells to adapt to varying oxygen and nutrient supply. Although tumour cells are able to compensate for nutrient starvation for a while, if NAD(P) levels are low then both glycolysis and glutaminolysis are inhibited and tumour apoptosis occurs (6). A similar mechanism for tumour cell apoptosis is induced by chemotherapeutic drugs in which decreased NAD(P) level results in the inability of tumour cells to recycle NAD.

M2-PK is a target of different oncoproteins with totally different physiological mechanisms such as the pp60v-src kinase (29) and HPV-16 E7 (26). The pp60v-src kinase phosphorylates M2-PK in tyrosine. The E7 oncoprotein of the human papilloma virus type 16 directly binds to M2-PK. Thus the tetrameric form of M2-PK is dissociated to dimeric form during transformation of normal cells to oncoprotein-expressing cells (4;6;20;24;26;29).

1.3   Pancreatic cancer and tumour microenvironment

Clinical investigations carried out over the last 20 years have clearly shown that the prevalence of hypoxic tissue areas (i.e. areas with O_2 tensions [pO_2 values] ≤ 2.5 mm Hg) is a characteristic pathophysiological property of locally advanced solid tumours such as

pancreatic cancer (30). Up to 50 – 60% of locally advanced solid tumours may exhibit hypoxic and/or anoxic tissue areas that are heterogeneously distributed within the tumour mass (30).

1.3.1 Hypoxia

Cells exposed to hypoxic conditions respond by reducing their overall protein synthesis, which leads to restrained proliferation and eventually to cell death. There is abundant evidence suggesting that hypoxia can slow down or even completely inhibit tumour cell proliferation *in vitro* (31). Furthermore, sustained hypoxia can change the cell cycle distribution and the relative number of quiescent cells, which in turn can lead to alterations in the response to radiation and many chemotherapeutic agents. The degree of inhibition depends on the severity and duration of hypoxia, as well as on the coexistence of other microenvironmental inadequacies (e.g. acidosis, glucose depletion). The response of cells exposed to hypoxia in terms of cell cycle is in most cases a G1/S-phase arrest. Hypoxia levels necessary to induce a disproportionate lengthening of G1 or an accumulation of cells in this cycle phase are in the range of 0.2 – 1 mm Hg. Above this "hypoxic threshold" the environmental O_2 status appears to have only negligible effects on proliferation rate. Under anoxia, most cells undergo immediate arrest in whichever phase of the cell cycle they are (32). In addition to hypoxia-mediated changes in tumour cell proliferation, hypoxia can induce programmed cell death (apoptosis) both in normal and in neoplastic cells. P53 accumulates in cells under hypoxic conditions (through a hypoxia-inducible factor 1α [HIF-1α]-dependent mechanism) and induces apoptosis.

However, hypoxia also initiates p53-independent apoptosis pathways including those involving genes of the BCL-2 family and others (32). Below a critical energy state, hypoxia/anoxia may result in necrotic cell death, a phenomenon seen in many human tumours and experimental tumour models. Hypoxia-induced proteome changes leading to cell cycle arrest, differentiation, apoptosis, and necrosis may explain delayed recurrences, dormant micrometastases, and growth retardation in large tumour masses (32). In contrast, hypoxia-induced proteome and/or genome changes in the tumour and/or stromal cells may promote tumour progression via mechanisms enabling cells to overcome nutritive deprivation to escape from the "hostile" environment and to favour unrestricted growth. Sustained hypoxia and microenvironmental impoverishment in a growing tumour may also lead to cellular changes that can result in a more clinically aggressive phenotype (33;34). During the process of hypoxia-driven malignant progression, tumours may develop an increased potential for local invasive growth (35) perifocal tumour cell spreading (33;35;36) and regional and distant tumour cell metastasis (35;37;38).

1.3.2   Acidic environment

Acidification of tumour cells is a consequence of upregulated aerobic glycolysis with increased lactate, H^+ and CO_2 production. The intracellular pH value in tumour cells is usually in the range of 7.0 – 7.2 (39). The intracellular H^+ ions and lactic acid are pushed out of the cell by membrane-bound Na^+/H^+ exchangers and H^+/lactic acid co-transporters while the CO_2 diffuses rapidly across the plasma membrane and gets converted to

carbonic acid by membrane-bound ectoenzyme carbonic anhydrases (40). Uptake of the weak base HCO_3 – via a member of the Na+-dependent and Na+-independent Cl–/HCO_3 – exchangers contributes to intracellular alkalinisation (40;41). The resulting extracellular pH in most solid tumours is usually acidic (5.8–7.2) – (42). The pH value used for acidic condition in our study was within this range. Studies indicate that extracellular acidic environment helps in tumour invasion by the killing of normal cells and clonal selection of tumour cells by caspase-mediated activation of p53 dependent apoptosis (43).

1.3.3 Glucose deprivation

Glucose deprivation in solid tumours occurs when they outgrow their nutrient supply. It does not occur in isolation and is accompanied by hypoxia (44). The effect of hypoxia on malignant progression is mediated by a series of hypoxia-induced proteomic and genomic changes activating angiogenesis (45), anaerobic metabolism (30), and other processes that enable tumour cells to survive or escape their oxygen and nutrient-deficient environment (46). These changes are mediated by a cytoplasmic protein called hypoxia-induced factor (HIF-1α) (3). The metabolic shift mediated by HIF-1α is characterised by activation of genes for glucose transporters (GLUT-1) and various glycolytic enzymes including pyruvate kinase type M2 (45). Another effect of glucose deprivation which is independent of HIF-1α is the conversion of the tetrameric form of pyruvate kinase M2 to the monomeric form the biologic significance of which has not been elucidated (47). It has been suggested that most pancreatic cancer cells with constitutive expression of HIF-1α protein adapt themselves to hypoxia and glucose deprivation by increased

glucose uptake and anaerobic metabolism (48). Although this adaptation to adverse conditions is seen in other solid tumours it is predominant in pancreatic cancers (48) which are relatively avascular and hypoxic (49).

2 Chapter 2: Overview of Tumour M2-PK in GI Cancers

2.1 Introduction

Gastrointestinal cancer is one of the commonest causes of cancer death in Europe (50;51). In the UK, colorectal cancer accounts for 12% of all cancers. It is the second most common cancer among women after breast cancer and the third most common in men after lung and prostate cancer (52). Stomach and pancreatic cancer account for 3% of all reported cases of cancer (52). The high mortality of GI cancers may relate to their advanced stage at diagnosis and early detection is an important way of reducing cancer mortality. Current tumour markers have a low sensitivity for detecting cancer and their role is limited to detecting recurrence after surgery or monitoring response to treatment. Even the most commonly used GI tumour marker, CEA, has been repeatedly questioned regarding its clinical usefulness (53;54).

Tumour M2-PK, the inactive dimeric form of the M2 isoenzyme of pyruvate kinase (a glycolytic pathway enzyme), was first described in 1985 by Eigenbrodt as a characteristic metabolic tumour marker (16;19;23). Initial studies in patients with cancers of the lung, pancreas, liver, kidney and breast showed increased activity of pyruvate kinase type M2 in blood as well as cancer tissues and its role is emerging in the management of GI cancers (17;55-59). It can be measured in both blood and faeces. The review aims to provide a critical review of the current literature on Tumour M2-PK as a marker of gastrointestinal cancer.

2.2   Methods

A literature search was conducted for the period from 1980–2005 using PubMed and

NeLH databases using the following keywords: Tumour M2-pyruvate kinase, faecal

Tumour M2-PK, tumour metabolism, tumour markers and carcinoembryonic antigen. A

total of 56 references relevant to Tumour M2-PK were retrieved. 38 references were

reviews, book chapters, and bibliographic links from the reviews on Tumour M2-PK

biochemistry, assay and measurement (4-6;16-23;25-29;55;57-73). 18 references were

the clinical trials involving circulating/faecal Tumour M2-PK and GI cancer (56;74-90).

7 of these 18 clinical studies were related to faecal Tumour M2-PK in GI cancer (76-

78;80;82;87;90). Of the remaining 11 studies for plasma/serum Tumour M2-PK, 3 studies

were in non-English language and have been excluded (75;79;81). Full papers on 8

studies with serum/plasma Tumour M2-PK and GI cancer were reviewed (56;74;83-

86;88;89). The sensitivity, specificity, positive predictive value (PPV) and negative

predictive value (NPV) was calculated for Tumour M2-PK for individual GI cancer types

and in comparison to other cancer markers. Three (84;88;89) of the eight studies which

all used the same diagnostic cut-off value of 15 U/ml for EDTA plasma Tumour M2-PK

were used for a small meta-analysis. Only one full published English language paper (78)

on faecal Tumour M2-PK was available. The rest of the data on faecal Tumour M2-PK

was obtained from 2 clinical trials (77;90) 2 published abstracts (80;82) and 2 German

studies with English abstract (76;87).

2.3   Current GI tumour markers – Roles and Limitations

2.3.1   Carcinoembryonic antigen (CEA)

This glycoprotein has a structural similarity to the adhesion proteins: ICAM-1 and ICAM-2 (91;92) suggesting a role in cancer invasion and dissemination (93;94). It can be measured in the serum and its clinical use has been investigated in GI cancers. It is less frequently elevated in early stage (Duke's A and B) colon cancers, the stages at which early detection is most likely to result in curative surgery. In a study by Wang et al., the proportion of patients with increased serum CEA concentration (>5ng/ml) in Duke's A and Duke's B stage disease were 25% and 39% respectively compared to 71% in Duke's C stage (95). However, as pointed out by Fletcher (96) sensitivity in symptomatic subjects is likely to be higher as compared to asymptomatic subjects because the former group is likely to have advanced disease. Serum CEA can also be increased in other forms of cancer and in multiple benign disorders (97). A high preoperative serum CEA level is associated with a poor outcome in colorectal cancer (95;98-103). Unfortunately, no clinical benefit has been demonstrated by the use of adjuvant chemotherapy based solely on increased preoperative CEA concentration (53). Elevated CEA levels following bowel cancer resection is also correlated with an adverse outcome (53). In a landmark study Moertel et al. demonstrated that CEA monitoring following bowel cancer resection had a 59% sensitivity rate for recurrence but with a 16% false-positive rate (104). In a randomised prospective study Ohlsson et al. (105) showed no difference in 5-year survival rate or cancer-specific survival rates between an intensive CEA-based follow-up

and a group with no follow-up. However, recent meta-analyses of randomised trials suggest that intensive CEA, CT scan and colonoscopy-based postoperative surveillance improves 5-year survival rates by approximately 10% compared with less intensive follow-up (106-108). Current guidelines by the National Institute for Health and Clinical Excellence (NICE) therefore recommend the measurement of CEA along with serial imaging following colorectal cancer resection (109).

2.3.2   Carbohydrate antigen 19-9 (CA19-9)

This is an oligosaccharide related to the Lewis A blood-group substance (54). It has been proposed as a sensitive marker for pancreatic, gastric and hepatobiliary malignancies (110). CA19-9 is elevated in nearly 80% of advanced pancreatic cancer patients. However, the false-positive rates are also high at 20–30% in benign hepatobiliary and pancreatic diseases (111). Other benign conditions associated with elevated CA19-9 levels include pneumonia, pleural effusion, renal failure and SLE (110). Recent reviews and multicentre studies (82;112) have questioned the clinical significance of elevated levels of CA19-9. Confident discrimination between benign and malignant disease can not be made on the basis of a solitary elevated CA19-9 (> 33 U/ml) measurement (112). Elevated levels are associated with advanced disease at presentation and with disease progression during follow-up (113). The clinical role of the tumour markers CEA and CA19-9 in gastrointestinal cancer diagnosis and management are limited and new serological markers are required.

2.4   Quantification of Tumour M2-PK

2.4.1   Blood

Tumour M2-PK can be detected by a highly sensitive enzyme-linked immunosorbent assay (ELISA) which allows the quantitative measurement of Tumour M2-PK in EDTA-plasma samples. The test is based on two monoclonal antibodies which specifically react with Tumour M2-PK and do not cross-react with the other isoforms of pyruvate kinase (types L, R, and M1) (67;70;114). Tumour M2-PK is adsorbed onto microtitre wells coated with a specific monoclonal antibody. It is quantified after incubation with a biotinylated second monoclonal antibody and with streptavidine-peroxidase conjugate (114). The mean intra-assay coefficient of variance is 3.5% and the mean inter-assay CV is 5.3% (63;68). A reference concentration of ≤ 15.0 U/ml in EDTA-plasma corresponds to specificity of 90% for a control group of patients without cancer (n=393) – (68); also see Figure 2). A study involving 695 healthy controls showed specificity of 95% at a diagnostic cut-off value of 17.5 U/ml in EDTA plasma sample (115). The Tumour M2-PK concentration in these healthy individuals ranged from 2–30 U/ml with a median value of 6 U/ml. Tumour M2-PK concentrations have been shown to be affected by haemolysis of blood sample (median value: 50.5U/ml), icterus (median value: 39.1 U/ml) and lipaemia (median value: 30.8 U/ml). However, a correlation with the severity of these conditions has not been reported (115).
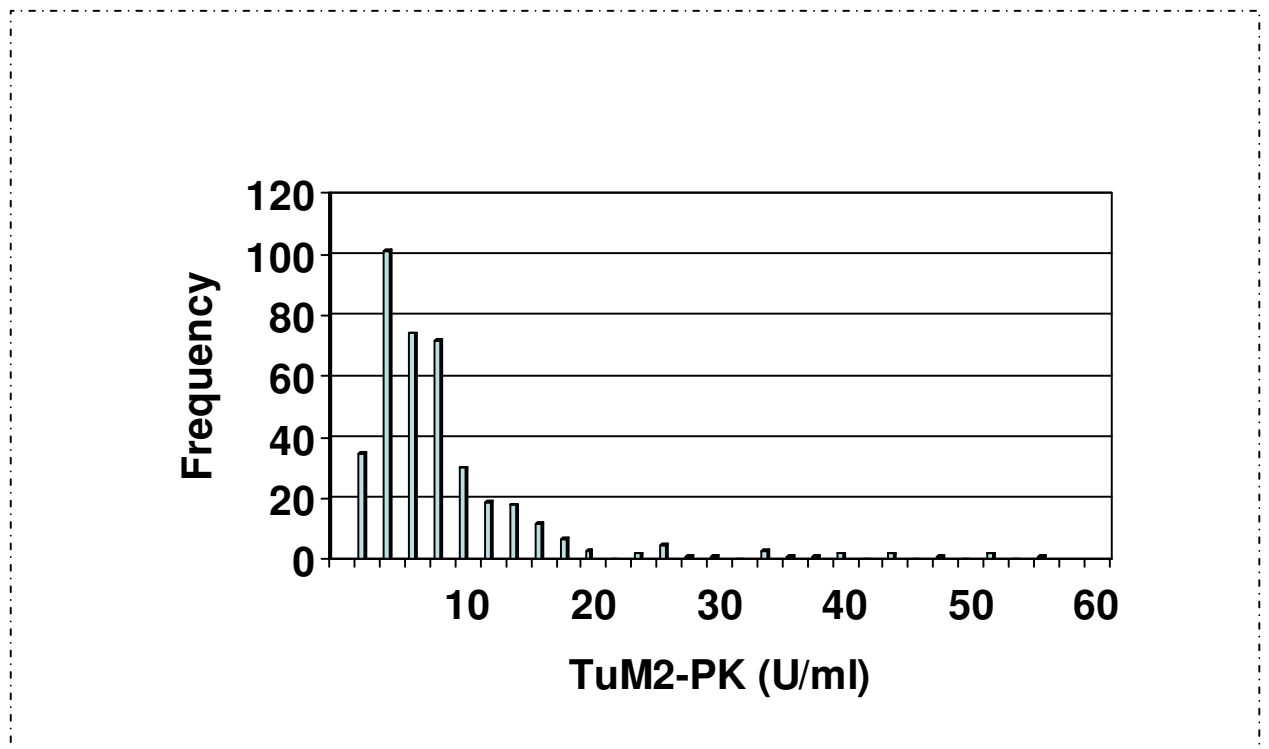
Figure 2: Distribution of Tumour M2-PK levels in individuals without cancer (n=393)

2.4.2   Stool

Tumour M2-PK can be measured in stool by a similar ELISA technique using the same

monoclonal antibody as used in serum/plasma assay. A reference concentration of 4 U/ml

corresponds to a specificity of 83% for a control group of subjects aged 50 to 89 yrs

(116). The intra-assay mean coefficient of variance (CV) was 7.9% and the inter-assay mean CV was 7.3% (116).

2.5   Factor affecting Tumour M2-PK in plasma

2.5.1   Benign diseases

Tumour M2-PK levels in EDTA plasma have been found to be elevated in bacterial infection as opposed to severe sepsis and polytrauma (66). Other benign conditions reported to have Tumour M2-PK elevations include: Rheumatic diseases (69), Diabetic nephropathy (117), Chronic cardiac failure (65), Inflammatory bowel disease (89) and acute and chronic pancreatitis (86). Plasma Tumour M2-PK, at cut-off value of 25 U/ml is elevated in 39% of patients with diabetic nephropathy (117). In chronic cardiac failure (CCF), the median Tumour M2-PK level in plasma of patients with NYHA (New York Heart Association) grade-2 disease was 24 U/ml, with grade-3 disease was 30 U/ml and with grade-4 disease was 46 U/ml. The diagnostic cut-off value for CCF was 5 U/ml. How often it was elevated in both controls and patients with CCF was not mentioned in this study (65). The mechanism suggested for the rise in plasma Tumour M2-PK value in patients with heart disease was the increased glycolyis to meet the metabolic demand related to the increased ventilation and neurohormonal activation, for example, seen in chronic cardiac failure. An alternative explanation was that the increased bilirubin and triglycerides levels commonly observed in CCF patients had caused analytical interference with the Tumour M2-PK assay. These postulations were not investigated although the author had ruled out the impaired renal function seen in CCF as a cause of

elevated plasma Tumour M2-PK levels (65). Oehler et al. (66) studied the expression of pyruvate kinase type M2 in neutrophils of polytrauma patients. Using western blotting for identifying M2-PK expression, they noticed strong expression of M2-PK in 62% of polytrauma patients as compared to none of the healthy volunteers. Oremek et al. (69) showed elevated levels of plasma Tumour M2-PK (diagnostic cut-off 17.5 U/ml) in different types of rheumatic diseases. It was elevated in 82% of rheumatoid arthritis patients, 82% of seronegative spondyloarthritis patients and 63% of patients with collagen disorders. The overall median value of plasma Tumour M2-PK in rheumatic diseases was 26 U/ml. Plasma Tumour M2-PK (diagnostic cut-off 15 U/ml) was elevated in 68% of patients with inflammatory bowel disease with a median value of 12 U/ml (89), 68% of patients with acute pancreatitis with a median value of 22 U/ml and 67% of patients with chronic pancreatitis with a median value of 11 U/ml (cut-off 8.9 U/ml) (86). These levels were significantly higher as compared to the median levels in respective controls. The cause of this rise in Tumour M2-PK value with benign disease has not been elucidated in any of these studies. The mechanism suggested is an increased glycolysis to meet the metabolic demand related to the stress of trauma and inflammatory reaction (86). No correlation between plasma Tumour M2-PK levels and the severity activity index (SAI) or CRP levels was found in these inflammatory conditions (89). Cross-reactivity of monoclonal antibodies with the tetrameric form of M2-pyruvate kinase cannot explain these results as the two monoclonal antibodies used in these studies are highly specific to the dimeric form. The level of Tumour M2-PK in EDTA plasma should

therefore be interpreted with caution in GI tumours associated with these benign conditions.

2.5.2 Tumour stage

As with most tumour markers, the concentration of Tumour M2-PK tends to increase with disease stage. Zhang et al. (88) showed an increase in plasma Tumour M2-PK levels with increasing tumour stage in gastric (compared with TNM stage), colorectal cancers (compared with Duke's stage) and pancreatic cancers (compared with TNM stage) (86). The level of Tumour M2-PK in patients with pancreatic cancer (n=60) differed significantly between those with stage I-II disease and those with distant metastasis (stage IV). Among non-GI cancers the association between Tumour M2-PK levels and disease stage has also been found (73;118). In lung tumours the sensitivity of Tumour M2-PK was observed to be 28% in stage I, increasing progressively to 73% in stage IV. A similar correlation is seen in renal cancer staging (Robson staging) with serum/EDTA plasma Tumour M2-PK increasing in sensitivity from 60% in stages I and II to 100% in stage IV. Faecal levels of Tumour M2-PK showed a strong correlation with TNM and Duke's staging in colorectal cancer (78). Faecal Tumour M2-PK has a higher sensitivity than plasma Tumour M2-PK in determining cancer stage in colorectal cancer (78;84).

2.5.3 Sample stability for the tumour assay

The level of Tumour M2-PK in blood can be influenced by the mechanical stress of

shaking the sample, the type of anticoagulant (EDTA, heparin, citrate), duration before the blood sample is centrifuged and the temperature at which the centrifuged sample is stored. Hugo et al. (63) observed a high reproducibility of Tumour M2-PK levels in EDTA plasma but not with serum or citrated/heparinised plasma blood samples from 10 healthy volunteers. Shaking or leaving the samples at room temperature for several hours prior to centrifugation led to a two- to threefold increase of Tumour M2-PK in serum and heparin-plasma samples. In contrast, the quantification in EDTA-plasma and citrate-plasma were absolutely stable after 24 hours (63). Lymphocytes were found to be a potential source for the increased concentration in serum and citrate-plasma (63). After centrifugation the EDTA-plasma sample is stable for three days at 4ºC or for up to one year at -20ºC (76;87). There are no known factors that can interfere with the faecal Tumour M2-PK levels. Excessive dilution of stool can lower the faecal M2-PK level. Therefore, a formed stool sample should always be analysed. Undiluted stool extracts can be stored at 4–8˚ C for one day or up to 4 weeks at -20˚ C without losing their stability (116).

2.5.4 Tumour pathology

There have been no GI cancer studies so far correlating M2-PK levels with the tumour size, grade and histological type. In renal cell carcinoma (RCC) patients (n=40), a significant correlation was found between serum Tumour M2-PK and RCC grade (50% in G1-RCC, 70% in G2-RCC and 86% in G3-RCC) (73). No correlation was found between serum Tumour M2-PK levels and histological type or tumour diameter.

Similarly in lung cancer neither plasma Tumour M2-PK nor immunohistochemical staining showed significant correlation with the histological type or differentiation of cancer but the concentration of Tumour M2-PK in EDTA plasma correlated well with tumour staging (71).

2.6   Tumour M2-PK: Role as a GI cancer marker

2.6.1   Faecal M2-PK in screening for GI cancer

Following the completion of a pilot project based on centres in Scotland (Fife, Tayside and Grampian) and England (Coventry and Warwickshire) in which around 120,000 patients aged 50 – 69 years old were enrolled (119), the UK Department of Health announced the introduction of national colorectal cancer screening which began to "roll out" from 2006 in England for men and women aged 60 – 69 and from March 2007 in Scotland for those aged 50 – 74 (120). Under these programmes patients were offered a guaiac faecal occult blood (FOB) test every two years, with positive FOB test results further investigated by diagnostic colonoscopy. A similar approach is also currently being assessed in Australia (121). Randomised trials of screening by FOBT have been shown to reduce the disease specific mortality by 15 – 18% although screening for cancer remains controversial due to the large number of false-positive results (122-124). The data from the Nottingham study showed a positive predictive value of only 12% (false- positive rate 88%) for colorectal cancer in individuals who underwent subsequent colonoscopy after FOBT (123). Sigmoidoscopy, colonoscopy or combinations are the other current practices of searching for and removing adenomatous polyps to prevent colorectal cancer

(125) but they are limited by poor patient compliance, complications and cost
effectiveness (126;127). Therefore newer screening tools for colorectal cancer are under
evaluation and may take their place in future guidelines. Hardt et al. showed that Tumour
M2-PK can be detected in the faeces of GI cancer patients (77;90). Symptomatic patients
undergoing colonoscopy for various reasons had faecal Tumour M2-PK measured. The
faecal level of Tumour M2-PK was higher in patients with histology-proven colorectal
cancers as compared to controls (non-colorectal cancer patients). The sensitivity of faecal
Tumour M2-PK at a cut-off value of 4 U/ml was 73% with a specificity of 78%. The
false-positive rate was 15%. However, this low false-positive rate should be viewed with
caution when comparing it to the high false-positive rate for Haemoccult faecal blood test
used in the Nottingham study and the Danish trial which were based on a large
asymptomatic population (123;124). Faecal Tumour M2-PK levels were higher with
more advanced disease. The sensitivity increased from 57% in case of T1 cancer, 78% in
T4 and 90% in patients with distant metastasis (78). Two recent studies also showed a
high sensitivity (92%) of faecal Tumour M2-PK for detecting colorectal cancer (80;82).
Using a cut-off of 3.33 U/ml, Koss et al. found a specificity of 92% (80). At a cut-off of 4
U/ml McLoughlin et al. found a similarly high sensitivity of 95% (82). These studies also
looked at the sensitivity for the detection of polyps, finding a sensitivity of 63% for
adenoma (82), 63% for polyps >1cm (80) and 25% for polyps <1cm (80). One study has
compared faecal Tumour M2-PK with a guaiac and an immunological FOB test (87).
Sensitivity of the guaiac FOB test was only 27% for colorectal cancer and 10% for

polyps, whereas it was 77% and 48% respectively for faecal M2-PK and 91% and 19%

respectively for the immunological FOB test. Specificity was 89%, 72% and 94%

respectively. Small meta-analyses of studies with faecal Tumour M2-PK reported an

overall sensitivity of 77.9% for the detection of colorectal cancer and specificity ranging

from 74.3 – 83.3%. Overall sensitivity for adenomatous polyps was 45.9%, increasing to

61.1% for those >1cm (76). There has been no randomised trial comparing faecal M2-PK

with FOBT or colonoscopy as a screening tool in terms of efficacy, cost effectiveness,

feasibility and reducing the cancer-related mortality.

2.6.2 Plasma M2-PK in detection of different GI cancers

In this review the data of eight clinical studies was analysed related to Tumour M2-PK

and GI cancer (56;74;83-86;88;89). The diagnostic cut-off values for Tumour M2-PK

used in these studies ranged from 8.9 U/ml to 28 U/ml. Three studies (84;88;89) used the

same cut-off value of 15 U/ml for Tumour M2-PK in EDTA plasma and were chosen for

meta-analysis of histologically proven GI cancers.

Oesophageal cancer (Table 2.1)

Three studies (one prospective and two retrospective) were found related to histologically

proven oesophageal cancer (83;84;89). One study combined data for gastric and

oesophageal cancer (89). The plasma Tumour M2-PK concentration in oesophageal

cancer ranged from 3.2 to 397 U/ml with a mean value of 42 U/ml. The controls used in

these studies were non-malignant disease subjects. The mean control value was 9.3 U/ml.

The diagnostic cut-off value of 15 U/ml (published cut-off) was used in two of the studies

(84;89) with a specificity of 89% while the other study (83) used 19.8 U/ml cut-off value

with a specificity of 95%. When data from the 2 oesophageal cancer studies with the

same diagnostic cut-off level for plasma Tumour M2-PK was analysed, 107 patients with

201 controls were evaluated with an overall sensitivity of 59%, specificity of 89%, PPV

of 74% and NPV of 80%. The overall sensitivity, PPV and NPV of plasma Tumour M2-

PK was higher as compared to those of CEA (14 – 25%, 45 – 75% and 49 – 78%

respectively), CA72-4 (12 – 53%, 38 – 92% and 62 – 64% respectively) and

CA19-9 (28 – 43%, 54 – 86% and 54 – 80% respectively). The ranges represent the

lowest and the highest value for these tumour markers in the 3 studies. Because of

different cut-off values the data from the individual studies could not be combined. The

specificity of CEA, CA72-4 and CA19-9 was not clearly stated in these studies.

Table 2.1: Studies comparing the tumour markers: Tumour M2-PK, CEA, CA19-9 and CA72-4 in oesophageal cancers

Reference	Study detail	Tumour marker (Cut-off value)	Sensitivity %	PPV %	NPV %
[Schulze et al. 2000]	**Retrospective study**	Plasma Tumour M2-PK (15 U/ml)	59	76	77
n= 87		CA72-4 (4 U/l)	12	38.4	62
Controls=141		CEA (5µg/l)	15	45	63
Specificity=89%*		CA19-9 (25 U/L)	43	70	71
[Schneider et al. 2003]	**Prospective study**	Plasma Tumour M2-PK(19.8U/ml)	55.8	92	65
n= 86		CA72-4(3.2U/l)	53.5	92	64
Controls=76		CA19-9 (23U/L)	27.9	85.7	54
Specificity=95%*		CEA (8.3µg/l)	14.5	75	49
[Hardt et al. 2000]	**Retrospective study**	Plasma Tumour M2-PK(15 U/ml)	60	66.6	87
n=20 †		CEA (3µg/l)	25	50	78
Controls=60		CA19-9 (37 U/L)	33	53.8	80
Specificity=90%*					

† Esoph/Gastric cancers, *The specificity of CEA, CA72-4 and CA19-9 was not stipulated in these studies.

Gastric cancer (Table 2.2)

Five studies (two prospective and three retrospective) were reviewed with data relevant to histology-proven gastric cancer and Tumour M2-PK (56;83;84;88;89). One study combined data for gastric and oesophageal cancers (89). Serum Tumour M2-PK measurement rather than EDTA plasma concentration was measured in one study (56). Tumour M2-PK levels in gastric cancer ranged from 2 – 965 U/ml with mean value of 43 U/ml. The controls used in these studies were mainly healthy donors. The mean control value of Tumour M2-PK was 9.3 U/ml. The diagnostic cut-off value for Tumour M2-PK in plasma was 15 U/ml in three of the studies, 19.8 U/ml in one study (83) and 22 U/ml in another (56) with specificity ranging from 89 – 95%. When data from the 3 gastric cancer studies with the same diagnostic cut-off level for plasma Tumour M2-PK were analysed (84;88;89) 211 patients with 221 controls were evaluated giving an overall sensitivity of 64%, specificity of 89%, PPV of 85% and NPV of 72%. The sensitivity, PPV and NPV of CA72-4 (35 – 91%, 14 – 95% and 34 – 100% respectively) is superior to CEA (24 – 38%, 6 – 80%, and 44 – 99% respectively) and CA19-9 (33 – 49%, 8-93%, and 52-99% respectively), the efficacy of Tumour M2-PK (57 – 67%, 10 – 94% and 44 – 99% respectively) was comparable. The range of values is the least and the best value for sensitivity, PPV and NPV for these tumour markers in the 5 studies. Because of different cut-off values, the data from the individual studies could not be combined. The specificity of CEA, CA72-4 and CA19-9 in these studies was again not stipulated. Low sensitivity and PPV was found in one study (56) which used serum Tumour M2-PK rather than

EDTA plasma and a high diagnostic cut-off. The cut-off values of CEA, CA19-9 and

CA72-4 in this study were historical.

Table 2.2: Studies comparing the tumour markers: TuM2-PK, CEA, CA19-9, CA72-4 and CA50 in gastric cancers

Reference	Study detail	Tumour marker (Cut-off value)	Sensitivity%	PPV%	NPV%
[Oremek et al. 1997]	**Retrospective study**	Serum Tumour M2-PK (22U/ml)	58	9.6	99
n=12		CA-72-4(4U/L)	91	14.3	100
Controls=666		CA19-9 (65U/L)	49.5	8.3	99
Specificity=90%*		CEA (10µg/l)	38	6.4	99
		CA50 (50U/L)	47.2	8.2	99
[Schulze al. 2000]	**Retrospective**	Plasma Tumour M2-PK (15U/l)	67	84	74
n=137		CA72-4(4U/l)	41	76.8	62
Controls=141		CEA (5µg/l)	26	70	55
Specificity=89%*		CA19-9 (25U/L)	45	79	62
[Schneider et al. 2003]	**Prospective study**	Plasma Tumour M2-PK (19.8U/ml)	57	94	58
n=122		CEA (8.3µg/l)	23.8	80	44
Controls=76		CA72-4(3.2U/l)	60.7	95	60
Specificity=95%*		CA19-9 (23U/L)	45.5	93	52
[Hardt et al. 2000]	**Prospective study**	Plasma Tumour M2-PK(15U/ml)	60	66.6	87
n=20 †		CEA (3µg/l)	25	50	78
Controls=60		CA19-9 (37 U/L)	33	3.8	80
Specificity=90%*					
[Zhang et al. 2004]	**Retrospective study**	Plasma Tumour M2-PK(15U/ml)	57	94	44
n=54		CA72-4(4U/l)	35.3	90	34
Controls=20					
Specificity=90%*					

† Esoph/gastric cancer

*The specificity of CEA , CA72-4 and CA19-9 was not stipulated in these studies.

Colorectal cancer (Table 2.3)

Four studies (two prospective and two retrospective) evaluated Tumour M2-PK and colorectal cancer patients (83;84;88;89). The level of plasma Tumour M2-PK in colorectal cancer patients was in the range of 2 – 986 U/ml with a mean value of 44 U/ml. The controls used in these studies were either healthy blood donors or patients with non-malignant disease. The mean value of Tumour M2-PK in controls was 9.6 U/ml. The diagnostic cut-off used in these studies was either 15 U/ml or 19.8 U/ml in EDTA plasma. Three studies used 15 U/ml cut-off level (84;88;89) and included 251 patients with colorectal cancer and 221 controls with a sensitivity of 57%, specificity of 89%, PPV of 86% and NPV of 65%. The overall specificity of Tumour M2-PK ranged from 89% – 95% with sensitivity, PPV and NPV (50 – 76%, 81 – 95% and 35 – 87% respectively). Tumour M2-PK was better compared to CEA (sensitivity 34 – 71%, PPV 80 – 95% and NPV 30 – 84%) and CA19-9 (sensitivity 27 – 55%, PPV 50 – 95% and NPV 29 – 77%). The range of values is the least and the best value for sensitivity, PPV and NPV for these tumour markers in the 4 studies. The specificity of CEA and CA19-9 were not clarified in all 4 studies.

Table 2.3: Studies comparing the tumour markers: Tumour M2-PK, CEA and CA19-9 in colorectal cancer

Reference	Study detail	Tumour marker (Cut-off value)	Sensitivity%	PPV%	NPV%
[Schulze al. 2000]	**Retrospective**	Plasma Tumour M2-PK (15U/ml)	50	83.5	60
n= 163		CEA (5µg/l)	42	81	57
Controls=141		CA19-9 (25U/L)	27	50	46
Specificity=89%†		TuM2-PK+CEA	67	87.3	70
[Schneider et al. 2003]	**Prospective study**	Plasma Tumour M2-PK (19.8U/ml)	47.8	96.7	35
n=250		CEA (8.3µg/l)	33.6	95	30
Controls=76		CA19-9 (23U/L)	30.4	95	29
Specificity=95%†					
[Hardt et al. 2000]	**Prospective study**	Plasma Tumour M2-PK (15U/ml)	76.5	81	87
n=34		CEA (3µg/l)	71	80	84
Controls=60		CA19-9 (37 U/L)	55.2	75	77
Specificity=90%†					
[Zhang et al. 2004]	**Retrospective study**	Plasma Tumour M2-PK (15U/ml)	68.5	95	51
n=54		CEA (3µg/l)	43.12	92	37
Controls=20					
Specificity=90%†					

† The specificity of CEA , CA72-4 and CA19-9 was not stipulated in these studies.

2.6.3 Combining Tumour M2-PK with other GI markers (Table 2.4)

Combining Tumour M2-PK with the conventional tumour markers increases its diagnostic

efficacy, as shown in a study by Schulze (84). In oesophageal cancer combining Tumour M2-PK

with CEA increases the sensitivity, PPV and NPV from 59%, 76% and 77% respectively to 65%, 78% and 80% respectively. In gastric cancer, it increased from 67%, 84% and 74% respectively to 82%, 87% and 97% respectively when Tumour M2-PK was combined with CA72-4. In colorectal cancer, combining Tumour M2-PK with CEA increases the sensitivity, PPV and NPV from 50%, 83% and 60% respectively to 67%, 87% and 70% respectively.

Table 2.4: Combining Tumour M2-PK with other GI cancer markers

Cancer Type [Reference]	Tumour Marker	Specificity %	Sensitivity %	PPV%	NPV%
Oesophageal cancer	Tumour M2-PK+CA19-9	89	65	78	80
[Schulze et al. 2000]	Tumour M2-PK (15U/ml)	89	59	76	77
	CA19-9(25U/l)	89	43	70	71
Gastric Cancer	Tumour M2-PK+CA72-4	89	82	87	97
[Schulze et al. 2000]	Tumour M2-PK (15U/ml)	89	67	84	74
	CA72-4 (4U/l)	89	41	77	62
Colorectal cancer	Tumour M2-PK+CEA	89	67	87	70
[Schulze et al. 2000]	Tumour M2-PK (15U/ml)	89	50	83	60
	CEA (5µg/l)	89	42	81	57

2.6.4 Plasma Tumour M2-PK levels in post treatment surveillance.

There has been only one study assessing Tumour M2-PK levels and the response to therapy as far as GI cancers are concerned. Ventrucci et al. (86) showed a rise in plasma Tumour M2-PK levels shortly (within 2 weeks) after pancreaticoduodenectomy for pancreatic cancers. This immediate post-operative rise was attributed to accelerated

glycolysis due to healing (66). There has been only one study so far monitoring the serum Tumour M2-PK levels after the resection of cancer. In this study, with only 6 patients followed after renal cell carcinoma resection, Tumour M2-PK normalised 11 weeks after surgery and showed rising levels 2 months before computed tomography detected recurrence (73). In studies with advanced breast and lung cancer patients Tumour M2-PK levels in plasma decreased within 4 weeks after the start of palliative chemotherapy and rose again with disease progression (58;72). In another study with lung cancer patients, plasma Tumour M2-PK concentration reflected the course of the disease and correlated well with tumour progression or remission following treatment (71).

2.7   Summary and conclusion

Tumour M2-PK can be quantified in blood with a specificity of 90 – 95% at a diagnostic cut-off value of 15 – 17.5 U/ml and in stool with a specificity of 83 – 95% at a cut-off value of 3.33 – 4 U/ml. The stability of Tumour M2-PK is best in EDTA plasma for 24 hrs at room temperature and is not influenced by any mechanical stress. The quantification in blood/stool is by highly sensitive enzyme-linked immunosorbent assay (ELISA) using two monoclonal antibodies specific to Tumour M2-PK. It can be elevated in benign conditions including chronic cardiac failure, diabetic nephropathy, rheumatic diseases, inflammatory bowel disease and pancreatitis. The inclusion of these benign conditions as non-cancer controls can result in false-positive rates ranging from 38 – 82%. Studies on gastric and colorectal cancer show a good correlation between plasma/faecal Tumour M2-PK and disease stage (78;84;88). Although no prospective

data is available on plasma Tumour M2-PK, faecal Tumour M2-PK has sensitivity of 64% to detect early stage (T1, T2) colorectal cancers. As a screening tool for bowel cancer, the overall sensitivity of faecal Tumour M2-PK is 73% with false-positive rate of 15% in symptomatic subjects. It has not yet been validated in a large-scale screening of an asymptomatic population. In our meta-analysis Tumour M2-PK showed good diagnostic accuracy for oesophageal/gastric and colorectal cancers with PPV of 86 – 88%. Recently Tumour M2-PK has been established as an important marker of transformed and highly proliferating cells during progression of the metaplasia-dysplasia-adenocarcinoma sequence in Barrett's oesophagus (64). The diagnostic accuracy of Tumour M2-PK was better than CEA and CA19-9 in oesophageal and colorectal cancer and was comparable to CA72-4 in gastric cancer and CA19-9 in pancreatic cancer. Combination of these tumour markers increases their diagnostic strength especially in pancreatic cancers. The current literature on Tumour M2-PK and GI cancer is limited but would justify further investigation of this novel cancer marker. Faecal Tumour M2-PK has a potential role in bowel cancer screening. A proper screening trial is required on an asymptomatic population comparing faecal Tumour M2-PK with Haemoccult test and validating its efficacy, cost effectiveness, feasibility and influence cancer specific mortality. There is limited information yet on the utility of Tumour M2-PK as a prognostic marker, as a marker of malignant transformation or in assessing tumour recurrence or response to treatment. Large multi-centre trials are, therefore, needed to define its clinical role.

Hypothesis and Aims

The hypothesis behind the work done in this thesis was:

'Tetramer-dimer switch-over of Tumour M2-PK and its localisation to mitochondria or nucleus in pancreatic cancer cells is a metabolic adaptation response to the altered tumour microenvironment'

Aims:

The following aims were pursued in this thesis:

(i) *To investigate the available studies on Tumour M2-PK in pancreatic cancer in the form of a meta-analysis.*

(ii) *To measure the levels of Tumour M2-PK in pancreatic cancer cell lines and to study the influence of altered tumour microenvironment on these levels.*

(iii) *To evaluate the tetramer-dimer switch-over of Tumour M2-PK in pancreatic cancer cells under altered culture conditions and to study its correlation with apoptosis.*

(iv) *To study the localisation of Tumour M2-PK at the subcellular level under the altered tumour microenvironment.*

3 Chapter 3 Tumour M2-PK in Pancreatic Cancer

3.1   Background

Pancreatic cancer accounts for 3% of all reported cases of cancer. It is the fifth leading cause of cancer death in Western countries (52;128). It is the 11th most common cancer in the UK, with an average of 20 cases diagnosed every day (52). The prognosis is extremely poor with five-year survival rate of less than 5% (129). Surgical resection represents the best chance for cure, but only 10 – 20% of patients are eligible for resection (130) and approximately 25% of patients who undergo laparotomy will have unresectable tumour (131;132). Early diagnosis to improve the dismal prognosis remains challenging. Most of the symptoms related to this malignancy occur only after disease advancement to an unresectable stage (133).

CA 19-9 has been proposed as a useful marker for pancreatic cancer (134;135). Elevated levels are associated with advanced disease at presentation and disease progression during follow-up (113). Confident discrimination between benign and malignant disease cannot be made on the basis of a solitary elevated CA19-9 (> 33 U/ml) measurement (112).

As the current pancreatic cancer markers have a limited role in diagnosis and disease monitoring, a review of Tumour M2-PK was carried out with the intention of performing a meta-analysis to maximise the clinical data derived from the current trials.

3.2   A Meta-analysis

3.2.1   Methods

Inclusion criteria

All studies in English language comparing the sensitivity and specificity of Tumour

M2-PK with CA19-9 were considered for inclusion.

Data source

The following databases were searched:

Pubmed (1951 – September 2006)

Embase (1974 – September 2006)

CENTRAL (Issue 3, 2006)

Science Citation Index (SCI) Expanded (1980 – September 2006)

References of identified studies were searched for identification of further references.

Search strategy

The following search was used for Pubmed:

("Pyruvate Kinase"[MeSH] OR pyruvate kinase) AND (m2 or "m-2" or "m 2") AND

("Neoplasms"[MeSH] or neoplasm or neoplasms or cancer* or tumor or tumors or tumour

or tumours) AND English [lang] AND "humans"[MeSH Terms]

Equivalent search strategy was used for other databases.

Evaluation of quality of included studies

Since the quality of the included studies can overestimate or underestimate the diagnostic

odds ratios (136;137), the following information was extracted from each study:

1. Design: Case-control design overestimates diagnostic odds ratio (136).

2. Differential verification: Different reference test performed based upon diagnostic test in question overestimates diagnostic odds ratio (136).

3. Blinding of assessors: If assessors are not blinded, diagnostic odds ratio is overestimated (136).

4. Description of population: If population is not described, diagnostic odds ratio is overestimated (136).

5. Description of diagnostic test: If the diagnostic test is not described, diagnostic odds ratio is overestimated (136).

6. Description of reference test: If the diagnostic test is not described, diagnostic odds ratio is underestimated (136). However, we do not expect the description of histopathology or imaging methods in any study.

7. Prospective or retrospective data collection: Collection of data retrospectively overestimates diagnostic odds ratio (136).

Statistical methods

Since different studies use different cut-off values (56;74) it was intended that a meta-analysis of diagnostic odds ratio (138) was performed. For this purpose, we calculated using the reported sensitivity, specificity, number with disease and the number without disease. Since the sensitivity (a/(a+c)), people with pancreatic cancer (a+c), specificity (d/(b+d)) and people without pancreatic cancer (b+d) are reported in the studies, it is possible to calculate the true-positivity (TP), false-positivity (FP), false-negativity (FN) and true-negativity (TN) – (a,b,c,d respectively in Figure 3.1).

	Histopathology	
	Positive	Negative

TuM2-PK		
Positive	**a** True positive	**b** False positive
Negative	**c** False negative	**d** True negative

Sensitivity = a/(a+c)
Specificity = b/(b+d)

Figure 3.1: Two-by-two contingency table for TuM2-PK sensitivity and specificity

The diagnostic odds ratio (DOR) for each study was then calculated by using the formula (139):

DOR = (TP/FP)/(FN/TN).

The 95% confidence interval of the DOR was calculated by finding out the anti-log of the expression log DOR±1.96SE(log DOR), where SE(log DOR) was calculated using the formula (139):

SE (log DOR) = [(1/TP) + (1/TN) + (1/FP) + (1/FN)] $^{1/2}$.

The DOR and 95% confidence interval thus calculated was pooled using the statistical software Stats Direct 2.5.6 (140) . The fixed-effect model (141) was used if the statistical heterogeneity measured by I^2 as calculated by Higgins (142) was less than 25; otherwise, the random-effects model (143) was used. Bias was explored using funnel plot (144).

Visual asymmetry was used to explore bias. Egger's linear regression method was used for statistical evaluation of bias (144).

The summary estimate DOR and 95% CI was calculated for each of the two tests Tumour M2-pyruvate kinase and CA19-9 and compared to see whether one test was statistically superior to the other (p< 0.05) by evaluating the overlap of 95% confidence intervals. If there was no significant variation in the diagnostic performance with threshold (i.e. diagnostic odds ratio is asymmetrical or the DOR varies with the threshold used) as estimated by the Littenberg and Moses method (138), the equation for the receiver operator characteristics (ROC curves) was calculated using the relation (138):

Sensitivity = 1/ [1+ [1/ ((1-specificity)/specificity)*DOR)]].

If there was a significant variation in the diagnostic performance with threshold, the equation for the ROC curve was calculated using the relation (138):

Sensitivity = 1/ [1+ [1/ (exp (a/(1-b)) * ((1-specificity)/specificity) ^ ((1+b)/ (1-b)))]]

where a and b are the estimates in the linear regression equation of the Littenberg and Moses method (138).

A subgroup analysis for pancreatic cancer versus healthy volunteers and pancreatic cancer versus benign pancreatic disorders was also performed.

3.2.2 Results

A total of 258 references were retrieved from Pubmed (78), Embase (75), Central (17) and SCI Expanded (88). 118 duplicates were removed and 132 references were excluded by reading the titles and abstracts. Full text was obtained for eight references (56;74;83-86;89;145) of seven studies. No further references were identified by searching the references of identified studies. All the seven studies could provide data for meta-analysis.

The characteristics of the included studies are tabulated in Table 3.1. The quality of the included studies is stated in Table 3.2. As expected, no study described the reference test in detail.

The sensitivity, specificity, true-positive, false-positive, false-negative, true-negative, diagnostic odds ratio and 95% confidence intervals are tabulated in Table 3.3.

All the seven studies reported the sensitivity and specificity of Tumour M2-pyruvate kinase, while only three studies (83;86;145) included the specificity of CA19-9. Only one study (89) reported the sensitivity and specificity of Tumour M2-pyruvate kinase at different cut-off levels (15 Units/ml) and (18 Units/ml). Since the diagnostic odds ratio (DOR) was better for the cut-off level of 15 Units/ml, the sensitivity and specificity corresponding to this cut-off level was used for the meta-analysis. One study (86) reported the sensitivity and specificity of Tumour M2-PK in distinguishing pancreatic cancer from other GI cancers, benign pancreatic disorders and other benign gastrointestinal diseases. However, this study reported the number of people positive for TuM2-PK for different conditions individually and so it was possible to calculate the sensitivity and specificity of TuM2-PK in distinguishing pancreatic cancer from other benign disorders. The meta-analysis of DOR for Tumour M2-PK is shown in Figure 3.2.

Table 3.1: Characteristics of included studies

Study	Reference test	Controls	Cut-off level of M2PK in units/ml	Sample size Pancreatic cancer	Sample size Control	Method of calculating cut-off level of M2PK	Cut-off level of CA19-9 in units/ml	Method of calculating cut-off level of CA19-9
Oremek et al. 1997	Histopathology	Healthy volunteers	22.5	64	666	Corresponding to a specificity of 90% in ROC	65	Literature
Cerwenka et al. 1999	Histopathology	Healthy volunteers, benign pancreatic disease	28	38	128	Corresponding to a specificity of 90% in ROC	37	Literature
Hardt et al. 2000	Not stated	Healthy volunteers	15	14	60		23	
Schulze et al. 2000	Not stated	Healthy volunteers	15	26	141	Literature	25	Not stated
Schneider et al. 2003	Histopathology		19.8	24	76	Corresponding to a specificity of 95% in ROC	23	Corresponding to a specificity of 95% in ROC
Ventrucci et al. 2004	Histopathology, imaging	Healthy volunteers, other benign GI disorders including benign pancreatic diseases*	8.9	60	95*	Best cut-off value in ROC	60	Best cut-off value in ROC
Siriwardana et al. 2005	Histopathology	Patients with suspected pancreatic cancer but histologically proven to have no cancer	27	77	69	Not stated	38.5	Not stated

*Patients with other cancers were excluded from analysis

Table 3.2: Quality of included studies

Study	Design	Differential verification	Blinding of assessors	Population described	Description of diagnostic test	Description of reference test	Data collection
Oremek et al. 1997	Case-control	No	Not stated	Yes	Yes	No	Prospective
Cerwenka et al. 1999	Case-control	No	Not stated	No	Yes	No	Prospective
Hardt et al. 2000	Case-control	No	Not stated		Yes	No	Prospective
Schulze et al. 2000	Case-control	No	Not stated	No	Yes	No	Retrospective
Schneider et al. 2003	Case-control	No	Not stated	Yes	Yes	No	Prospective
Ventrucci et al. 2004	Case-control	No	Not stated	Yes	Yes	No	Prospective
Siriwardana et al. 2005	Cohort	No	Not stated	Yes	Yes	No	Prospective

Table 3.3: Sensitivity and specificity of TuM2-PK and CA19-9

Study	Tumour marker	Sensitivity (%)	Specificity (%)	True positivity (n)	False Positivity (n)	False negativity (n)	True negativity (n)	Diagnostic odds ratio (95% CIs)
Oremek et al. 1997	TuM2PK	71	90	45	67	19	599	22.03 (12.14, 40.00)
	CA19-9	68.5	NA	-	-	-	-	-
Cerwenka et al. 1999	TuM2PK	79	90	30	13	8	115	33.86 (12.82, 89.40)
	CA19-9	65	NA	-	-	-	-	-
Hardt et al. 2000	TuM2PK	71.4	90	10	6	4	54	22.47 (5.36, 94.23)
	CA19-9	83	NA	-	-	-	-	-
Schulze et al. 2000	TuM2PK	73	89	19	16	7	125	26.47 (9.60, 72.96)
	CA19-9	85	NA	-	-	-	-	-
Schneider et al. 2003	TuM2PK	72.9	95	17	4	7	72	12.78 (3.25, 50.24)
	CA19-9	87.5	95	21	4	3	72	33.25 (6.78, 163.03)
Ventrucci et al. 2004	TuM2PK	85	41	51	50	9	45	141.16 (63.47, 318.95)
	CA19-9	75	81	45	16	15	79	42.33 (19.16, 93.50)
Siriwardana et al. 2005	TuM2PK	66	60	51	28	26	41	46.59 (23.74, 91.41)
	CA19-9	71	73	55	19	22	50	48.26 (23.38, 99.58)

TuM2PK: Tumour M2-Pyruvate Kinase, CA19.9: Carbohydrate Antigen 19.9, NA: Not available

Summary meta-analysis plot [random effects]

Study	Odds Ratio (95% CI)
Oremek 1997	22.03 (12.14, 40.00)
Cerwenka 1999	33.86 (12.82, 89.40)
Hardt 2000	22.47 (5.36, 94.23)
Schulze 2000	26.47 (9.60, 72.96)
Schneider 2003	12.78 (3.25, 50.24)
Ventrucci 2004	141.16 (62.47, 318.95)
Siriwardhana 2005	46.59 (23.74, 91.41)
combined	35.00 (19.66, 62.30)

odds ratio (95% confidence interval)
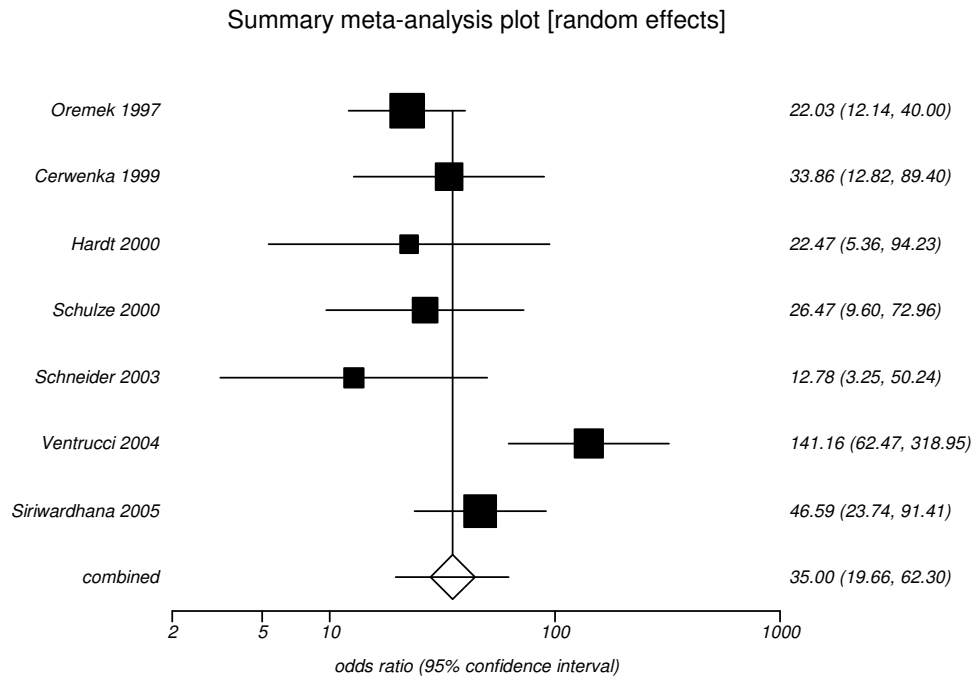
Figure 3.2: Meta-analysis Forest Plot (M2-PK)

Since I^2 was 64.6%, the random-effects model was used for the meta-analysis. The summary estimate of the diagnostic odds ratio for Tumour M2-PK was 35.00 (95% CI = 19.66 – 62.30) and that of CA19-9 was 44.04 (95% CI = 26.53 – 73.10). There was significant overlap of the 95% confidence intervals in the two tumour markers.
The funnel plot is shown in Figure 3.3. Although visual inspection showed some asymmetry, there was no statistically significant bias (P = 0.6944).

Bias assessment plot
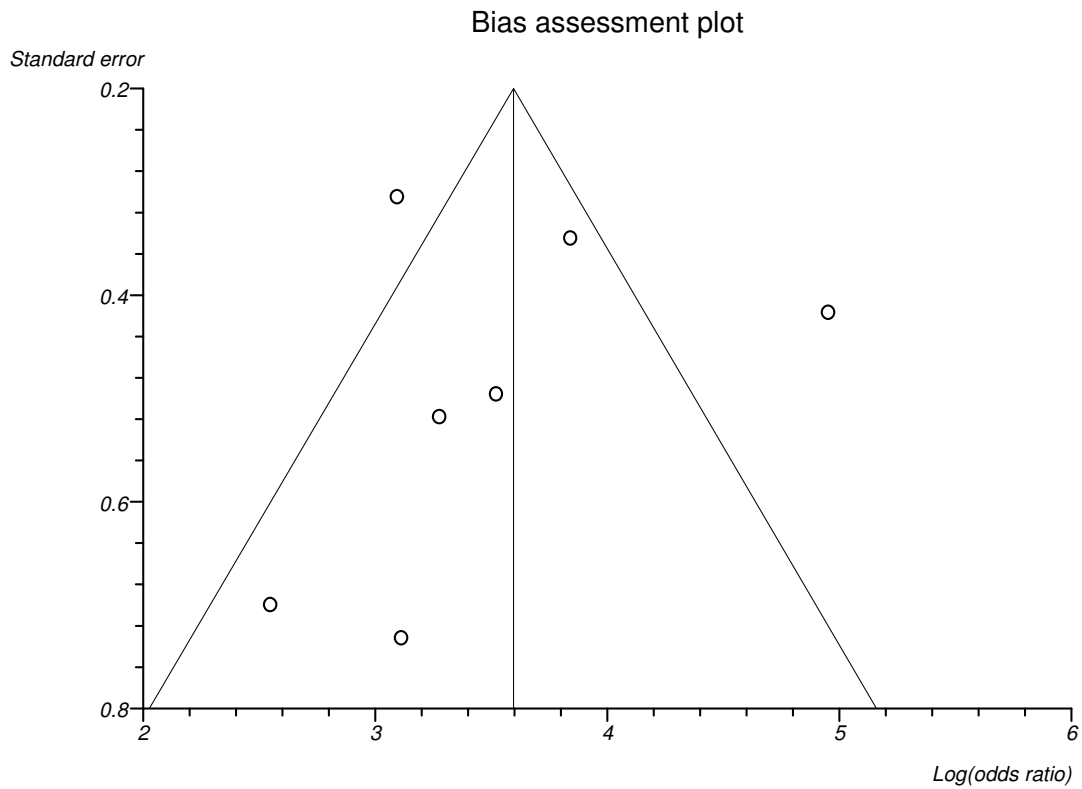
Figure 3.3: Funnel plot for the studies bias assessment

There was significant variation in the diagnostic performance with threshold as estimated

by the Littenberg and Moses method (138). The appropriate equation for the ROC was

used to calculate the ROC. The ROC for Tumour M2-PK is shown in Figure 3.4. This

corresponds to a sensitivity of 81% for a specificity of 65%.

ROC TuM2-PK
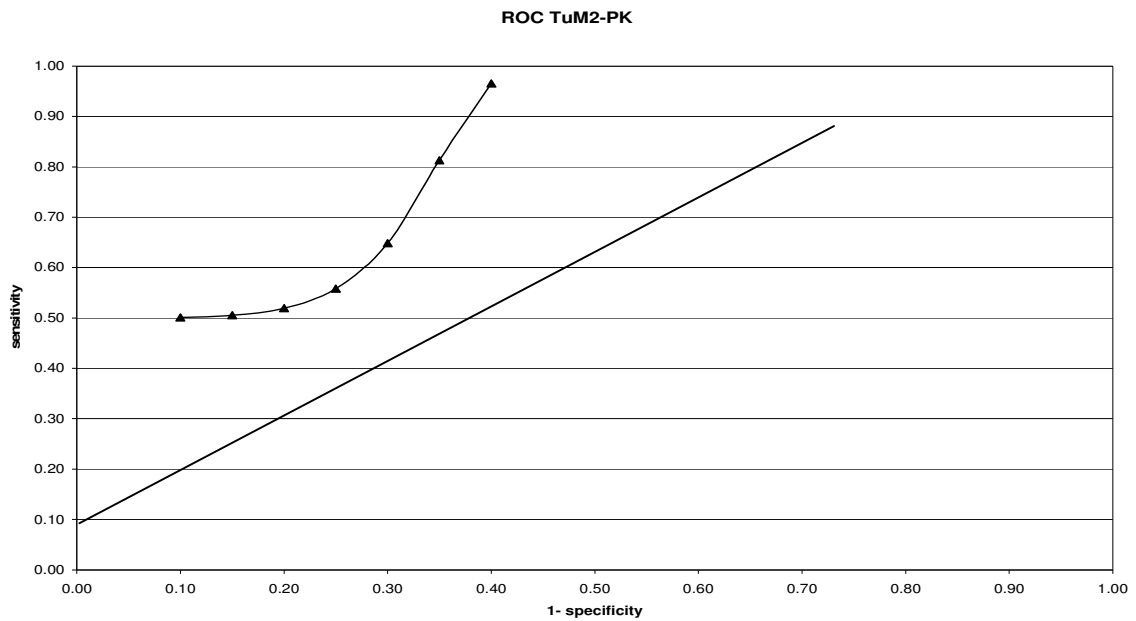
Figure 3.4: Receiver Operating Characteristics curve of summary estimate

A subgroup analysis to determine the role of Tumour M2-pyruvate kinase in identifying

people with pancreatic cancer from healthy volunteers was performed. Five studies

(56;74;83;84;89) reported the sensitivity and specificity of Tumour M2-pyruvate kinase

in pancreatic cancers compared with healthy volunteers. The DOR was 23.40 (95% CI =

15.48 – 35.36). There was no statistical heterogeneity as the I^2 was 0%. Since there was

significant variation in the diagnostic performance with threshold, the sensitivity and

specificity was calculated by the Littenberg and Moses method. This corresponded to a

sensitivity of 94% for a specificity of 55%.

Another subgroup analysis was performed to determine the role of Tumour M2-pyruvate kinase in identifying pancreatic cancer from benign pancreatic disorders. Only two studies (86;145) reported this. Therefore, a meta-analysis was not performed. The sensitivity and specificity was 85% and 35% in the study (86) which reported the number of people positive for Tumour M2-pyruvate kinase for different conditions individually. In the other study (145) the specificity and sensitivity were 66% and 60%.

Two studies (84;86) reported the sensitivity and specificity of Tumour M2-pyruvate kinase alone and in combination with CA19-9. Of these two, one study (84) reported only the sensitivity without reporting the specificity. The combination of Tumour M2-pyruvate kinase and CA19-9 identified 96% of the cancers. In the other study (86) the combination identified pancreatic cancer with a sensitivity of 97% which was higher than Tumour M2-pyruvate kinase (sensitivity 85%) or CA19-9 (sensitivity 75%) when used alone. The specificity was 38%. However, the later study included other malignancies also.

3.2.3 Discussion

Tumour M2-pyruvate kinase is not an organ-specific cancer marker and is elevated in different type of cancers. The different specificities reported in the published studies used in our meta-analysis are due to the type of control populations or the diagnostic threshold used. Because of the different thresholds used in the different studies (Table 3.1) diagnostic odds ratio (DOR) was calculated. Summary ROC is a useful method of meta-analysis when different studies use different thresholds for calculating the sensitivity and specificity of a diagnostic test (138). Since the ROC curve was asymmetrical–identified by the Littenberg and Moses method–(138), the appropriate formula was used to calculate the summary ROC curve. Most of the studies were of satisfactory quality with no significant bias in the evaluation of diagnostic test. Although there was some asymmetry in the funnel plot it was not statistically significant. Histological confirmation was the reference test used in most of the studies based on which the sensitivity of Tumour M2-pyruvate kinase or CA19-9 was determined.

Different authors used different cut-off points to calculate the specificity and sensitivity. Hence, the Littenberg and Moses method (138) was used to perform the meta-analysis. The main limitation of this meta-analysis is that the information about sensitivity and specificity is lost because of calculation of the diagnostic odds ratio. Furthermore, the overall cut-off value for the test cannot be recommended. However, the main strength of

this meta-analysis is that it has summarised the evidence in literature regarding the diagnostic utility of Tumour M2-pyruvate kinase. The Forest plot results are reproducible in different centres with the DOR varying between 13 and 35 in 6 of the 7 studies. Tumour M2-pyruvate kinase may not be useful for screening of general population. However, it may be of more value in patients at high risk of pancreatic cancer. There are a few high risk groups that would be considered for screening e.g. Peutz-Jeghers syndrome, relatives of Familial pancreatic cancer (FPC), Hereditary pancreatitis, Intraductal Papillary Mucinous Neoplasms (IPMN) and chronic pancreatitis (146). FPC groups are as rare (147) as familial chronic pancreatitis which has a high incidence of malignant transformation (148). Chronic pancreatitis has a small but significant high risk (148) but the role of Tumour M2-pyruvate kinase in distinguishing pancreatic exocrine cancers from benign pancreatic diseases is limited with only two studies (86;145) comparing Tumour M2-pyruvate kinase levels in pancreatic exocrine cancers and benign pancreatic diseases.

The diagnostic odds ratio was higher for CA19-9 than Tumour M2-pyruvate kinase although the difference was not statistically significant. This meta-analysis therefore suggests that Tumour M2-pyruvate kinase has equivalent diagnostic utility to CA19-9 in distinguishing patients with pancreatic cancer from healthy individuals. A potential role for Tumour M2-pyruvate kinase would be in combination with CA19-9 in patients with jaundice where elevated CA19-9 levels are elevated in the presence of biliary obstruction independent of the aetiology. In such cases measurement of Tumour M2-pyruvate kinase

would be of utility since its level in plasma is not influenced by elevated bilirubin levels (85;86). Due to the limited number of studies involving combination of markers, it was difficult to calculate the DOR for Tumour M2-pyruvate kinase and CA19-9 combination. However, these studies indicate that combining these two markers identify more patients with pancreatic cancer than when used alone.

Recently several protein markers have been identified which are the product of over or altered gene expression in pancreatic cancer (149). These include macrophage inhibitory cytokine-1 (MIC-1), synuclein-gamma, mesothelin, osteopontin and S100A4 which have been investigated as potential markers for pancreatic cancer, but their efficacy as serum markers remains undetermined (150-154). Tumour M2-pyruvate kinase, on the contrary, has a better reproducibility of diagnostic efficacy in pancreatic cancer either alone or in combination at different centres. This meta-analysis would suggest further trials comparing levels of Tumour M2 pyruvate kinase, either alone or in combination with CA19-9, in patients with exocrine pancreatic cancer and patients with high risk for pancreatic cancer.

3.2.4   Conclusion

Tumour M2-PK has a potential role as a marker of pancreatic cancer. It can reliably distinguish healthy individuals from patients with pancreatic cancer. Further experimental studies are required to clarify the significance of elevated levels of Tumour M2-PK in pancreatic cancer with reference to tumour biology, tumour metabolism and tumour microenvironment.

4 Chapter 4 Materials and Methods

List of experimental techniques used in this thesis:

1. **Isolation and culture of cell lines**.

2. **Immunoblotting for total M2-PK protein**–this method was used to demonstrate the total M2-PK protein in cells before dimeric M2-PK can be measured.

3. **ELISA for Tumor M2-PK assay**–this method demonstrates the dimeric form of M2-pyruvate kinase. It is an accurate, highly specific and easily reproducible method (63).

4. **Pyruvate kinase activity assay by spectrophotometry**–this method measures pyruvate kinase activity and is a validated method as described before (155;156).

5. **Bradford assay–**a validated reproducible method for measuring total protein content in the cell sample (157).

6. **Active Caspase 3, 7 and 8 assay**–this method allows measurement of activity of initiator (Caspase 8) as well as effector (Caspase 3 and 7) caspases in the adherent and floating cells in culture. It is a fast and reliable method and has been validated before (158).

7. **Annexin V-FITC and Propium iodide staining and FACS analysis–** Annexin V staining was used for early apoptosis while Propium iodide was used as a marker of necrosis. FACS analysis was used to quantify

early or late apoptosis and necrosis.

8. **Immunofluorescence–**this method is used for localising M2-PK in relation to cell organelles especially nucleus and mitochondria to predict its role other than tumour metabolism.

4.1 Cell lines description

Panc-1 is a Human Caucasian pancreatic adenocarcinoma cell line of ductal origin purchased from European Collection of Cell Culture (ECACC) catalogue number 87092802 while Colo 357 is a continuous human cell line derived from a lymph node metastasis of a pancreatic adenocarcinoma (Table 4.1). Dr N. R. Lemoine, Centre for Molecular Oncology and Imaging, Institute of Cancer, Barts and The London School of Medicine and Dentistry, University of London, London, UK, gifted these. These cell lines were chosen for the experiments because of difference in their origin, proliferation rate and metastatic potential (Table 4.1). Moreover, these were readily available in our laboratory as continuous monolayer culture because of ongoing research work on pancreatic cancer.

Table 4.1: Characteristics of Panc-1 and Colo 357 pancreatic cancer cell lines.

Cell line characteristics	Panc-1	Colo 357
-Source of tumour cells[a]	Primary tumour	Lymph node metastasis
-Histology and grade[a] of primary tumour	PDAC, G3	PDAC, G1-G2
-Ultrastructural grading*[a]	3	2
-Cytokeratin marker[a]		
CK-7	-	+++
Vimentin	+++	+
-Cell doubling time of monolayer cultures (hrs)	52 [c]	21 [d]
- Gemcitabine sensitivity[b]	Resistant	Sensitive

* Based on cellular and nuclear polymorphism, cell membrane structure, cell organelles, mucin granules. - Negative, + <10%, +++ >50% of the stained cells, PDAC pancreatic ductal adenocarcinoma. [a] Sipos et al.(159) , [b] Schniewind et al.(160), [c] Lieber et al.(161) and [d] Morgan et al. (162)

MCF7: It is a Human Breast epithelial cell line purchased from ECACC (Catalogue number 86012803). This cell line expresses Tumour M2-PK as described previously (163) and is used as a positive control for Tumour M2-PK quantification.

HMEC-1 (Human microvascular endothelial cells) cell line was used as a non-cancer cell control and was kindly donated by Dr Shiyu Yang, Department of Anatomy, Royal Free and University College Medical School, UCL, London.

4.2　Cell culture techniques

Panc-1 and Colo 357 cell line were maintained in culture in DMEM (Dulbeco's Modified Eagle's Medium – Gibco Catl. No. 21969-035) while MCF 7 was maintained in EMEM (Earl's Minimum Essential Medium – Gibco Catl. No. 10370047) supplemented with Penicillin/Streptomycin solution (1%), 2mM glutamine and 10% foetal bovine serum (FBS). Cell culture was maintained at 21% O_2, 5% CO_2 and at 37 ˚C until 70 – 80% confluent and then subcultured for experiments. For each experiment, 2 million cells were plated on 75 cm^2 flask and cultured for 24 hrs, 48 hrs, 72 hrs and 96 hrs without changing the medium. Adherent cells were removed from the flask using 0.25% Trypsin EDTA (Gibco) and counted under an inverted phase contrast microscope (Nikon TMS-F, Japan) in a hemacytometer (Bright Line™ Hemacytometer Z359629, Sigma, UK). Cells were then washed thrice in cold PBS, centrifuged at 800 rpm and pelleted. Cell pellets were stored at -80 ˚C for Tumour M2-PK extraction.

4.3　Culture conditions

4.3.1　Acidic pH condition

Preparation of acidic (pH 6.5) medium:

Acidic medium was prepared by adding 1 mM of MES and 25 mM of HEPES (Sigma-aldrich, UK) to DMEM without sodium bicarbonate (GIBCO, UK). Final pH of the medium was adjusted to 6.5 using 25 mM NaOH or HCl. Addition of MES and HEPES maintained the required acidic pH for 96 hrs in culture as described before (35). Panc-1 and Colo 357 cell lines were allowed to adhere to plate for 48 hrs in normal pH medium before exposure to acidic medium further for 24, 48, 72 and 96 hrs. Cells grown in normal culture conditions were used as controls and were cultured for the same duration with the replacement of medium after 48 hrs. The pH of the culture medium was monitored at 24, 48, 72 and 96 hrs using pH meter (Hanna pH meter 210, Bedfordshire, UK).

4.3.2　Glucose deprived condition

Panc-1 and Colo 357 cell lines were incubated for 48 hrs in normal medium in 75 cm^2 flask. A glucose-deprived condition (10 mg/dl) was created by adding 10% FBS to glucose-free DMEM. Because normal glucose concentrations in human peripheral blood range between 70 and 200 mg/dl, we used DMEM containing 100 mg/dl glucose. Thus, a concentration of 100 mg/dl is defined as ''normal glucose'' in our study. Oxygen concentration in tumour tissues is 1.25% (about one tenth of that in peripheral blood); thus, glucose concentration in tumour tissues could also be estimated to be around one

tenth of that in peripheral blood. Therefore, the cultures carried out under glucose-deprived conditions were done in glucose-free DMEM, supplemented with 10% FBS (the final concentration of glucose in this medium is 10 mg/dl as described before (164). After 48 hrs medium was changed to glucose-free DMEM (GIBCO, U.K) and cells were further incubated for 24, 48, 72 and 96 hrs. Further check on glucose concentrations during this period were not done as glucose levels were not expected to rise. Control cells were grown in normal medium for the same duration.

4.3.3   Hypoxic condition

Initial experiments were done using the AnaeroGen™ System (Oxoid Limited, Basingstoke, UK) until hypoxia chamber (Innova CO-48, New-Brunswick Scientific, New Jersey, USA, Picture 1) which maintains hypoxic condition with 1% O_2, 5% CO_2 and 94% Nitrogen, was available for later experiments. AnaeroGen™ System involves placing a paper sachet containing ascorbic acid into a 2.5 litre jar that rapidly reacts with air to reduce the oxygen level to 1% within 30 minutes and increases the carbon dioxide level to 10% (manufacturer's information sheet). An indicator resorufin (pink at atmospheric oxygen) is reduced to hydroresorufin (white colour) on exposure to 1% O_2. After incubating Panc-1 and Colo 357 cells in normal culture medium for 24 hrs in 100 x 20 mm Petri dishes, medium was replaced and the Petri dishes were placed in the anaerobic jar for 24, 48, 72 and 96 hrs at 37˚ C. Control cells were grown in normal medium for the same duration with the replacement of medium after 24 hrs.

Picture 1: Hypoxia chamber

The hypoxic condition in the jar was monitored throughout the culture duration by the anaerobic indicator while the inbuilt oxygen and CO_2 sensors were available to monitor hypoxic environment in the hypoxia chamber. The oxygen level in culture medium was monitored using commercially available kit from BD Biosciences.

4.4 Biochemical Assays

4.4.1 ELISA (Quantitative)

Principle

Tumour M2-PK in cell homogenate supernatant was measured using ELISA kit for plasma Tumour M2-PK (ScheBo® Tumour M2-PK™). The assay is a sandwich-type ELISA based on two monoclonal antibodies specific for Tumour M2-PK with no cross-reactivity to other isoforms of M2-PK. The primary monoclonal antibody is pre-coated to the ELISA plate. Tumour M2-PK in the standards and the cell homogenate supernatant (diluted only if the absorbance is beyond the standard curve) binds to the antibody and is thus immobilised on the plate. A second monoclonal antibody which is biotinylated, binds to Tumour M2-PK. The conjugates of peroxidase (POD) and streptavidin bind to the biotin moiety. The peroxidase then oxidises TMB (3, 3'5, 5'-tetra-methyl benzidine) substrate to give the final colour reaction for the absorbance which was determined spectrophotometrically.

Materials and Reagents

Reagents (Picture 2)

1. 12 ELISA-strips with 8 wells each, coated with a monoclonal antibody to human Tumour M2-PK 96 wells.

2. Sample-washing buffer concentrate (5x) 100 ml phosphate buffered saline, pH 7.2, with detergent.

Picture 2: Tumour M2-PK ELISA kit

3. Tumour M2-PK standards 1 to 4, ready-to-use, 700 µl each Tumour M2-PK in
   serum matrix with sodium azide.

4. Control, ready-to-use, 700 µl tumour M2-PK in serum matrix with sodium azide.

5. Second monoclonal antibody to tumour M2-PK conjugated to biotin, i.e. anti-
   tumour M2-PK bio, 150 µl in aqueous solution with sodium azide.

6. POD-Streptavidin, ready-to-use, light sensitive, 8 ml in aqueous solution.

7. Substrate solution, ready-to-use, light sensitive, 12 ml TMB in aqueous solution.

8. Stop solution, ready-to-use, 12 ml aqueous acidic solution.

Picture 3: ELISA plate-reader

9. ELISA-reader capable of reading absorbance at 450 nm. Reference wavelength: 620 nm (Picture 3).

10. Polystyrene test tubes (3 ml, 10 ml).

11. Graduated cylinder (500 ml).

12. Vortex mixer.

13. Adjustable precision pipettes: 0-50 µl, 50-200 µl, and 200-1000 µl.

14. Pipettes 2 ml, 5 ml and 10 ml and adjustable 8-channel pipette 50-250 µl.

Sample protocol

The frozen cell pellets stored at -80 ℃, then thawed at 37 ˚C and washed thrice with cold PBS (Phosphated Buffered Saline). Cells were resuspended in 1ml of cold extraction buffer (10 mM Tris, 1 mM NaF and 1 mM Mercaptoethanol, pH 7.4, Sigma-Aldrich) and homogenised using a glass-pestle homogeniser (Wheaton Science products, USA) using 20 strokes of pestle. The homogenised cells were then centrifuged at 40,000 g (Optima ™ TLX ultracentrifuge, Beckman Coutler™, USA) for 20 minutes at 4º C and the cell homogenate supernatant was used for ELISA.

Assay protocol

Preparations

-Preparation of sample-washing buffer

100 ml sample-washing buffer 5x (black cap) + 400 ml bidistilled water.

The diluted sample-washing buffer is stable for 6 months at 4 - 8 °C.

-Preparation of ELISA plate

ELISA plate was brought to room temperature before opening. Desired numbers of ELISA strips were left in the microplate frame. Unused ELISA strips were stored in the well-sealed plastic bag containing the desiccant. The study samples were brought to room temperature before plating.

-Preparation of the secondary antibody anti-tumour M2-PK bio (1:100)

Preparation of 1:100 dilutions of the biotin-conjugated second monoclonal anti-tumour M2-PK antibody was made depending on the number of strips to be used:

For 2 strips (1/6 plate):

15 µl anti-tumour M2-PK bio + 1.5 ml sample-washing buffer.

For 4 strips (1/3 plate):

25 µl anti-tumour M2-PK bio + 2.5 ml sample-washing buffer.

For 6 strips (1/2 plate):

30 µl anti-tumour M2-PK bio + 3.0 ml sample-washing buffer.

For 12 strips (1 plate):

60 µl anti-tumour M2-PK bio + 6.0 ml sample-washing buffer.

Dilutions of anti-tumour M2-PK-bio was stored at 4 – 8 °C and brought to room

temperature shortly before use.

Assay procedure

Incubation of samples and standards

-Pipetted 50 µl of sample-washing buffer into wells A1 and A2 as blanks.

-Pipetted 50 µl of each standard into strips 1 and 2 as duplicate.

Standard 1 =   5.0 U/ml

Standard 2 =   15.0 U/ml

Standard 3 =   40.0 U/ml

Standard 4 =   100.0 U/ml

-Pipetted 50 µl of control solution (20.0 U/ml ± 15 %) into wells F1 and F2.

-Pipetted 50 µl of undiluted samples into each of two adjacent wells and incubated for 60

minutes at room temperature.

-The wells were emptied and washed 3 times with sample-washing buffer (8 channel

pipette, 250 µl/well). The plate was inverted and tapped firmly on a clean paper towel to

remove any remaining liquid.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
B	STD1	STD1	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
C	STD2	STD2	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
D	STD3	STD3	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
E	STD4	STD4	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
F	CON	CON	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
G	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
H	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42

<-2 strips-->

<----- 4 test strips ------>

<----------------------- whole ELISA plate, 12 test strips ----------------------->

STD: standards
CON: control
S1-S42: Undiluted cell lysate supernatant samples

Figure 4: Possible plate layout

-Incubation with second antibody (anti-tumour M2-PK bio)

50 µl of the 1:100 biotin-conjugated second monoclonal antibody was added into each

well using 8 channel pipette and incubate for 30 minutes at room temperature.

Wells were emptied by inverting and tapping the plate and washed 3 times with sample-

washing buffer (8-channel pipette, 250 µl/well). Plate was inverted and tapped on a clean

paper towel to remove any remaining liquid in the wells at the end of each washing step.

-Incubation with POD-Streptavidin

50 µl/well of ready-to-use POD-Streptavidin was added and incubated for 30 minutes in the dark at room temperature. Wells were emptied by inverting and tapping the plate and washed 3 times with sample-washing buffer (8-channel pipette, 250 µl/well). Plate was inverted and tapped on a clean paper towel to remove any remaining liquid in the wells at the end of each washing step.

-Colour Reaction

Added 100 µl of ready-to-use substrate solution to each well. The mixture was incubated for 15 minutes in the dark at room temperature. This time was shortened when using an ELISA-reader which reads extinctions only up to OD 1.5 or 2.

-Stopping the colour reaction

The substrate reaction was stopped by adding 100 µl of stop solution per well and the contents were mixed by agitating the plate for 10 seconds.

Analysis of results

The optical density was measured at 450 nm with a microtitre plate-reader (Anthos 2020 microplate-reader, Austria) between 5 and 30 minutes after addition of the stop solution. Contents were mixed well before measuring. The reference wavelength of 600 nm was used to correct any error arising from the microplate.

The mean optical densities of all duplicates were calculated after subtracting the mean blank value. The concentration of standards versus their corresponding optical densities was plotted on a log-log XY graph to obtain a standard curve (Appendix 2). The absorbance was extrapolated to Tumour M2-PK concentration using ELISA software (Stingray, Dazdaq Ltd, East Sussex, UK). Tumour M2-PK in cell lysate supernatant was expressed as milli U/10^6 cells.

4.4.2   Pyruvate Kinase Activity assay

Principle

This assay measured the activity of the enzyme pyruvate kinase on its substrate

Phosphoenolepyruvate as described before (155;156). In the presence of NADH and ADP

this results in production of lactate in the presence of LDH.

Reagents (All reagents from Sigma-Aldrich, Dorset, UK)

1. KH_2PO_4/K_2HPO_4 (20 mM), pH 7.2

2. KCl (80 mM)

3. $MgCl_2$ (7.6 mM)

4. NADH (0.63 mg)

5. ADP (1 mM)

6. LDH (3 U/ml)

7. Phosphoenolpyruvate (44 mM)

Protocol

-Negative control: Normal saline

-Positive control: Solution with known concentration of PK

-Solution 1 was prepared by mixing KH_2PO_4/K_2HPO_4 (20 mM), KCl (80 mM), $MgCl_2$

(7.6 mM), NADH (0.63 mg), ADP (1 mM) and LDH (3 U/ml) at pH 7.2

-Solution 2: Phosphoenolpyruvate (44 mM)

200 µl of solution 1 was added to 4 µl sample and reaction was started by adding

10 µl of solution 2.

Analysis of results

Activity was calculated by monitoring the absorbance fall at 340 nm at 37º C by a

photometric analyser (EPOS 5060, Eppendorf GmbH, Hamburg, Germany). Activity was

expressed in Enzyme units/x10^6. The total activity was adjusted by multiplying with the

dilution factor and cell density using the equation:

Measured activity x dilution factor

PKactivity (U/million cells) = _____

Total cell density

4.4.3   Western Blot (Semi-quantitative) measuring total M2-PK

Principle (Illustration 1)

The isoform of M2-PK will not affect the total M2-PK protein expression. This process

involves:

– The separation of sample proteins by polyacrylamide gel electrophoresis (PAGE).

– The transfer of the separated proteins from the gel onto a thin support membrane. The

membrane binds and immobilises the proteins in the same pattern as in the original gel.

– The membrane (or "blot") is then exposed to a solution containing antibodies that

recognise and bind to the specific protein of interest.

– The antibodies bound to the membrane are detected by any of a variety of techniques,

usually involving treatment with a secondary antibody.

Materials and Reagents

1. Laemlli Sample Buffer (Sigma-Aldrich, UK, Catl. No. S3401-1VL).

2. NuPAGE 4-12% Bis-Tris Gel 1.0mm x10 wells (Invitrogen, UK, Catl. No.
   NP032).

3. Western Blot Tank (XcellSure Lock, Novel Experimental Technology, UK).

4. NuPAGE MOPS SDS Running Buffer 20x (Invitrogen, UK, Catl. No. NP0001).

5. Ladder protein (SeeBlue® Plus2 Prestained, Invitrogen, UK, Catl. No. LC5925).

6. Power pack (Savant PS4000A plus).

7. PVDF membrane (0.2 µm) (Bio-Rad, UK, Catl. No. 162-0176).

8. Blotting cards.

9. Transfer Buffer.

10. Blotter (Trans-blot SD Semi dry transfer cell).

11. 100% Methanol.

12. Dried skimmed milk (Marvel).

13. Primary anti-tumour M2-PK anti-Human Mouse Monoclonal Antibody (DF-4, ScheBo®Biotech, Giessen, Germany) and Anti-Actin Rabbit polyclonal antibody (A2066, Sigma-Aldrich, Dorset, UK).

14. Secondary Anti-Mouse Goat Peroxidase conjugated and Anti-Rabbit Goat Polyclonal Antibody (Pierce Biotechnology, UK, Catl. Nos. 34075 and 31210 respectively).

15. SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, UK, Catl. No. 34075).

16. Fuji Super Rx, Fuji Photo Film (UK) Ltd.
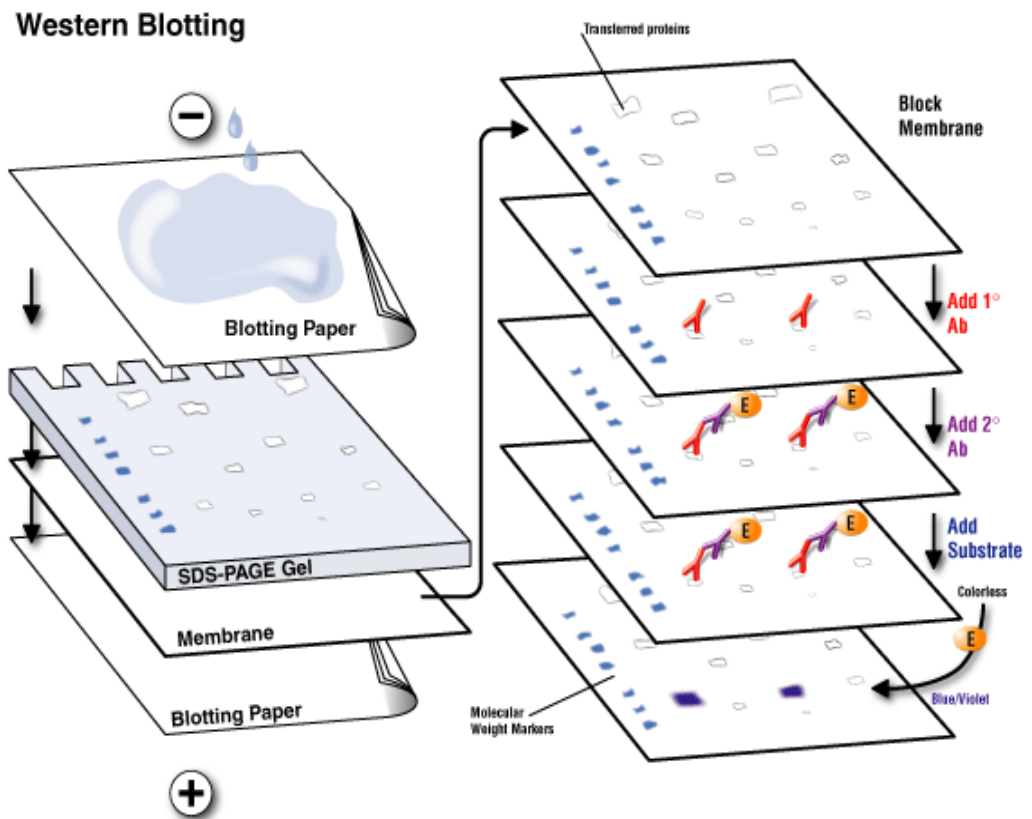
Western Blotting

Illustration 1: Principle of Western blotting

Sample preparation

Protein extraction

To prepare whole-cell extracts, cells were trypsinised from culture flasks, washed with

cold PBS and homogenised in ice-cold buffer containing 10 mM Tris/HCl, pH 7.4, 1 mM

NaF, 1 mM Mercaptoethanol and a Complete Mini EDTA-free Protease Inhibitor

Cocktail Tablet (Roche diagnostics, UK) using a Dounce tissue grinder (Wheaton Science

products, USA) with 20 strokes of pestle. The homogenised cells were then centrifuged at

40,000 g (Optima ™ TLX ultracentrifuge, Beckman Coutler™, USA) for 20 minutes at 4º

C. The sediment was discarded and supernatant was preserved.

Protein measurement (Bradford assay)

Principle: The Bradford assay is a protein determination method that involves the

binding of Coomassie Brilliant Blue G-250 dye to proteins (157). The dye exists in three

forms: cationic (red), neutral (green), and anionic (blue) (165;166). Under acidic

conditions, the dye is predominantly in the doubly protonated red cationic form (Amax =

470 nm) – (166). However, when the dye binds to protein, it is converted to a stable

unprotonated blue form (Amax = 595 nm). It is this blue protein-dye form that is detected

at 595 nm in the assay using a spectrophotometer or microplate reader.

H+   H+

Cation   ↔   Neutral form ↔   Anion

470 nm (red)   650 nm (green)   595 nm (blue)
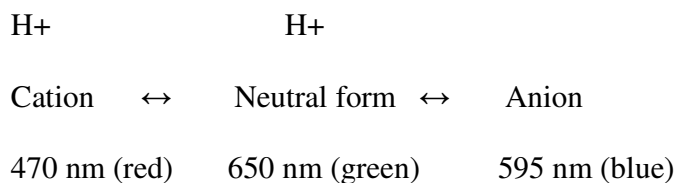
Reagents (Quick Start™ Bradford Protein Assay Kit, Bio-Rad, Hemel Hempstead, UK,

Catl. No. 500-0202)

➢ Seven concentrations of bovine serum albumin (BSA) – (2, 1.5, 1, 0.75, 0.5, 0.25,
   0.125 mg/ml) used as standards.

➢ Coomassie Brilliant Blue G-250 dye solution containing methanol and phosphoric
   acid.

➢ 96 wells micotitre plate.

➢ 8 channel pipette (250 µl).

➢ Plate-reader (595 nm).

Protocol for Bradford assay:

Standards and dye solution were brought to room temperature.

5 µl of standards and samples were pipetted into each well.

250 µl of dye solution added into the wells using 8 channel pipette and mixed gently by moving the plunger up and down a few times.

The plate is incubated at room temperature for at least 5 minutes and then absorbance is read on the microplate-reader using 595 nm filter.

A standard curve was created by plotting the 595 nm values (y-axis) versus their concentration in µg/ml (x-axis). The unknown sample concentration was determined using the standard curve. If the samples were diluted, the final concentration of the unknown samples was adjusted by multiplying the dilution factor used.

Western blot protocol

-15µl of 2x Laemlli Sample buffer was added to 15 µl of sample containing 20 µg of protein.

-This was heated for 5 minutes at 95º C, vortexed and then heated for a further 5 minutes. The mixture was centrifuged for few seconds at low speed.

-Samples were loaded along with the ladder protein and run on NuPAGE Gel @ 25 mA and 150 V for approximately 1 hour in MOPS Running Buffer.

-For each gel, 7.5x8.5 cm PVDF membrane and thick cards (x2) of the same size as membrane were cut. The membrane was rolled into Sterilin tube containing 100% methanol to avoid it getting dry.

-Wet membrane was placed in a large weigh boat containing transfer buffer enough to soak the membrane. The boat was placed on a tilting platform.

-The gel was removed from plastic covering and placed in weigh boat. The stacking gel teeth and the lower portion of the gel which was stuck to the plastic covering were removed. The gel was covered with transfer buffer and placed on a tilting platform.

-Thick blotting cards were soaked in transfer buffer and placed on the blotter.

-Membrane was then layered onto card, followed by gel and second piece of card making sure no air bubble was trapped between the gel and membrane and cards by rolling falcon tube across surface.

-Blotter was gently sealed and set to run at 25 V and 75 mA for 45 minutes. Success of transfer was ensured by observing transfer of ladder from gel onto membrane.

-Membrane was washed briefly with bi-distilled water and blocked with 5% Marvel in PBS for 30 minutes.

-Membrane was washed briefly with PBS and incubated overnight with Primary Antibody (1:2000) to M2-PK and Actin in 5% Marvel PBS at 4º C on a tilting platform.

-Membrane was washed thrice for 5 minutes and incubated in Secondary Antibodies (1:1000) in 5% Marvel PBS for 1 hour at room temperature.

-Membrane was washed for further 5 minutes thrice and incubated in SuperSignal West Dura Extended Substrate (1:1 mixture of Luminol and peroxidase substrate) for 5 minutes at room temperature. Excess of mixture was drained and membrane was covered in plastic film making sure no air bubbles were trapped.

-The membrane was then exposed to X-ray film for different periods to get the best signal band.

Analysis of results

The size of the protein band was identified by comparing the band position with reference to SeeBlue® Plus2 pre-stained standards (Invitrogen, Paisley, UK) run along with samples during electrophoresis. The M2-PK band corresponds to 58 Kda as described before (167). Actin was used as a loading control protein.

Density of each band was measured by densitometry in Gel Doc 2000 (BioRad, Milan, Italy) using a computer software Quantity one® (BioRad, California, USA).

Density of the M2-PK was standardised against the Actin band in the form of M2-PK: Actin ratio.

4.5   Apoptosis measurement

4.5.1   Caspase 3/7 and 8 activity assay

Principle

The Caspase-Glo® Assay (Promega, Southampton, UK) is a homogeneous luminescent assay that measures caspase activity. The assay provides a proluminogenic caspase substrate in a buffer system optimised for caspase activity, luciferase activity and cell lysis. The addition of a single Caspase-Glo® Reagent in an "add-mix-read" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal. The signal generated is proportional to the amount of caspase activity present. The Caspase-Glo® Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which generates the stable "glow-type" luminescent signal and improves performance across a wide range of assay conditions.

Materials and Reagents

-Caspase-Glo® 3/7 or 8 Buffer (Promega, Southampton, UK)

-Caspase-Glo® 3/7 or 8 Substrate (lyophilised – Promega, Southampton, UK)

-White-walled multiwell plates adequate for cell culture and compatible with the luminometer being used (Labsystems Cliniplate, Fisher Scientifc, Loughborough, UK)

-Multichannel pipet

-Plate shaker, for mixing multiwell plates

-Luminometer (Fluoroskan Ascent FL, ThermoLabsystem, Finland) capable of reading multiwell plates

Protocol

-13,000 Colo 357 and Panc-1 cells from culture platted in duplicate in each well of 96-well white-walled plates.

-Cells were allowed to incubate under normal, acidic pH, glucose deprived or hypoxic medium (100 μl/well) for 24 hrs, 48 hrs, 72 hrs and 96 hrs.

-Caspase-Glo® 3/7 or 8 buffer and lyophilised Caspase-Glo® 3/7 or 8 substrate was allowed to equilibrate to room temperature before use.

-The contents of the Caspase-Glo® 3/7 or 8 buffer bottle were transferred into the amber bottle containing Caspase-Glo® 3/7 or 8 substrate. The contents were mixed by swirling or inverting until the substrate was thoroughly dissolved to form the Caspase-Glo® 3/7 or 8 reagent.

-The 96-well plate containing cells was removed from the incubator and allowed to equilibrate to room temperature.

-100 µl of Caspase-Glo® 3/7 or 8 reagent was added to each well of a white-walled 96-well plate containing 100 µl of blank, negative control cells or treated cells in culture medium.

-After incubating the mixture for 2 hrs at room temperature, the luminescence of each sample was measured in a plate-reading luminometer.

Precautions:

-Because of the sensitivity of this assay, care was taken not to touch pipette tips to the wells containing samples to avoid cross-contamination.

-Plate was covered with a plate sealer or lid.

-Contents of wells were mixed gently using a plate-shaker at 300 – 500 rpm for 30 seconds.

-Room temperature fluctuation was taken into account to avoid its interference in the luminescence reading.

4.5.2 Bax and Bcl-2 measurement

Bax and Bcl-2 protein expression was measured by immunoblotting (as described in section 5.4.3) using mouse monoclonal antibody (1:200 dilution) and rabbit polyclonal antibody (1:200 dilution) respectively (Santa Cruz laboratories, CA, USA). This method was used to measure apoptosis because at the beginning of the experiment it was not known whether the apoptosis to altered microenvironment would be caspase or Bcl-2 protein dependent as different types of apoptotic pathways in pancreatic cancer have been described in the literature (168). Bax is an apoptotic protein while Bcl-2 is an anti-apoptotic protein regulating mitochondrial permeabilisation (168) . Their effect on mitochondrial permeability pores is caspase independent(168) .

4.5.3 Annexin V-FITC and Propium Iodide staining

Principle

In normal viable cells phosphatidyl serine (PS) is located on the cytoplasmic surface of the cell membrane. Upon induction of apoptosis, rapid alterations in the organisation of phospholipids in most cell types occurs leading to exposure of PS on the cell surface. Recognition of PS by phagocytes *in vivo* results in the removal of cells programmed to die, thus apoptosis is not commonly associated with the local inflammatory response which accompanies necrosis. *In vitro* detection of externalised PS can be achieved

through interaction with the anticoagulant Annexin V. In the presence of calcium, rapid high affinity binding of Annexin V to PS occurs. PS translocation to the cell surface precedes nuclear breakdown, DNA fragmentation, and the appearance of most apoptosis-associated molecules making Annexin V binding a marker of early-stage apoptosis. In this assay a fluorescein isothiocyanate (FITC) conjugate of Annexin V is used allowing detection of apoptosis by flow cytometry or by fluorescence microscopy. Since membrane permeabilisation is observed in necrosis, necrotic cells will also bind Annexin V-FITC. Propidium iodide is used to distinguish between viable, early apoptotic, and necrotic or late apoptotic cells. Necrotic cells will bind Annexin V-FITC and stain with propidium iodide while propidium iodide will be excluded from viable (FITC negative) and early apoptotic (FITC positive) cells. In the absence of phagocytosis final stages of apoptosis involve necrotic-like disintegration of the total cell; thus cells in late apoptosis will be labelled with both FITC and propidium iodide.

A RAPID protocol kit (AnnexinV-FITC kit, PF032, Calbiochem, UK) has been used for Annexin V-FITC binding directly in tissue culture media.

This obviates the need for tedious centrifugation and wash steps which increase the occurrence of mechanical membrane disruption. In addition, since apoptosis is a dynamic process that is ongoing once cells are removed from culture conditions and continues throughout experimental processing, the RAPID protocol was used for the detection of cells in early apoptosis.

Material and reagents

-Annexin V-FITC: 200 μg/ml recombinant Annexin V conjugated to fluorescein

isothiocyanate (FITC)

-5X Binding Buffer:

-Media Binding Reagent: a reagent designed to enhance binding of Annexin V to PS in

tissue culture media

-Propium Iodide: 30μg/ml

-PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4-7H2O, 1.4 mM KH2PO4, adjust

pH to 7.4)

-2 – 20 μl, 20 – 200 μl and 200 – 1000 μl precision pipetters with disposable tips

-Microcentrifuge tubes

-Adjustable speed microcentrifuge (Eppendorf, Cambridge, UK)

-Flow cytometer (Beckman Coulter EPICS® XL-MCL, High Wycombe,

Buckinghamshire, UK).

Protocol

Media from flask of adherent cells was transferred to a 25 ml universal container and

placed on ice and later on mixed to the trypsin detached cells.

Note: This media contained cells that have become detached from the flask during the cell

death process.

Cells in flask were gently washed with 10 ml PBS. PBS removed after 3 washes. Added 1

– 2 ml 0.5X trypsin in flask and incubated just until cells appear detached by microscopic

evaluation. Cells were released from flask with firm tapping and gently resuspended in

media to approximately 1 x 10^6 cells/ml. Transferred 0.5 ml of cell suspension to a

microfuge tube. To this added 10 μl of Media Binding Reagent. Subsequently added 1.25 μl Annexin V-FITC and incubated for 15 mins at room temperature (18–24º C) in the dark. This was centrifuged at 1000 x g for 5 mins at room temperature. Media in the supernatant discarded. Cells gently resuspended in 0.5 ml cold 1X Binding Buffer. Added 10 μl Propidium Iodide. Samples placed on ice and away from light till analysed by flow cytometry immediately (within 1 hour).

 Flowcytometry:

A flow cytometer equipped with 488 Argon laser was used which detects FITC signal at wavelength 518 nm and PI signal at wavelength 620 nm. Annexin V analysis was visualised using forward- and side-scatter detectors set to analyse whole-cell population. The whole cells were gated and then plotted as log annexin-V-FITC intensity versus log PI intensity. Untreated cells, with binding buffer only, PI only, and Annexin V only, used as negative controls while 1 mM Stausporine treated (1:200 dilution with media) cells, with binding buffer only, PI only, and Annexin V only, used as positive controls were run to set appropriate detector gains, compensation, and quadrant gates. Five thousand cells were analysed for each sample. Quadrant analysis of the sorted cells was performed on computer software (Coulter System II software™ Version 3.0). The test samples were evaluated by the following categories: cells negative for both PI and Annexin V staining were live cells; PI-negative, Annexin V–positive staining cells were early apoptotic cells; PI-positive Annexin V–positive staining cells were primarily cells in late stages of apoptosis where membrane integrity was lost while PI-positive, Annexin V–negative were considered necrotic cells.

4.6 Immunofluoroscence for localisation of Tumour M2-PK

Materials and Reagents

-Superfrost glass slides (manufacturer)

-Glass cover slip 22 mm (VWR, UK, Catl. no. 631-0159)

-Poly-L-lysine 5 mg (Sigma-Aldrich, UK, Catl. no. P6282)

-0.1 M Sodium borate

-Paraformaldehyde

-100% Methanol

-Normal goat serum (Sigma-Aldrich, UK, Catl. no. G9023)

-Primary antibody – anti-tumour M2-PK anti-human mouse monoclonal antibody (DF-4, ScheBo®Biotech, Giessen, Germany).

-Primary antibody – Complex IV subunit I mouse monoclonal antibody (COX-1) (Mitosciences, Oregon, USA)

-Secondary goat anti-mouse IgG Alexa Flour 488 (green) (Molecular probes, Invitrogen, Paisley, UK, Catl. no. A21131) for anti-COX-1 primary antibody and Alexa Flour 594 (red) for anti-tumour M2-PK primary antibody (Molecular probes, Invitrogen, Paisley, UK, Catl. no. A21125).

-Citiflour/PBS/Glycerol (Agar Scientific, Essex, UK, Catl. no. R1320)

-DAPI (4',6-Diamidino-2-phenyindole, dilactate) – (Sigma-Aldrich, Dorset, UK, Catl. no. D9564)

Protocol:

Poly-lysine coating of coverslips

5 mg poly-L-lysine resuspended to a 1mg/ml solution using 5ml of 0.1 M sodium borate buffer, pH 8.11. The solution was vortexed and left for few minutes to fully resuspend and filter sterilised (0.2 µm). This was dispensed into 12 x 400 µl aliquots (autoclaved 1.5ml eppendorfs) and frozen at -20º C until use. For coating a 22 mm cover slip, 200 µl poly-lysine was used. (N.B. Poly-lysine gets precipitated upon thawing. It should, therefore, be warmed and vortexed well before use). Heat-sterilised glass coverslips were placed in the centre of the wells of 6-well plate using heat-sterilised forceps and 200 µl of poly-lysine was dispensed onto each coverslip (lots of small drops of poly-lysine were placed around the edge of coverslip to make it easier to cover the whole surface). Coverslips covered with poly-lysine were left for 1 hour and then thrice washed with 2 ml sterile ddH$_2$O. The coverslips were allowed to dry onto 24-well plate for approximately 20 minutes and then replaced into wells of a 6-well plate.

Seeding of cells

500 µl of cell (Panc-1 and Colo 357) suspension with appropriate cell density (pilot experiments were carried out to determine the optimum cell density that would give around 50% cell confluence on the coverslip) was placed on each coverslip. Thus for experiment involving 72 hrs exposure of cells to different condition, seeding density of 7,500 cells per coverslip was used. Cells were allowed to adhere to the sterile coverslips coated with poly-lysine for 2 hrs. Once the adherence of cells to coverslip was confirmed under light microscopy, 500 µl of cell suspension on each coverslip was topped up with 2

ml of desired culture medium for the normal, acidic pH, glucose deprived or hypoxic culture conditions for 72 hrs.

Preparation of 4% paraformaldehyde

2 g paraformaldehyde was carefully added to 40 ml of ddH_2O in a 100 ml beaker and stirred for 15 mins whilst heating it to approximately 60º C. To this added 8–12 drops of 0.1 M NaOH until the paraformaldehyde was dissolved. The final volume was made up to 50 ml by adding 5 ml of x10 PBS. This was mixed thoroughly and the pH was adjusted to be <8.0. The mixture was filtered through a filter paper into a screwtop with the aid of a funnel. The filtered solution was stored for up to 1–2 weeks at 4º C and brought to 37º C just before use.

Immunostaining for fluorescence microscopy

Washing of coverslips: At the end of the experiment cultured medium from each well of 6-well plate was aspirated and the coverslips were washed thrice in beakers containing 25 ml PBS (not supplemented with $MgCl_2$ and Ca Cl_2). Coverslips were drained between washes on tissue paper.

Fixation: Coverslips were fixed in fresh pre-warmed 4% paraformaldehyde in PBS for 20 mins and subsequently washed thrice with 25 ml PBS and drained between washes on tissue paper. Before permeabilisation transfer the coverslips in a rack to a container with PBS so as to avoid drying.

Permeabilisation: The coverslip rack was transferred to container with 100% methanol at -20º C for 15 mins. Subsequently the rack with coverslips was replaced quickly in container with PBS to make sure that the cells do not dry out (methanol evaporates quickly).

Blocking with 10% normal goat serum: Coverslips were washed thrice in 25 ml PBS, drained between washes on tissue paper and blocked with 300 µl of 10% normal goat serum in PBS for 30 mins at 37° C in a humidified atmosphere.

Incubation with primary antibody: At the end of blocking, each coverslip was drained and incubated with 80 µl of diluted primary antibody in PBS and 2% normal goat serum for 45 mins at 37° C in a humidified atmosphere. The appropriate dilution for each primary antibody was determined by initial piloting experiments (1:200 for tumour M2-PK and 1:75 for COX-1)

Incubation with secondary antibody: The coverslips were washed thrice in a beaker with 25 ml of PBS, drained between washes on tissue paper and incubated with 80 µl of diluted (1:100) secondary antibodies (goat anti-mouse IgG Alexa Flour 488 (green) for anti-COX-1 primary antibody and Alexa Flour 594 (red) for anti-tumour M2-PK primary antibody) in PBS and 2% normal goat serum for 45 mins at 37° C in a humidified atmosphere. Coverslips were finally washed in 25 ml PBS thrice, drained between washes on tissue paper and placed in rack in a container with PBS.

Mounting coverslips: coverslips were drained thoroughly on tissue paper and mounted on superfrost glass slide in Citiflour/glycerol/PBS mounting media supplemented with DAPI. Any extra mounting media was blotted off the coverslip on tissue paper before sealing the edge with varnish. The mounted slides were stored at 4° C in the dark until examination under fluorescence microscope. Confocal microscope (Zeiss LSM 510 Meta system, Welwyn Garden City, UK) was used to see co-localisation of M2-PK with mitochondria or nucleus.

4.7   Statistical analysis

The mean inter-assay and mean intra-assay coefficients of variation (CV) were calculated from nine separate measurements of 6 samples on 9 different days to measure the reproducibility of the test results. All values were expressed in Mean ±S.D unless stated separately. Tumour M2-PK and total M2-PK values from independently performed experiments at 24, 48, 72 and 96 hrs were combined to calculate the adjusted mean in accordance with Analysis of Serial measurement (169).   Mean values of Tumour M2-PK between different culture conditions were compared by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test.

For comparison of the different cultivation conditions one-way analysis of covariance with cell density as covariable was performed using the statistical program package BMDPV1 (W. J. Dixon, 1992, UCLA, Los Angles, California). Since the distribution of the data was skewed to the right, a logarithmic transformation of the data was performed and the results are presented as geometric mean and dispersion factor ($\overline{x}_g \bullet DF^{\pm1}$). This is the delogarithmic form of the arithmetic mean and deviation of the previously logarithmically transformed data.

Pearson correlation coefficient was used for correlation between cell density and Tumour M2-PK in different tumour microenvironment.

5 CHAPTER 5 Measurement of Tumour M2-PK in Tissue Culture and Comparison of Levels in Two Phenotypically Different Human Pancreatic Cancer Cell Lines

5.1 Introduction

All proliferating cells and especially tumour cells are characterised by the expression of the pyruvate kinase isoenzyme type M2 which may occur in a highly active tetrameric form and a nearly inactive dimeric form (20;170). In contrast to normal proliferating cells, tumour cells have mainly the inactive dimeric form Tumour M2-PK. The dimerisation of M2-PK is caused by direct interaction of M2-PK with different oncoproteins (26;29;171). The dimeric form of M2-PK is released from tumours into the blood and stool of most GI cancer patients (20;77;78;89;172) and the quantification of Tumour M2-PK in EDTA plasma and stool is used for the early detection of tumours and in evaluating the response to cancer therapies (77;78;89).

Clinical studies have shown marked variability in the Tumour M2-PK levels between individuals with the same cancer type (172) and a greater understanding of its expression and control is required. Recently, it has been shown that low levels of Tumour M2-PK is associated with cisplatin resistance in gastric cancer (173) while higher levels are associated with P53 induced apoptosis (174). A tissue culture model allows the study of Tumour M2-PK control mechanisms in a controlled environment. Two dimensional gel electrophoresis (2-DE) – (173) and Matrix-associated laser desorption ionisation-mass spectroscopy (MALDI-MS) were the methods used (174) in previous studies to identify M2-PK protein in cancer cell lines. Gel permeation allows the quantification of the tetramer : dimer ratio (4). ELISA is a reliable assay for measuring Tumour M2-PK in blood and stool (63;90) but its use in tissue culture homogenate has not been reported. Measuring M2-PK in cancer cells could help define a role in cancer monitoring and could

lead to therapeutic modalities. By the time pancreatic cancer is diagnosed it is often unresectable (133). Many genomic and proteomic based markers have been identified in pancreatic cancer (149) but targeting these proteins or genes for treatment has always been challenging. Tumour M2-PK is elevated in plasma of patients with pancreatic cancer (56;86;175). There are no previous reports of measuring Tumour M2-PK in pancreatic cancer cell lines. The aim of this study is to measure variability of Tumour M2-PK levels between two different pancreatic cancer cell lines and to correlate Tumour M2-PK levels with cell proliferation.

5.2   Materials and Methods

5.2.1   Cell culture:

Cell line Panc-1 was obtained from the European collection of cell culture (ECCAC) while Colo 357 was gifted by Dr N. R. Lemoine, Centre for Molecular Oncology and Imaging, Institute of Cancer, Barts and The London School of Medicine and Dentistry, University of London, London, UK. These cell lines were chosen because of difference in their biologic phenotype as mentioned in the cell lines description in Chapter 5 (section 5.1). The HMEC-1 (human microvascular endothelial cells) cell line was used as a non-cancer cell control while the MCF-7 (human breast cancer) cell line was used as positive control since M2-PK was previously identified in this cell line by Gel permeation. HMEC 1, Panc-1 and Colo 357 cell lines were cultured in DMEM (Dulbeco's Modified Eagle's Medium, Gibco, UK) while MCF 7 was maintained in EMEM (Earl's Minimum Essential Medium, Gibco, UK) supplemented with Penicillin/Streptomycin solution (1%), 2 mM glutamine and 10% foetal bovine serum (FBS). Cell culture was maintained at 21% O_2,

5% CO_2 and at 37 ˚C till 70-80% confluent. Two million cells were plated on 75 cm^2

flask and cultured for 24 hrs, 48 hrs, 72 hrs and 96 hrs without changing the medium.

Adherent cells were removed from the flask using 0.25% Trypsin EDTA (Gibco, UK).

5.2.2   Measurement of cell proliferation

Suspended cells in culture medium were counted under an inverted phase contrast

microscope (Nikon TMS-F, Japan) in a haemocytometer (Bright Line™ Haemocytometer

Z359629, Sigma, UK). The cells were counted in four different fields of the chamber and

the count was averaged to give overall cell count per cubic mm. After counting cells were

washed with cold PBS three times, pelleted by centrifuging at 800 rpm. Cell pellets were

stored at -80 ˚C for Tumour M2-PK assays. The number of cell doubling after 96 hrs

culture was calculated by the equation as described previously (176;177).

Number of cell doublings = (Log (F)-Log (I))/Log2, where F is final density at a

particular time in cell culture and 'I' indicates initial seeding density.

5.2.3   Cell homogenisation for M2-PK measurement

The frozen cell pellet was thawed, resuspended in 1 ml of cold homogenisation buffer

(10mM Tris, 1 mM NaF and 1Mm Mercaptoethanol, pH 7.4, Sigma-Aldrich) and

homogenised using a glass pestle homogeniser (Wheaton Science products, New Jersey,

USA). The homogenised cells were then centrifuged at 40,000 g for

20 minutes and the cell homogenate was used for the total M2-PK protein expression by

Western blot and the Tumour M2-PK by ELISA.

5.2.4 Total M2-PK protein by immunoblotting

This method was used in the beginning to see whether M2-PK protein is expressed in the cell lines. 20 µg of sample protein was separated on 10% SDS polyacramide gel. For details see Chapter 4, section 4.4.3.

5.2.5 Measurement of Tumour M2-PK

Tumour M2-PK was measured using an ELISA kit commercially available for Tumour M2-PK measurement in plasma (ScheBo® Biotech AG, Giessen, Germany). For details see Chapter 4, section 4.4.1.

5.2.6 Statistical analysis

The mean inter- and intra-assay coefficients of variation (CV) were calculated from 9 separate measurements of 6 samples on 9 different days. All values were expressed as Mean ±S.E unless stated separately. The results were analysed as described in Chapter 4 section 4.7. Correlation between cell density and Tumour M2-PK concentration was evaluated using the Pearson correlation coefficient.

5.3 Results:

5.3.1 Cell Proliferation of different cell lines

The proliferation rate with time as measured by direct cell count for the different cell lines is shown in Figure 5.1. The non-tumour cell line HMEC-1 remained viable but did not expand in culture over the 96 hr period. The Colo 357 pancreatic cancer cell line grew more rapidly than Panc-1 ($p<0.001$) which was similar in growth rate to the MCF-7 breast cancer cell line.
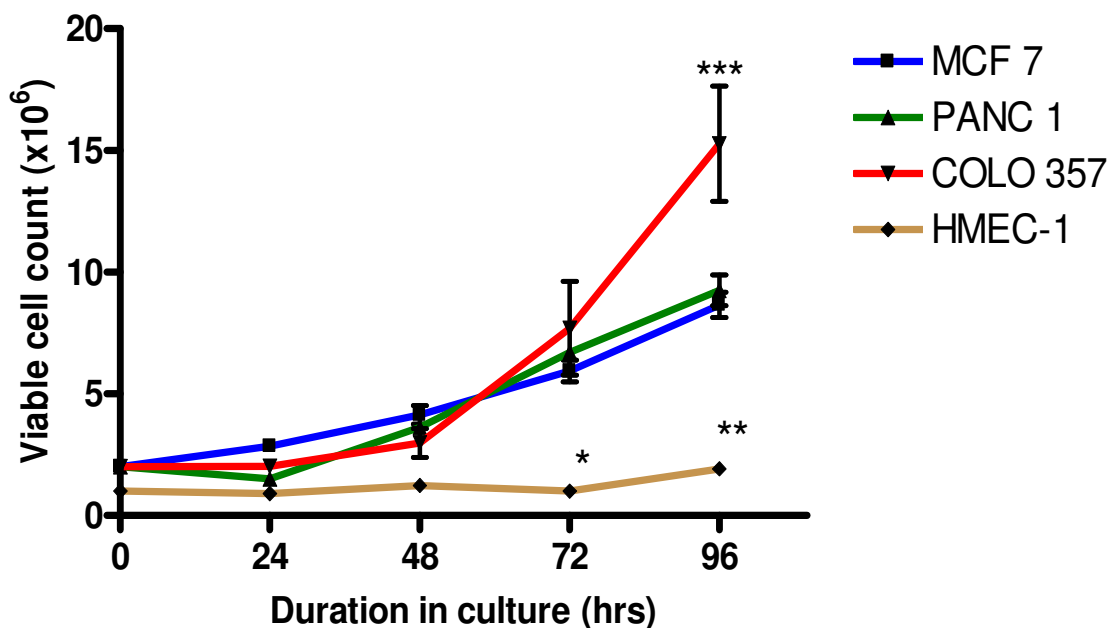
Figure 5.1: Cell proliferation of different cell lines. With the seeding density of 2 million cell, culture was maintained for 96 hrs without change of culture media in a 75 mm^2 flask. Viability check on adherent and suspended cells in culture was done by trypan blue exclusion and the total viable cells were counted under phase contrast microscope using haemocytometer. At 96 hrs, the number of cell doublings for HMEC-1, MCF-7, Panc-1 and Colo 357 cell lines were 0.8, 2.1, 2.2 and 2.9 respectively. A significant difference was seen between the proliferation rate for different cell lines at 72 and 96 hrs of culture duration.

5.3.2 Tumour M2-PK in cell culture medium.

Figure 5.2 shows the pilot data from Tumour M2-PK measurement in cell culture

supernatant. As the data was non-standardisable and levels were low, no further attempt

was made to study Tumour M2-PK levels in cell culture medium.
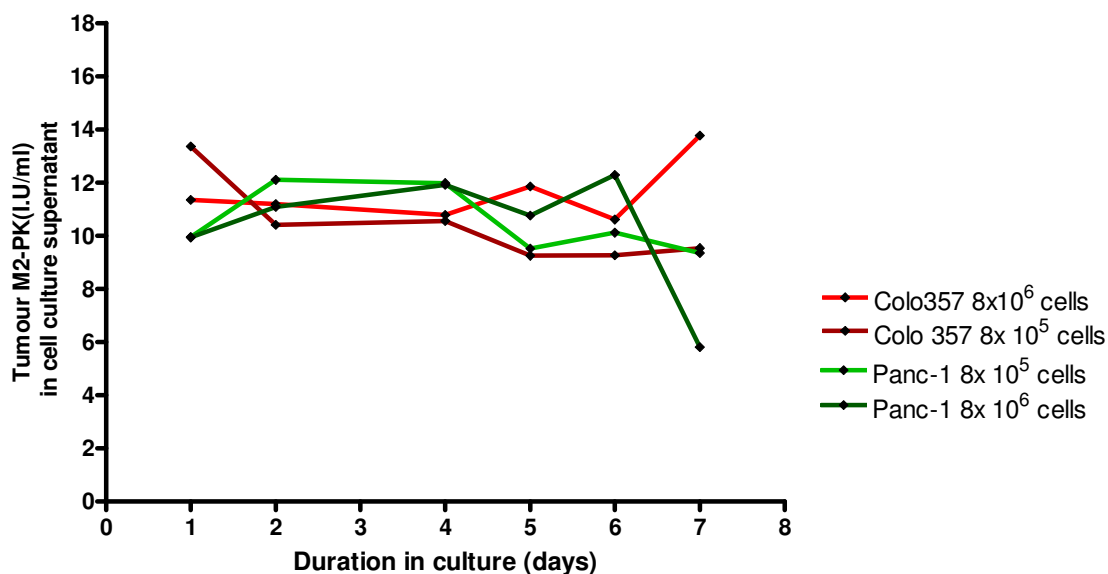
Figure 5.2: Tumour M2-PK Levels in cell culture supernatant (DMEM with Pyruvate) in Colo 357 and Panc-1 cell lines. Legends indicate the cell seeding density in 75cm^2 flask. Results from single experiment only.

Effect of pyruvate in culture medium on Tumour M2-PK

Since the study involved measuring M2-pyruvate kinase in tissue culture, it was

important to rule out the influence of pyruvate, used in most of the culture medium, on

M2-pyruvate kinase levels. Therefore, initial experiments used pyruvate-free or pyruvate-

containing culture medium. No difference in cell proliferation and Tumour M2-PK level

was observed (Figure 5.3). Since pyruvate is an essential component of culture medium,

all subsequent experiments were done in DMEM with pyruvate.
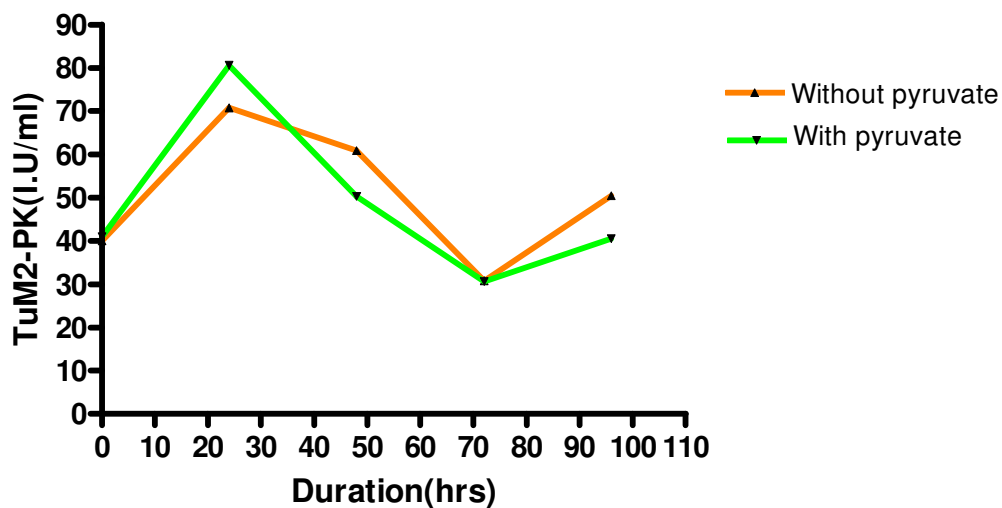
a)
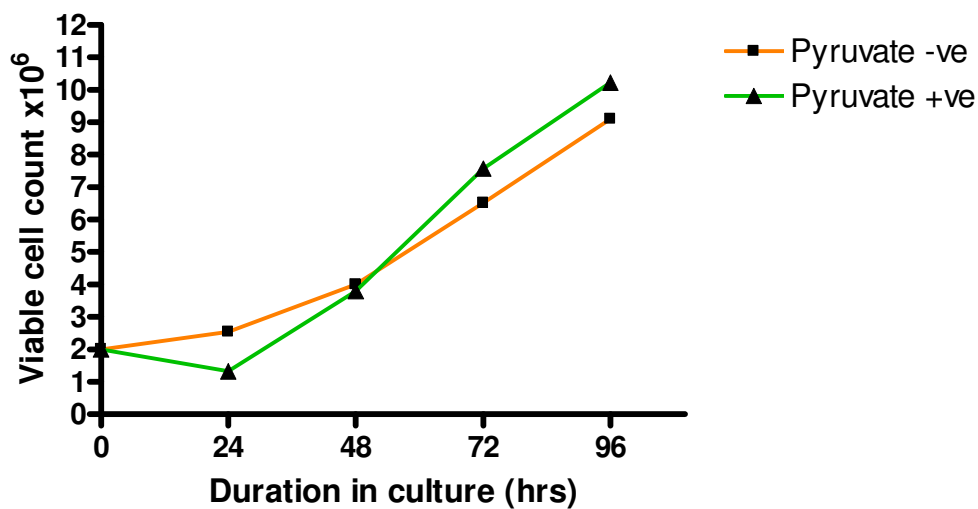
(b)

Figure-5.3: (a) Tumour M2-PK levels in cell homogenate as measured by ELISA and (b) Cell proliferation in Panc-1 cells in DMEM with pyruvate and without pyruvate. Seeding density $2x10^6$ cells /flask. No difference in Tumour M2-PK levels and cell proliferation was seen in pyruvate or pyruvate free medium [Results from single experiment only].

Effect of centrifugation speed on Tumour M2-PK levels

Pilot experiments were carried out to determine the optimum centrifugation speed to

obtain maximum yield of Tumour M2-PK. Figure 5.4 shows that ultracentrifugation at

40,000 g for 20 minutes with homogenisation buffer gave better yield of Tumour M2-PK

as compared to 16,000 g for 10 minutes with PBS.
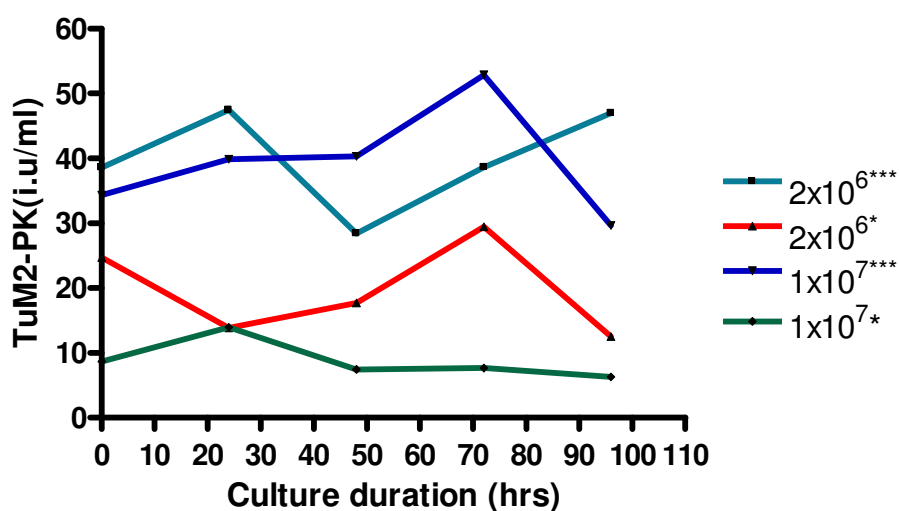
Figure 5.4 Tumour M2-PK levels in Panc-1 cell line. [*] Cell homogenate centrifuged at 4º C at 16000 g x 10 min with PBS and [***] Cell homogenate centrifuged at 4º C at 16,000 g or 40,000 g x 20 min with homogenisation buffer. These are the results from a single experiment.

5.3.3 Total M2-PK in different cell lines

Immunoblotting was used to semiquantitatively measure total M2-PK protein in the cell homogenate. DF4, a mouse anti-human monoclonal antibody specific against M2-PK was used. This antibody is not isoform specific and hence detects only total M2-PK protein. This method was used to see whether all the cell lines express M2-PK before Tumour M2-PK is measured.

The cell lysis buffer that is conventionally used in western blot is RIPA buffer (RadioImmuno Precipitation Assay Buffer). A comparison of band density of M2-PK from lysate obtained by using either RIPA buffer or homogenisation buffer (Tris, NaF, mercaptoethanol based buffer) is shown in Figure 5.5. M2-PK protein band was much thicker in homogenisation buffer than in RIPA buffer. Therefore, the former was used in all the experiments for M2-PK measurements by immunoblotting. M2-PK band was also best seen when the transferred protein on PVDF membrane was incubated overnight with primary antibody (DF4) at 4º C on a tilted platform.

 Total M2-PK protein expression using western blot at 48 hrs of culture duration was significantly higher in the Colo 357 than the Panc-1 or the control cell lines (Figure 5.6).
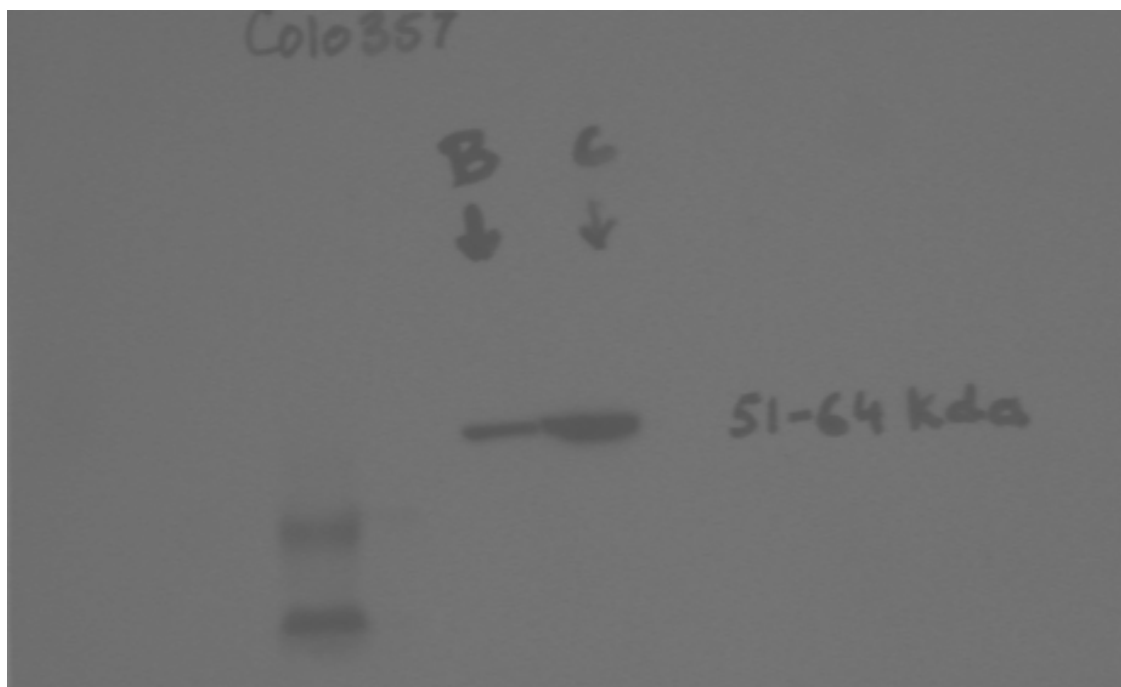
Figure 5.5 Immunoblotting of M2-PK protein in different buffers
M2-PK band was 58 KDa in size. (B) band denotes cell lysed in RIPA buffer and (C) band denotes cell homogenate in Tris, NaF and mercaptoethanol based buffer. Same amount of protein (25 µg) from 48 hrs cell culture homogenate sample was loaded into each well.

5.3.4   Levels of Tumour M2-PK in different cell lines

The mean inter- and intra-assay coefficient of variance (CV) for Tumour M2-PK as measured by ELISA in 6 different samples on 9 different days was 20.8% (11.9–30.15%). and 4.3% (0.18–16.6%) respectively. Tumour M2-PK levels in HMEC-1, MCF 7, Colo 357 and Panc-1 cell culture homogenate are shown in Figure 5.7.

Colo 357 cells showed significantly higher level of Tumour M2-PK compared to HMEC-1 (P<0.001), Panc-1 cells (p<0.001) and MCF-7 cells (p<0.01). Levels in Panc-1 cell

lines were higher than the non-cancer cell line HMEC-1 while lower than MCF-7 cells but not statistically significant.
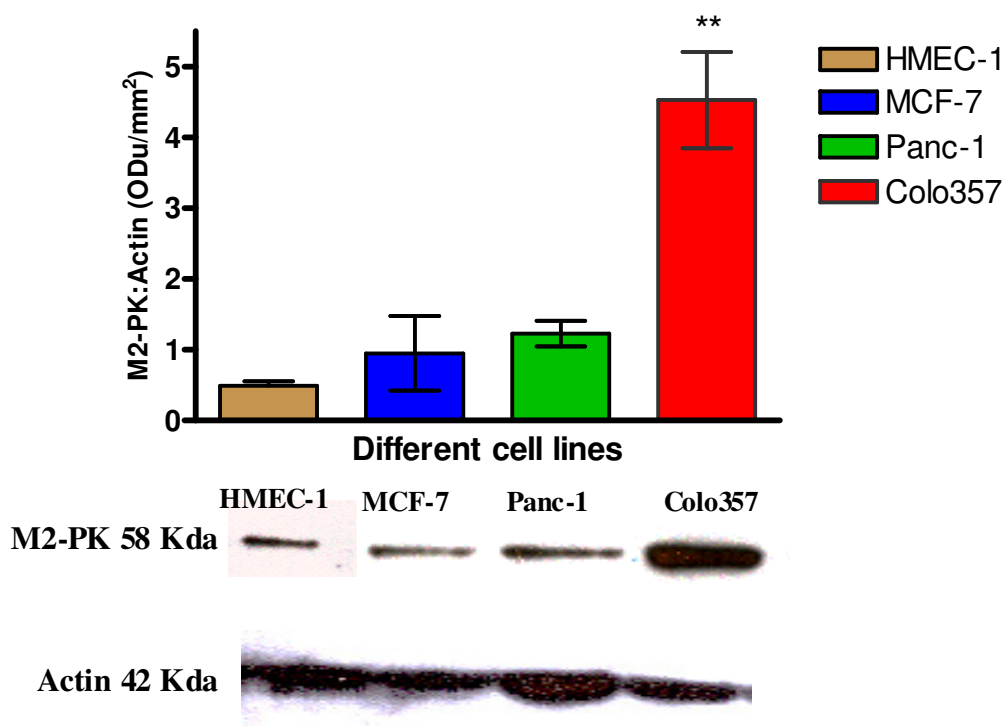
Figure-5.6: M2-pyruvate kinase (M2-PK) protein expression in different cell lines at 48 hrs of culture by western blot. M2-PK level was significantly elevated in Colo 357 cells (** $p<0.01$) compared to HMEC-1, MCF-7 and Panc-1 cells. Actin was used as loading control protein.

5.3.5   Tumour M2-PK and cell density

The correlation between Tumour M2-PK level and cell density was assessed using Pearson's correlation coefficient. This demonstrated that Tumour M2-PK decreased with increase in cell density for the both the pancreatic cancer cell lines:

(Panc-1: $r=-0.382$, $p=0.06$ and Colo 357: $r= -0.528$, $p<0.05$) – (Figure 5.8).
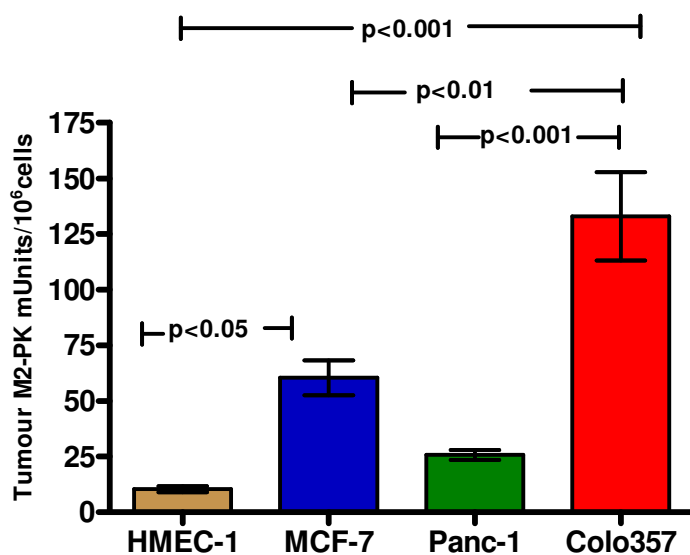
Figure 5.7: Comparison of Tumour M2-PK levels in two different pancreatic cancer cell lines Colo 357 and Panc-1. MCF 7 (Human epithelial breast cancer cell) was used as the positive control) and HMEC-1(Human microvascular endothelial cells) as the non-cancer control. Initial cell seeding density was 2 million cells per flask. The results shown above are from five independently performed experiments.
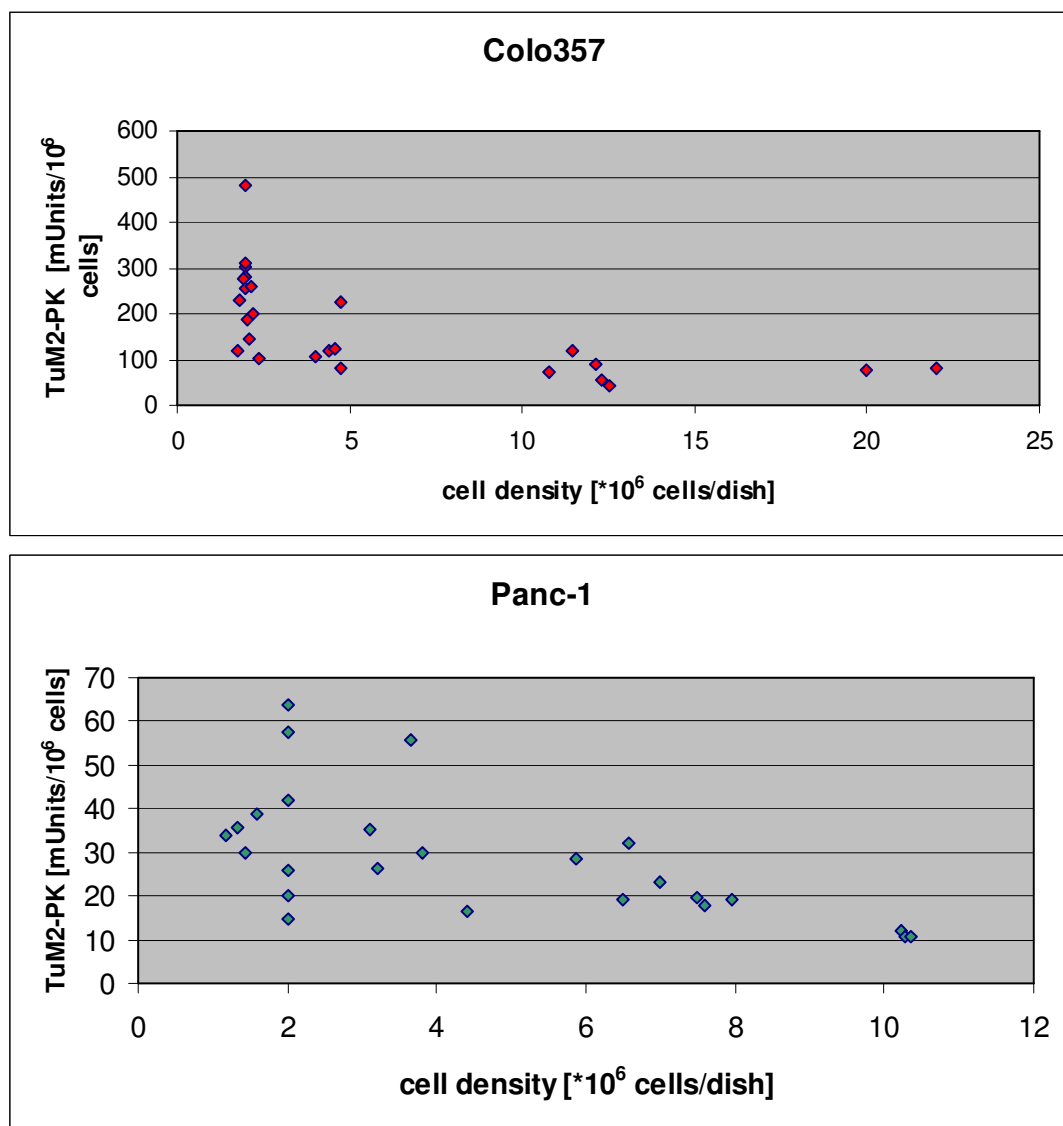
Figure 5.8: Correlation between Tumour M2-PK and cell density in pancreatic cancer cell lines. Tumour M2-PK levels were plotted against their corresponding viable cell count when the Panc-1 and Colo 357 cells with the seeding densities of 2×10^6 were cultured for 24, 48, 72 and 96 hrs without substrate replenishment [Results of 5 independent experiments].

5.3.6   Discussion

This is the first study on Tumour M2-PK in pancreatic cancer cell lines. In the absence of any previous data, it was important to study the variability of Tumour M2-PK levels with in-vitro factors including presence or absence of pyruvate in culture medium, type of buffer and centrifugation speed during cell homogenisation. Tumour M2-PK measurement in cell culture medium was attempted in pilot experiments but abandoned due to very low levels and technical difficulty in standardisation of results. Therefore, Tumour M2-PK was measured in cell homogenate and not in cell culture supernatant. It is known that Tumour M2-PK is released into the circulation *in vivo* in large amounts because it can be measured clinically in the serum of cancer patients by ELISA (63;114;172). However, we have found low levels of Tumour M2-PK in the culture medium of the cancer cell lines. Possible explanations could be the dilution effect of using large amount of culture medium as the cell culture was maintained in 75 cm^2 flask, Tumour M2-PK is released into the culture medium only when the cells lyse or die, Tumour M2-PK is produced in large quantity or certain co-factors maximize its expression *in vivo* rather than *in vitro*.

Pyruvate is an important component of culture medium. It is a key product of phosphoenolepyruvate (PEP) breakdown by the enzyme pyruvate kinase (20). We assessed the influence of pyruvate on Tumour M2-PK levels by using pyruvate-free culture medium. Our results showed no influence of the presence of pyruvate in culture medium on Tumour M2-PK levels. Previous studies have shown that it is the metabolites above PEP mainly 1,6 fructose biphosphate which regulates Tumour M2-PK (163;178).

Higher levels of 1,6 fructose biphosphate decrease Tumour M2-PK activity while lower levels increase the activity (20;24) .

Metabolic parameters such as intracellular metabolites, flux rates and enzyme activities vary with cell density (163). Therefore this study investigated the effect of cell density on Tumour M2-PK concentration. This study has observed an inverse relationship between cell density and Tumour M2-PK levels. This type of inverse correlation of Tumour M2-PK levels related to cell density has been reported for several other cell lines (163). The possible clinical implication of this finding would suggest that there will not be a linear correlation between tumour size and Tumour M2-PK in clinical practice. This is also supported in one of our clinical studies (179) and previous such studies on Tumour M2-PK (73).

However, results of *in vitro* conditions should be interpreted with caution in view of inherent limitation factors in this study. Firstly, monolayer cultures are optimally supplied with nutrients and oxygen with reduced cell-cell contacts and grow on an artificial surface. These conditions are highly artificial and may affect both the cell differentiation and the proliferation rate. Secondly, examining only two cell lines does not allow generalisation about pancreatic cancer cells as these cell lines may not necessarily be representative. Thirdly, there may be certain *in vivo* factors affecting tumour cell proliferation and metabolic activity which is independently controlled by different signal transduction pathways (180). Lastly, cancer cells *in vitro* are subjected to varying microenvironment which may influence the proliferation rate and tumour metabolism (32;181).

The amount of M2-PK protein in Panc-1 cells was similar to that in MCF-7 cells, a cell line that is derived from a Human Caucasian breast cancer. However, in Panc-1 cells Tumour M2-PK levels were about two times lower than in MCF-7 cells which indicates that the tetramer:dimer ratio in MCF-7 cells is more shifted towards the dimeric form than in Panc-1 cells. The non-tumour cell line HMEC-1 contained the same amount of M2-PK protein like the tumour cell lines Panc-1 and MCF-7 cells but less dimeric M2-PK. This corresponds to the finding that in tumour cells the tetramer: dimer ratio is shifted towards the dimeric form when compared to normal proliferating cells (170). This study revealed a significant difference in proliferation rate between Colo 357 and Panc-1 cell line as reported previously (161;162).

This difference in proliferation rate was reflected in different levels of M2-pyruvate kinase as measured by both ELISA and Western Blotting. Higher levels of Tumour M2-PK in a rapidly proliferating pancreatic cancer cell of metastatic origin may reflect an important role of Tumour M2-PK in cancer growth and spread. Elevated levels and activity of M2-PK have also been seen in other cancer cell lines with high metastatic potential such as MHCC97 (Human hepatocellular carcinoma) and H.Ep.2 (Human laryngeal carcinoma) – (25;182) clinical studies on pancreatic (56;74) and renal cancer (73). The significant difference in Tumour M2-PK level between the two pancreatic cancer cell lines, namely Panc-1 and Colo 357, observed in this study therefore needs further study of cell lines with known molecular genetics to correlate growth and apoptosis rates with M2-PK expression.

5.4   Conclusion

Tumour M2-PK can be measured in cell homogenates using ELISA and the levels are inversely proportional to cell density. The higher levels of Tumour M2-PK were found in the cancer cell line with higher metastatic potential suggesting a correlation with growth and spread of cancer that should be further investigated. The variability of levels of Tumour M2-PK with cell density in cell culture may be a response to the tumour microenvironment that requires further investigation.

6   CHAPTER 6   The Effect of Tumour Microenvironment on M2-Pyruvate Kinase Level in Human Pancreatic Cancer Cell Lines

6.1　Introduction

Increased proliferative activity in solid tumours leads to increased glucose and oxygen consumption resulting in local tumour hypoxia and reduced glucose (44). Glycolysis is upregulated in response to hypoxia leading to increased lactate production which reduces intracellular pH (181). Tumour cells adapt to an adverse environment by various mechanisms. One such mechanism is by exporting protons into the extracellular space by Na^+/H^+ exchangers (183). Another mechanism described is the upregulation of glycolytic enzymes mediated by hypoxia inducible factor (HIF)-1α, a transcription factor regulating gene responses to hypoxic stimuli (45). HIF-1α mediated alteration to glycolysis is most marked in pancreatic cancers (48) which are relatively avascular and hypoxic (49). The understanding of such altered metabolic phenotype on the biologic characteristics of pancreatic cancer is unclear.

Pyruvate kinase type M2 is known to switch over between the dimeric and the tetrameric form of M2-pyruvate kinase and has been proposed to be a metabolic adaptation mechanism in tumour cells to varying nutrient and oxygen supply conditions (20). The dimeric form of M2-pyruvate kinase is known to be elevated in patients with pancreatic cancer (56;86;175). However, clinical studies have shown marked variability in the Tumour M2-PK levels in patients with the same cancer type (172). The reason for its variability in levels is not known. It can be attributed to the *in vivo* heterogeneity within solid tumours in terms of their metabolic activity which is evident from *in vitro* studies which suggest difference in M2-pyruvate kinase activity in non-metastatic and metastatic cancer cell lines (25;182). The aim of this study is to investigate whether

altered microenvironment, especially hypoxia, acidic pH and glucose-deprived condition, has any role in variable expression and tetramer:dimer switch-over of M2-pyruvate kinase in pancreatic cancer cells.

6.2   Methods

6.2.1   Cell lines

Cell lines Panc-1 and Colo 357 were used as described in Chapter 4, section 4.1 and maintained in monoculture as described before (Chapter 4, section 4.2).

6.2.2   Acidic pH condition

Described in Chapter 4, section 4.3.1.

6.2.3   Glucose-deprived condition

Described in Chapter 4, section 4.3.2.

6.2.4   Hypoxic condition

Described in Chapter 4, section 4.3.3.

6.2.5   Cell viability and proliferation rate assessment

Described in Chapter 5, section 5.2.2.

6.2.6   Extraction of Tumour M2-PK

Described in Chapter 4, section 4.4.1.3.

6.2.7   Total M2-PK measurement

Described in Chapter 4, section 4.4.3.

6.2.8   Measurement of Tumour M2-PK

Described in Chapter 4, section 4.4.1.

6.2.9   Pyruvate Kinase activity assay

See Chapter 4, section 4.4.2.

6.2.10   Tetramer-dimer switch-over of M2-PK

Separate experiments were conducted in order to study the relation of Tumour M2-PK levels and total M2-PK expression with pyruvate kinase activity in terms of tetramer-dimer switch-over of M2-PK. Tumour M2-PK, total M2-PK and pyruvate kinase activity were measured in the same way as described in section 4.4.1, 4.4.3 and 4.4.2 respectively and in the cell population of the same passage in culture. Cells were maintained in monoculture until 70% confluent with subsequent replenishment of culture media and then exposed to altered culture conditions for 72 hrs. For the western blot, cell homogenate equivalent of 1 million cells for each culture condition was loaded into the gel rather than equal amount of protein so that the total M2-PK expression is comparable to the Tumour M2-PK level and M2-PK activity which were expressed in units/million cells.

6.2.11   Statistical analysis

See Chapter 5, section 5.9.

6.3   Results

6.3.1   Normal culture condition

In Colo 357, the number of cell doublings at 96 hrs in culture was higher than that in

Panc-1 cells (Table 6.1). Level of Tumour M2-PK and the total M2-PK expression was

also significantly higher ($p < 0.05$) in Colo 357 cells in comparison to Panc-1 cells (Table

6.2 and Figure 6.1). This was consistent with the findings observed in

Chapter 5.

6.3.2   Acidic pH condition

The mean pH in the culture medium at the end of 96 hrs incubation in acidic conditions was

6.7 ± 0.2 (n = 40) in comparison to 7.4 ± 0.5 (n = 32) in normal conditions. Cell doublings at

96 hrs were nearly totally suppressed in Panc-1 cells ($p < 0.05$) whereas in Colo 357 cells

acidification had no effect (Table 6.1).

On exposure to acidic pH (6.5) neither of the pancreatic cancer cell lines showed alteration in

total M2-PK expression (Figure 6.1). However, Colo 357 cells showed an increased

dimerisation of M2-PK while in Panc-1 cells a slight but non-significant decrease of the

dimeric form of M2-PK was found (Table 6.2).

6.3.3   Hypoxic condition

Hypoxia had significant impact on the cell proliferation of Colo 357 with sevenfold

decrease in the cell doubling at 96 hrs ($p < 0.01$) while Panc-1 cells remained unaffected

(Table 6.1). On exposure to hypoxia neither in Panc-1 nor in Colo 357 cells M2-PK

expression was affected (Figure 6.1). However, while Colo 357 cells showed Tumour

M2-PK levels significantly increased about two- and half-fold (p<0.05) Panc-1 did not

show a significant rise in levels (Table 6.2).

Table 6.1: Effect of different cultivation conditions on cell doublings of Colo 357 and

Panc-1 cells

Cultivation conditions	Colo 357	Panc-1
Control	4.9 ± 1.6	3.6 ± 1.6
Hypoxia	0.7 ± 0.1**	2.0 ± 2.0
Glucose deprivation	2.8 ± 1.5*	2.3 ± 1.3
Low pH	3.8 ± 1.5	0.8 ± 3.2*

The values represent mean ± S.D cell doublings at 96 hrs of 6 different experiments.
Significant values ** $p<0.01$, * $p<0.05$ in comparison to control.

6.3.4   Glucose-deprived condition

In Colo 357 cells, the number of cell doublings significantly decreased from 5 to 3 at 96

hours in culture (p<0.05) while in Panc-1 cell doublings were not significantly affected

(Table 6.1). In both cell lines glucose deprivation did not affect M2-PK expression

(Figure 6.1). However, a significant twofold increase in Tumour M2-PK levels was

measured in Colo 357 cells (p<0.05). The increase of Tumour M2-PK values in glucose-

deprived Panc-1 cells was not significant (Table 6.2).

Table 6.2: Effect of different cultivation conditions on Tumour M2-PK levels in Colo 357 and Panc-1 cells

Cultivation conditions	Colo 357 $\overline{x}_g \cdot DF^{\pm1}$ [mU/10^6 cells]	Panc-1 $\overline{x}_g \cdot DF^{\pm1}$ [mU/10^6 cells]	N
Control	79 • 1.3	33 • 1.3	20
Hypoxia	191 • 1.3*	62 • 1.2	20
Glucose deprivation	174 • 1.2*	54 • 1.2	20
Low pH	246 • 1.2**	23 • 1.2	20

Result of a one-way analysis of covariance with cell density as covariable. The values represent geometric mean values multiplied and divided by the dispersion factor.
* represents Control vs Condition: * $p < 0.05$ and **$p < .001$.
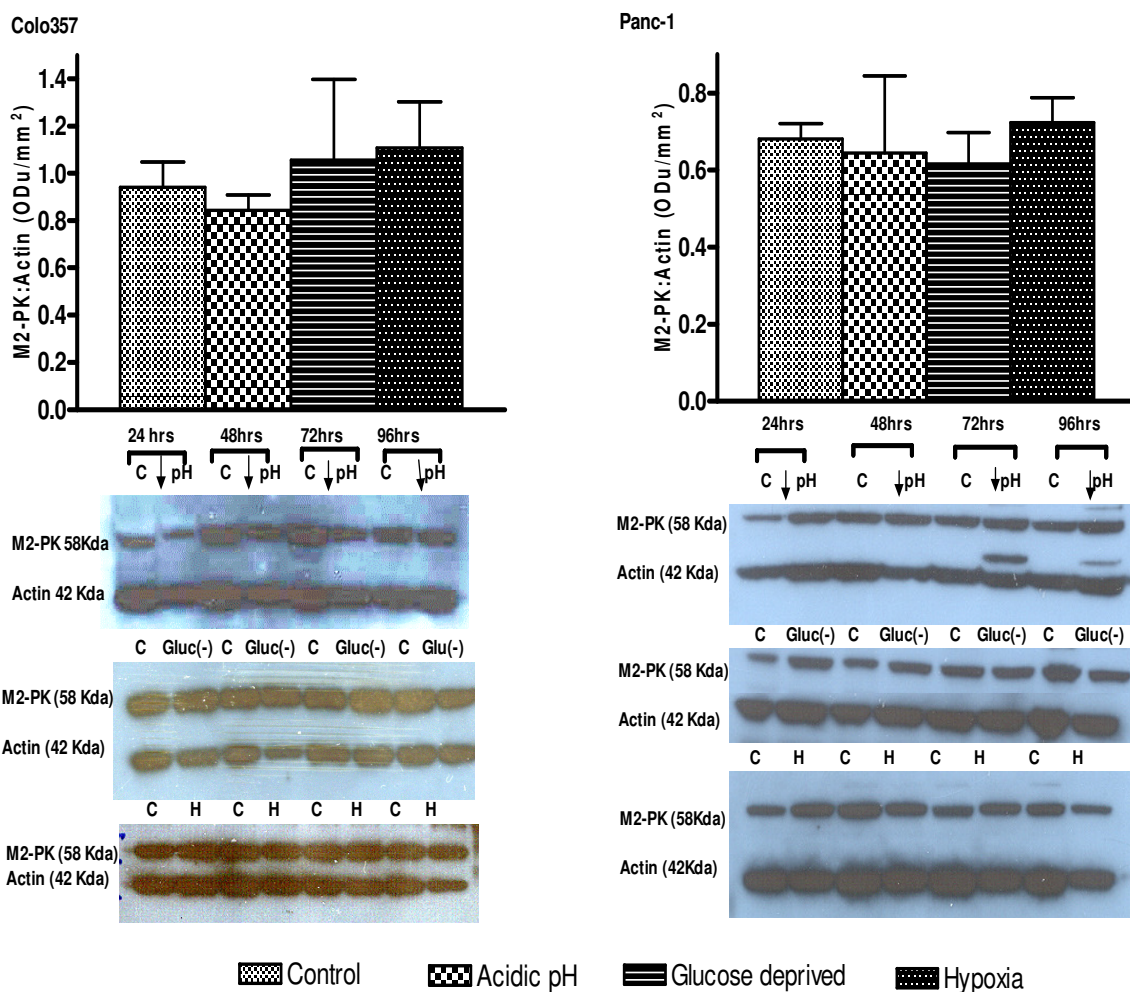
Figure 6.1: Total M2-PK expression in Colo 357 and Panc-1cells in different culture microenvironment (Seeding cell density was 2 million cells per dish for each condition) .The graphs represent mean± S.E value of 3 different experiments while the image shows the best representative immunobloting image). In each slot same amount of protein (20 µg) have been applied.

6.3.5   Comparison of total M2-PK, Tumour M2-PK level and pyruvate kinase in different culture conditions

These results are from separate experiments repeated to measure Tumour M2-PK, total M2-PK and pyruvate kinase activity at a given point of time in culture (72 hrs) in one million cells from the same passage in culture. The adjusted mean Tumour M2-PK level

as measured by ELISA was significantly higher ($p<0.01$) on exposure of Colo 357 cells to acidic pH condition. A non-significant increase in levels was observed under glucose-deprived condition. Levels did not vary significantly between normal and hypoxic conditions. In Panc-1 cell line Tumour M2-PK levels were not significantly different between normal, acidic pH or glucose deprived condition. A non-significant decrease in Tumour M2-PK was observed under hypoxic condition (Figure 6.2a, b). Levels were significantly higher in Colo 357 cell lines in comparison to Panc-1 in all the conditions (control $p<0.05$, acidic pH<0.001, glucose-deprived $p<0.001$ and hypoxia $p<0.01$). In contrast to dimeric M2-PK, total M2-PK protein expression did not differ between different culture conditions in both the cell lines. Total protein expression was higher in Colo 357 cells in comparison to Panc-1 (control $p<0.05$, glucose deprived $p<0.01$ and hypoxia $p<0.01$) – (Figure 6.2a, b).

Pyruvate kinase activity in Colo 357 cells was significantly increased with hypoxia when compared to normal conditions. Elevation in activity was also seen under acidic and glucose deprived conditions but this was not statistically significant. A similar trend was seen in Panc-1 cells comparing normal with altered culture conditions. When Pyruvate kinase activity was compared between Panc-1 and Colo 357 cells, no significant difference was observed under normal as well as altered culture conditions, although PK activity was higher in Colo 357 cells under acidic and hypoxic conditions (Figure 6.3).
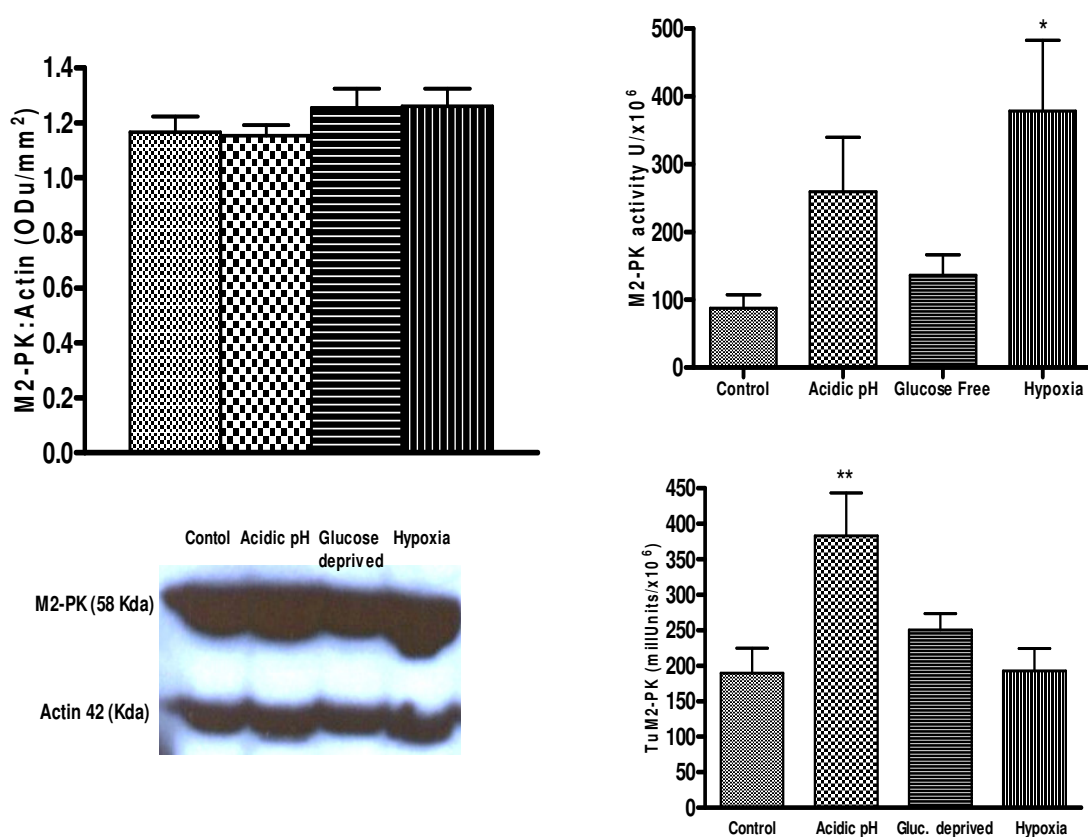
Figure 6.2a: Tumour M2-PK, total M2-PK protein expression and M2-PK activity in Colo 357 cells on exposure to different culture conditions
Cells were allowed to grow in normal culture condition until 70% confluence and then the culture medium was changed to expose the cells for different conditions for 72 hrs. For the western blotting cell homogenate of equal cell density (1 million) was loaded into SDS gel for each condition.

Figure 6.2b: **Tumour M2-PK, total M2-PK protein expression and M2-PK activity in Panc-1 cells on exposure to different culture conditions**
Cells were allowed to grow in normal culture condition till 70% confluence and then the culture medium was changed to expose the cells for different conditions for 72 hrs. For the western blotting cell homogenate of equal cell density (1 million) was loaded into SDS gel for each condition.

Figure 6.3: Comparison of pyruvate kinase activity in Panc-1 and Colo 357 cells under different microenvironment

6.4   Discussion

6.4.1   M2-PK with cell growth in normal condition

Under normal tumour condition, Colo 357 showed a significantly higher proliferation rate and Tumour M2-PK levels than Panc-1 cell lines. The difference in proliferation rate between the two cell lines observed in this experiment was consistent with the results of experiments in Chapter 6 and previous studies (161;162). Similarly, the difference in Tumour M2-PK levels observed between the two cell lines was also consistent with the results of experiments in Chapter 5 reflecting a fundamental difference in their biologic behaviour i.e. pancreatic cell line with high metastatic potential should have high inactive dimeric form of M2-PK in order to keep up high proliferation rate in tumour cells. However, it is difficult to justify this observation unless some more pancreatic cancer cell lines with different proliferation rate are also tested for Tumour M2-PK. Another limitation of this study was the method used for cell viability and proliferation rate assessment. This was done by direct viable cell count under microscope. There are more commercially available assays like MTT or Picogreen that could have been used in the current study. However, these assays are based on cells grown on microplate culture and cannot be used to calculate the proliferation rate for large cell population on culture flask. Given that the large numbers of cells were required for Tumour M2-PK level measurement and to standardise the measurement with corresponding cell numbers, assessment of cell proliferation by direct cell count at each timepoint in culture was found to be the most practical approach.

6.4.2   Cell growth with altered microenvironment cancer

Pancreatic cancer has an inherent property of an aggressive behaviour, metastatic potential and resistance to treatment. Hypoxic, acidic and nutritionally deprived areas are commonly seen in pancreatic cancer which renders the tissues resistant to apoptosis and hence treatment (48) (31). The altered microenvironment in cancer cells may lead to the arrest or impairment of cancer growth through molecular mechanisms, resulting in cellular quiescence, differentiation, apoptosis, and necrosis (184). Cells exposed to hypoxia are generally arrested at the G1/S-phase (20). On the other hand, the cells survive cell death by microenvironment-induced metabolic responses leading to low energy utilisation, high glycolytic rates and increased energy production (185). This study showed a reduced proliferation rate of both metastatic and non-metastatic pancreatic cancer cell lines with alteration to the tumour microenvironment associated with increased expression of a glycolytic enzyme, Tumour M2-pyruvate kinase indicating one such metabolic response to altered tumour microenvironment.

In the present study, I have simulated different nutrient supply conditions in solid tumours by culturing two different pancreatic cancer cells lines (Colo 357 and Panc-1) under hypoxia, glucose deprivation or low pH value. In Colo 357 cells, a twofold to threefold increase in tumour M2-PK levels was observed when the cells were cultured under low pH value, glucose deprivation or hypoxia. Since total M2-PK values were not changed under the different cultivation conditions the increase of Tumour M2-PK values points to a dimerisation of M2-PK. In comparison to this Panc-1 cells showed 1.6 – 1.9-fold increase in Tumour M2-PK levels under hypoxia and glucose deprivation although the increase was not significant.

6.4.3 Acidic pH condition

Acidification of tumour cells is a consequence of upregulated aerobic glycolysis with increased lactate, H^+ and CO_2 production. The intracellular pH value in tumour cells is usually in the range of 7.0 – 7.2 (39;42). The intracellular H^+ ions and lactic acid are pushed out of the cell by membrane-bound Na^+/H^+ exchangers and H^+/lactic acid co-transporters while the CO_2 diffuses rapidly across the plasma membrane and gets converted to carbonic acid by membrane-bound ectoenzyme carbonic anhydrases (40). Uptake of the weak base HCO_3^- via a member of the Na+-dependent and Na+-independent Cl/HCO_3^- exchangers contributes to intracellular alkalinisation (40). The resulting extracellular pH in most solid tumours is usually acidic (5.8 – 7.6) – (41). The pH value used for acidic condition in our study was within this range. Studies indicate that extracellular acidic environment helps in tumour invasion by killing of normal cells and clonal selection of tumour cells by caspase-mediated activation of p53 dependent apoptosis (186;187). Low pH levels correlated with a slight but non-significant decrease of the dimeric form of M2-PK in Panc-1 cells.

The dimerisation of M2-PK observed in Colo 357 cells at low pH value may reduce the amount of lactate produced and released from tumour cells into the environment thereby reducing further acidification. This may explain why in Colo 357 cells the cell doubling was not affected from low pH treatment whereas in Panc-1 cells which were unable to increase the dimeric form of M2-PK a nearly total suppression of cell proliferation took place. Thus upregulation of the dimeric form of M2-PK in acidic pH in Colo 357 cell lines may suggest a pattern in cancer survival.

6.4.4 Glucose-deprived condition

In glucose-deprived Colo 357 cells proliferation was slightly reduced but not totally suppressed. Glycolysis is the main energy production pathway in tumour cells even in the presence of oxygen. The reduced tumour cell proliferation under glucose-deprived condition was also observed in previous studies (163). The possible explanation may be that cell proliferation is an energy-consuming process, twofold to fourfold energy consumption of non-proliferating cells. Inhibition of cell proliferation saves energy and the ATP produced by optimal metabolism under glucose starvation is used by cells for survival rather than proliferation. Another possible reason may be the utilisation of alternate energy-producing pathways by tumour cells in glucose-starved condition (25). These pathways remain underutilised by the normal cells. Tumour cells have been shown to produce energy using pentose phosphate pathway (PPP) and glutaminolytic pathway (25). The decrease in proliferative activity may also be due to non-availability of glucose-derived carbon for the *de novo* synthesis of RNA and DNA. The significance of elevated levels of Tumour M2-PK under glucose-deprived condition observed in this study is not clear. Under glucose deprivation, a high amount of the dimeric form of M2-PK may represent the channelling of the glucose carbons available into synthetic processes. It may be one of the adaptation responses of tumour cells to glucose starvation in order to keep the cells at optimal proliferation rate for viability or it may be HIF-1 mediated upregulation of pyruvate kinase transcription. Although HIF-1, a transcription factor, is stabilised and activated in tumour cells exposed to hypoxia, a constitutive expression of HIF-1 was observed in most of the pancreatic cancer cells under normoxia by Akakura et al. (48). Once activated HIF-1 promotes the transcription of several genes such as glucose

transporters, glycolytic enzymes, and angiogenic factors (45). However, it is not known whether glucose deprivation alone stabilises and activates HIF-1. Thus, the limitation in explaining the observed increase in Tumour M2-PK level under glucose-deprived condition could have been overcome by measuring HIF-1 induced pyruvate kinase mRNA expression in the current study. This was not measured, as no significant increase in total M2-PK protein expression was observed under glucose-deprived condition as compared to normal condition. Another possible reason for elevated dimeric M2-PK in glucose-deprived condition could be the switch-over of tetrameric M2-PK to dimeric M2-PK as extrapolated from the results of dimeric M2-PK measurement by ELISA and total M2-PK measurement by western blot in normal and glucose-deprived condition. Tumour cells are known to have oscillatory dimer:tetramer ratio depending on fructose biphosphate levels (20;28). The dimerisation of M2-PK observed in glucose-deprived Colo 357 cells is presumably caused by a decrease of the key M2-PK regulator fructose 1,6-P2 and corresponds to results from Ashizawa et al., who showed in A431 cells that glucose starvation leads to a decrease in fructose 1,6-P2 levels and dissociation of the tetrameric form of M2-PK (47). When other sources for energy regeneration, i.e. glutaminolysis are available the dimerisation of M2-PK may enable tumour cells to proliferate even under low glucose supply.

6.4.5 Hypoxic condition

Under hypoxia mitochondrial respiration and glutaminolysis fail as energy source since both pathways depend on oxygen supply. In both cell lines total M2-PK protein content of M2-PK was not increased during hypoxia which may indicate that although the M-gene has hypoxia responsive site (188), hypoxia may not necessarily upregulate M2-PK

levels at post-translational level. In Colo 357 cells about twofold increase of the dimeric form of M2-PK under hypoxic conditions point out that the cells may be unable to shift to glycolytic energy regeneration at low oxygen supply. The dimerisation of M2-PK together with the inhibition of the oxygen-dependent energy regeneration by mitochondrial respiration and glutaminolysis may explain the stronger inhibition of cell proliferation in Colo 357 cells than in Panc-1 cells which did not show significant dimerisation under hypoxic conditions.

In this study, hypoxia increased levels of dimeric M2-PK. HIF-1 mediated increase in dimeric M2-PK could be one possibility but this explanation was not supported by elevation of total M2-PK protein expression under hypoxia. The possibility of coexistent acidic pH in inducing elevated Tumour M2-PK levels under hypoxia cannot be ruled out. This may be one of the main limitations of this study which could have been overcome by buffering the culture medium by long-acting buffering agents like MES or HEPES. Another possible limitation could be the oxygen levels in culture medium which could be a key factor to judge extracellular hypoxia. The average O_2 concentration measured in the culture medium was $7 - 10\%$ (data not shown in results) when the O_2 concentration in the chamber was 1%.

6.4.6   Correlation between Tumour M2-PK, Total M2-PK protein expression and pyruvate kinase activity

Total protein expression of M2-pyruvate kinase as measured by western blotting was unchanged in altered tumour microenvironment while dimeric M2-PK as measured by ELISA was elevated in both the cell lines in altered microenvironment. The monomeric isoform of M2-PK has been described previously by Ashizawa et al. in their study on

monomeric-tetrameric interconversion of M2-PK in response to various levels of glucose and fructose 1,6 biphosphate (47). Thus the total M2-PK, which remained unchanged with alteration in microenvironment in this study, will have a mixture of monomeric, dimeric and tetrameric isoform of M2-PK in different proportion. A question that remained unanswered in this experiment is: what proportion of dimeric M2-PK constitutes total M2-PK in each culture condition? If the proportion of dimeric M2-PK is small in all the conditions, any significant elevation of dimeric M2-PK seen in any of the above-mentioned altered culture conditions may not be of any biological significance. If the proportion is large, the elevated levels of dimeric M2-PK observed with altered microenvironment would suggest a tetramer-dimer switch-over, the biologic significance of which should be further investigated.

Similarly, the difference in concentration of dimeric form may not represent the influence of microenvironment but rather the timeframe of dimeric M2-PK synthesis. However, this possibility was ruled out as the cell population from the same passage was exposed to normal or altered culture condition for dimeric M2-PK measurement by ELISA.

Thirdly, this study has observed that altered tumour microenvironment influences the dimeric M2-PK rather than total M2-PK levels. Does this represent a qualitative rather than quantitative change in the M2-PK protein by the tumour microenvironment? Hence pyruvate kinase activity was measured to answer this query. Tetrameric form has high PK activity (conversion of phosphoenolepyruvate to pyruvate) while dimeric M2-PK is inactive isoform of M2-PK (20). In this study, a comparative elevation of pyruvate kinase activity in Colo 357 cell line under hypoxic culture condition with no significant change in the dimeric (tumour) M2-PK and total M2-PK levels would indicate monomeric to

tetrameric conversion. Similarly under acidic or glucose-deprived environment, an increased level of dimeric M2-PK associated with increase in PK activity and unchanged total M2-PK protein (in comparison to normal condition), a shift from monomeric to the dimeric and tetrameric M2-PK can be extrapolated. A similar monomeric to tetrameric conversion under acidic pH or glucose-deprived condition and monmeric/dimeric to tetrameric conversion under hypoxia could be extrapolated in Panc-1 cell lines. The findings in this study, especially under glucose-deprived condition, are in contradiction to the study by Ashizawa et al. where they showed tetramer to monomer conversion of M2-PK under low glucose concentration (47). While findings in this study are based on the extrapolation of the results from three different assays with only one assay using monoclonal antibody against dimeric M2-PK, Ashizawa et al. used monoclonal antibodies specific for monomeric or tetrameric form of M2-PK (47). These antibodies were indigenously produced by them and were not available for use commercially. Hence, our results need further verifications by a single assay measuring monomeric, dimeric and tetrameric M2-PK isoform by using their respective monoclonal antibodies.

6.5   Conclusion

Suppression of growth of pancreatic cancer cell lines by the altered tumour microenvironment is associated with increased levels of Tumour M2-PK and with possible interconversion between different isoforms of M2-PK. Whether elevated levels of Tumour M2-PK have any role in pancreatic cancer cell survival or apoptosis to altered tumour microenvironment requires further experiments.

7 CHAPTER 7 Tumour M2-Pyruvate Kinase and Apoptosis in Pancreatic Cancer Cells

7.1   Introduction

The inactive (dimeric) form of M2-PK is known to be elevated in pancreatic and other types of visceral cancer (172;175). M2-PK is elevated in cancer cells to channel the glycolytic metabolites towards nucleotide synthesis giving cancer cells a rapid proliferation rate compared to non-cancer cells (20). In Chapter 7 a predominance of dimeric M2-PK with suppression of cell growth and a possible tetramer-dimer interconversion on exposure of pancreatic cancer cells to altered culture conditions was observed. The biological significance of elevated dimeric M2-PK under these conditions is not known. It may represent a metabolic response to cellular quiescence or cell cycle arrest subsequently leading to apoptosis, necrosis or cell survival, or it could be a response to prevent cancers from outgrowing their blood supply. It has recently been observed that overexpression of M2-PK is associated with P53-mediated apoptosis (174) and tumour-specific M2-PK is involved in caspase and Bcl-2 independent apoptosis by translocating to nucleus, forming a nuclear death complex (189). Although these findings have not yet been verified by other investigators it clearly establishes a link between Tumour M2-PK and apoptosis.

The aim of this experiment is to simultaneously measure Tumour M2-PK and apoptotic markers in Panc-1 and Colo 357 cell lines under altered culture conditions and to verify any link between them. The apoptotic markers measured are membrane-bound Phosphotidyl serine binding of Annexin stain and cytoplasmic apoptotic markers of caspase-dependent (Caspase 3, 7 and 8) and caspase-independent pathway (Bax and Bcl-2) and are described in pancreatic cancer previously (168).

7.2   Methods

Cell lines and culture conditions, extraction of Tumour M2-PK and measurement of Tumour M2-PK were similar to that described in Chapter 4, sections 4.2, 4.3 and 4.4.1 except that the cells from the same culture dish and in same passage were used for Tumour M2-PK (ELISA), apoptosis markers (Annexin-PI, Caspase 3, 7, 8 and Bax) and anti-apoptotic marker (Bcl-2) measurement.

7.2.1   Annexin V-FITC and Propidium Iodide (PI) Staining for Viability Assessment

The protocol for Annexin V and PI staining is as described in the 'Materials and Methods' in Chapter 4, sections 4.6 and 4.7. The staining of cells with Annexin and PI was initially visualised using immunofluorescence to make a subjective assessment of the proportion of apoptotic or necrotic cells. Objective assessment was made subsequently by flocytometry. This method was used to quantify viable cells as well as cells in early or late apoptotic or necrotic phase on exposure to altered microenvironment.

7.2.2   Active Caspase 3/7 and 8 assay

The protocol for Caspase 3/7 and 8 assay is as described in Chapter 4 'Materials and Methods' section 4.5. This assay was used to determine if the apoptosis to altered tumour microenvironment is caspase-mediated.

7.2.3   Bax and Bcl-2 measurement

Bax and Bcl-2 protein expression was measured by immunoblotting using mouse monoclonal antibody (1:200 dilution) and rabbit polyclonal antibody (1:200 dilution) respectively (Santa Cruz laboratories, CA, USA). This method was used to measure apoptosis because at the beginning of the

experiment it was not known whether the apoptosis to altered microenvironment would be caspase or Bcl-2 protein-dependent as different types of apoptotic pathways in pancreatic cancer have been described in the literature (168). Bax is an apoptotic protein while Bcl-2 is an anti-apoptotic protein regulating mitochondrial permeabilisation (168). Their effect on mitochondrial permeability pores is caspase-independent (168) .

7.2.4   Statistical Analysis

All values were expressed in Mean ±S.D unless stated separately. Mean values were compared by one-way ANOVA. Any correlation between Tumour M2-PK, Caspase 3, 7 and 8 or Bax or Bcl-2 was verified by Pearson coefficient correlation.

7.3   Results

7.3.1   Tumour M2-PK levels in Colo 357 and Panc-1 cells

The results depicted in Figure 7.1 are a repetition of the results described in Chapter 6, section 6.4.6.
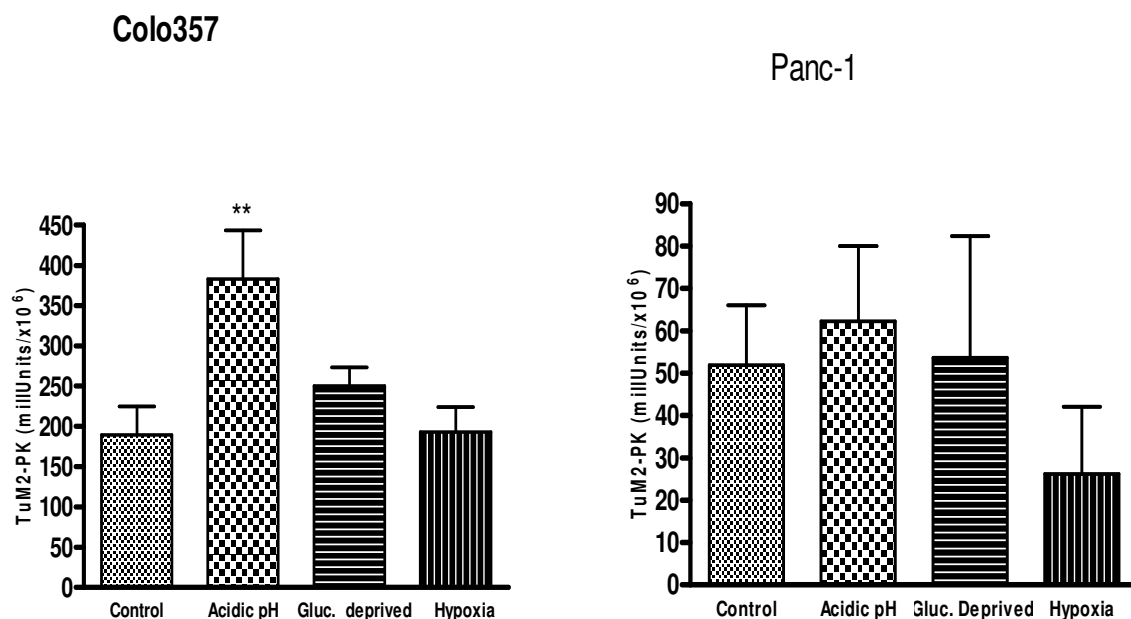
Colo357

Panc-1

Figure 7.1: Tumour M2-PK levels in Colo 357 and Panc-1 cells on exposure to different culture conditions
Cells were allowed to grow in normal culture condition till 70% confluence and then the culture medium was changed to expose the cells for different conditions for 72 hrs (repetition of results from section 6.3.5).

7.3.2 Caspase 3 and 8 (Figure 7.2)

Active Caspase 3/7 levels were not significantly different in different conditions in Colo 357 cells while significantly lower in acidic or glucose-deprived culture conditions compared to controls with Panc-1 cells. Active Caspase 8 levels did not differ significantly from different conditions in both the cell lines. However, Caspase 8 levels were significantly higher in Panc-1 than in Colo 357 on exposure to acidic pH ($p < 0.001$) and hypoxic ($p < 0.01$) condition while significantly lower in glucose-deprived conditions.
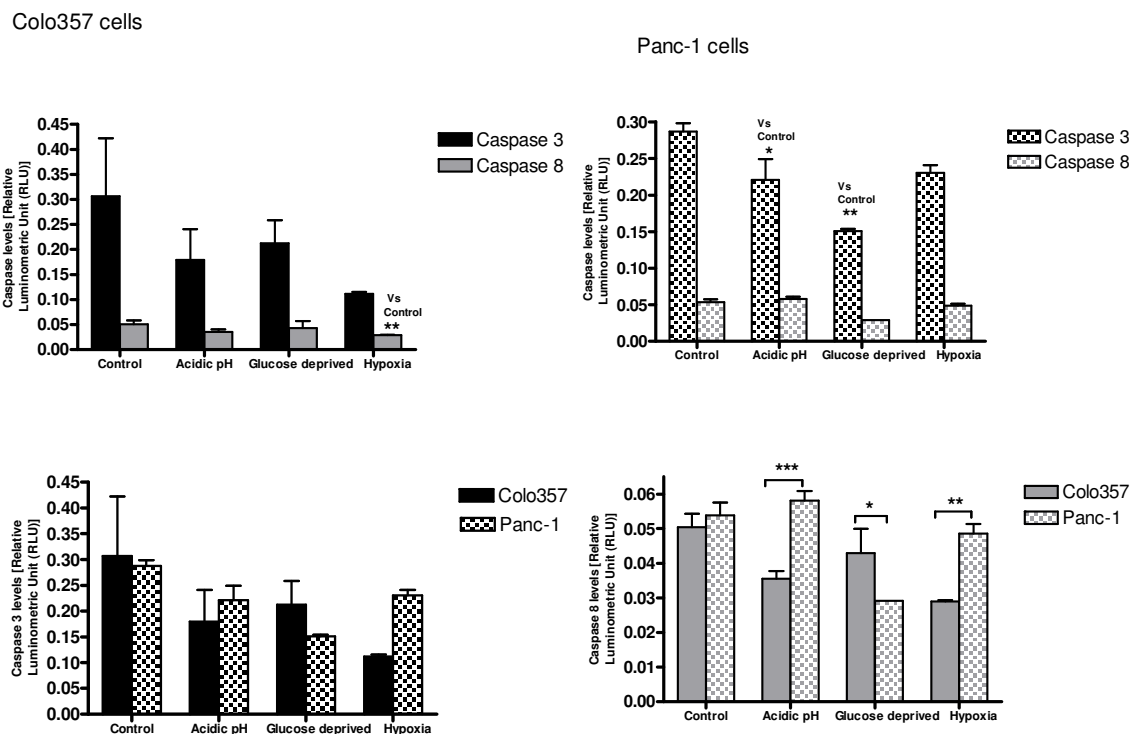
Figure 7.2: Active Caspase 3/7 and 8 levels in different culture conditions in two different cell lines

7.3.3 Bcl-2 and Bax (Figure 7.3)

Bcl-2 protein expression was similar between control, acidic pH and glucose-deprived

conditions in Colo 357 cell lines while lower in hypoxia. Panc-1 cell lines expressed

higher Bcl-2 on exposure to acidic pH condition compared to all other conditions. Colo

357 expressed higher Bcl-2 under glucose-deprived condition than Panc-1, while under

hypoxia Panc-1 expresed higher Bcl-2 than Colo 357. Bax expression did not vary

considerably between control and altered culture conditions.

BcL-2 protein expression in Pancreatic cancer cell lines

I----------------**Colo357**----------------I------------**Panc-1**----------------------I

Bcl-2

Bax

Actin

Control Acidic pH   Glucose   Hypoxia   Control Acidic pH   Glucose   Hypoxia
                deprived   **deprived**
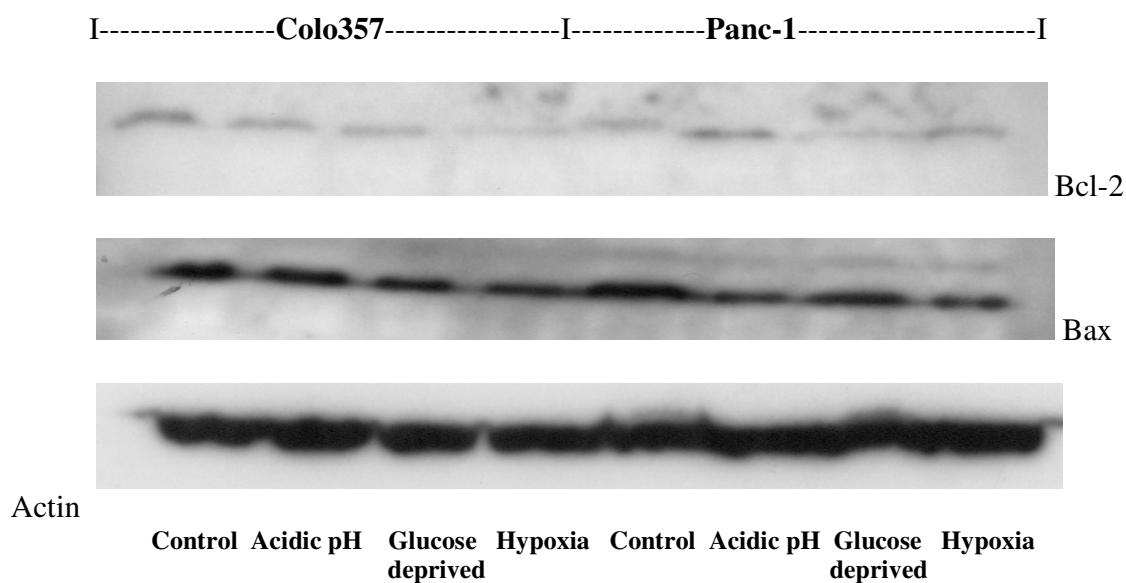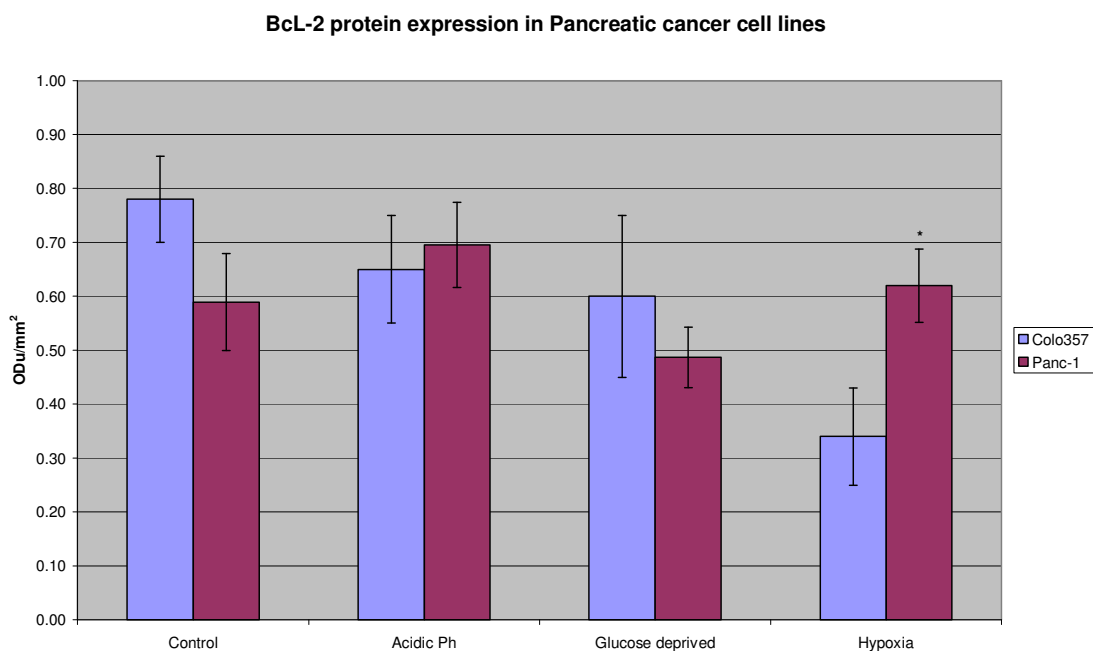
Figure 7.3: Bcl-2 and Bax protein expression in Colo 357 and Panc-1 cell lines on exposure of cells for 72 hrs to the altered culture conditions

Actin was used as a loading control. Total protein loading amount was 40 mg in each well. The exposure time of the film for obtaining Bax and Actin band was 5 seconds and for Bcl-2 was 8 minutes. Bcl-2 was visualised using mouse monoclonal antibody (1:200 dilution) while for Bax (1:200 dilution) and Actin (1:1000) rabbit polyclonal antibody was used.

7.3.4 Annexin V-FITC and Propium Iodide staining

The amount of apoptosis and necrosis is shown in Table 7.1. No significant difference in apoptosis, necrosis or viability was found with cells under normal culture conditions or exposed to an altered environment. Most of the cells were viable on exposure to altered culture condition except in hypoxia where Panc-1 cells showed significantly higher apoptosis and necrosis in comparison to Colo 357 cells ($p<0.05$) – (Figure 7.4).
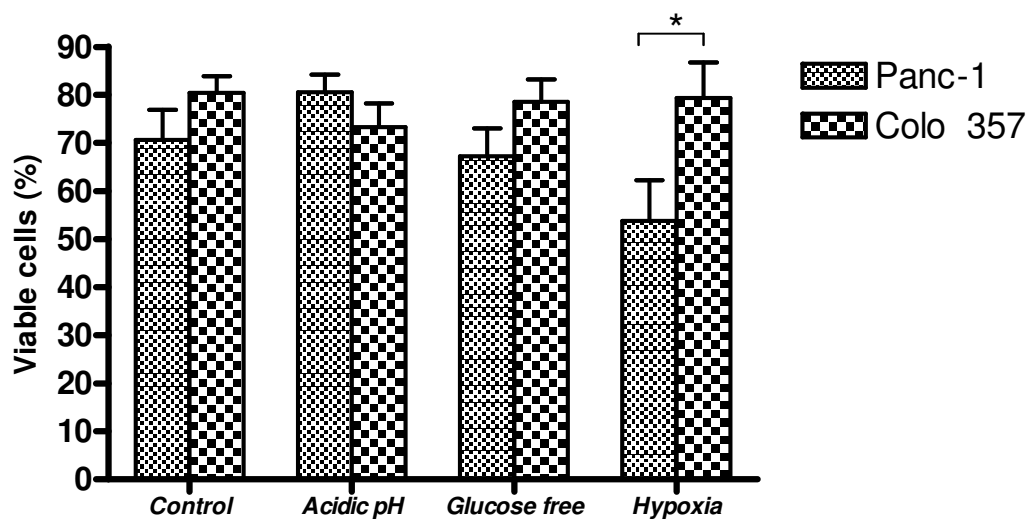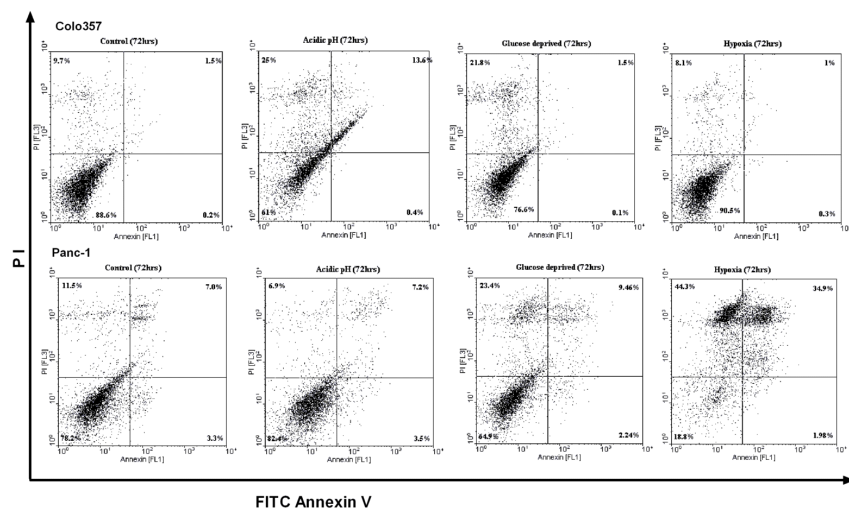
Figure 7.4: Comparison of cell viabilty (Annexin staining) of two pancreatic cancer cell lines under different microenvironment (result of 3 independent experiments)

Table 7.1: Viability and cell death in pancreatic cancer cell lines in different tumour microenvironment (assessed by FACS with FITC Annexin/PI staining). Result of 3 independent experiments.

Colo 357 (%)	Control (mean±S.E)	Acidic Ph (mean±S.E)	Glucose-deprived (mean±S.E)	Hypoxia (mean±S.E)
Viable	82.9±3.0	74.9±4.4	80.5±3.9	79.3±2.7
Early apoptotic	0.6±0.3	1.93±0.6	0.46±0.37	0.5±0.2
Late apoptotic	2.74±0.8	9.66±1.8	3.1±2.0	3.7±1.3
Necrosis	13.7±3.69	13.5±3.46	15.9±3.0	16.4±3.1
Panc-1 (%)	Control (mean±S.E)	Acidic Ph (mean±S.E)	Glucose-deprived (mean±S.E)	Hypoxia (mean±S.E)
Viable	70.6±6.3	80.5±3.6	67.2±5.8	53.8±8.4
Early apoptotic	1.76±0.4	1.4±0.5	1.6±0.4	1.9±0.53
Late apoptotic	5.5±0.7	5.7±0.7	8.3±0.9	12.3±4.8
Necrosis	22±7.1	12.4±3.7	22.9±5.7	32±5.5

7.3.5   Correlation between M2-PK, cell viability and apoptosis

The levels of Caspase 3/7, 8 and Bcl-2 or Bax expression did not correlate with Tumour

M2-PK levels or the M2-PK expression in both the cell lines. The cell viability (% of

viable cells on FACS analysis) on exposure to different culture conditions also did not

correlate with either Tumour M2-PK level or total M2-PK protein expression (Table 7.2).

Table 7.2: Correlation between Tumour M2-PK and total M2-PK with cell viability and apoptosis – the figures in table represent the Pearson coefficient correlation (r)

	Colo 357		Panc-1		P value
	Tumour M2-PK	M2-PK	Tumour M2-PK	M2-PK	
Caspase-3/7	0.02	0.10	-0.38	-0.31	
Caspase-8	-0.25	-0.25	-0.69	-0.52	>0.05
% Cell viability	0.056	-0.04	0.016	-0.17	
Bcl-2	0.23	-0.9	-0.05	0.14	

7.4   Discussion

Pancreatic cancer cells are usually resistant to hypoxia, acidic environment and glucose-deprived conditions (48). In this study pancreatic cancer cells were not affected by the altered *in vitro* culture conditions. No difference in the expression of any apoptotic or anti-apoptotic marker between normal and altered conditions was seen, suggesting that the cells were either resistant to the altered culture conditions or the apoptosis was not measurable entirely either due to the loss of apoptotic markers during the processing or due to the difference in timeframe between the occurrence and the measurement. The later possibility was evident from the observation of necrosis rather than apoptosis as the predominant mode of cell death to different culture conditions in this study. Apoptosis could have been measured earlier (<24 hrs) during the culture. However, given that the aim of the experiment was to correlate Tumour M2-PK levels with apoptosis it was considered to measure apoptosis at the time when Tumour M2-PK is measurable in culture (>24 hrs). The other possible explanation could be ATP depletion in the culture medium as the culture media was not replenished or changed throughout the exposure of cells to different culture conditions. As apoptosis is an ATP-dependent process (190) whatever mode of cell death observed was necrosis.

A similar timeframe difference between occurrence of apoptosis and elevation of dimeric M2-PK may explain the lack of correlation observed in this study. Thus a suitable culture model is required to correlate Tumour M2-PK and apoptosis.

7.5   Conclusion

Elevated levels of Tumour M2-PK under altered tumour microenvironment did not correlate with apoptotic markers in pancreatic cancer in the current experimental model. Suitable culture model is required to establish relationship between Tumour M2-PK and apoptosis in pancreatic cancer. This would involve measuring apoptosis under normal or altered culture conditions using M2-PK knockout or antisense RNA vector model or by inhibiting the tetramer-dimer ratio.

8 CHAPTER 8 Cellular Distribution of Tumour M2-Pyruvate Kinase Expression in Pancreatic Cancer Cell

8.1   Introduction

Several glycolytic enzymes have unexpected localisation to subcellular organelles in order to cater to roles other than glucose homeostasis (7). Enzymes commonly implicated are lactic dehydrogenase (LDH), glyceraldehyde-3-phosphate dehydrogenase (GAPD) and enolase-1 (ENO1) – (191;192). Nuclear translocation of these enzymes is associated with transcriptional regulation (8;189) and apoptosis (9) while mitochondrial localisation is incriminated in apoptotic regulation (7). Several studies have indicated that phosphorylation at tyrosine residue affects the subcellular localisation of glycolytic enzymes (171). M2-pyruvate kinase is phosphorylated at tyrosine residue in cancer cells (189). Nuclear localisation of M2-pyruvate kinase has been shown recently to be associated with various biological functions including apoptosis (193) and cell proliferation (194). This chapter has investigated the subcellular localisation of M2-pyruvate kinase in pancreatic cancer cells in normal and altered tumour microenvironment to give an indication of possible undetected roles of M2-PK based on its cellular location.

8.2   Materials and methods

8.2.1   Cell culture

See Chapter 4, Materials and Methods, section 4.8.

8.2.2   Immunostaining for fluorescence microscopy

See Chapter 4, Materials and Methods, section 4.8.

DAPI: 4',6-diamidino-2-phenylindole was used to bind nuclear DNA to visualise nucleus.

COX-1: Cytochrome C oxidase or Complex IV subunit I which is mitochondrial membrane- bound was used as a marker to stain mitochondria.

8.3   Results

8.3.1   Normal condition (Figures 8.1 and 8.2)

Under normal culture condition, Panc-1 and Colo 357 cells and nucleus appeared normal in size. Chromatin condensation as seen in Figure 8.1 may indicate early phase of nuclear division. Nuclear fragmentation was significantly low and may represent the stress processing during culture.

8.3.2   Acidic condition (Figures 8.1 and 8.2)

In acidic condition Colo 357 and Panc-1 cells appeared to be smaller and to grow in clumps when seen by confocal microscopy. Cells and nuclear morphology were well preserved. Some nuclei appeared to be fragmented. M2-pyruvate kinase showed reticular pattern (red colour) throughout the cytoplasm. No localisation of M2-PK was seen in either nucleus or mitochondria.

8.3.3   Glucose-deprived condition (Figures 8.1 and 8.2)

Colo 357 cells appeared to be a normal size with some cells having fragmented nuclei which may represent the processing injury or stress of glucose-deprived state within the cell. A conspicuously large cell (top right hand corner of Figure 8.1), with dense M2-PK staining, fragmented nuclei and low mitochondrial staining, suggests an apoptotic cell. The cellular and nuclear morphology in Panc-1 cells was not well defined. Mitochondria appeared condensed. M2-pyruvate kinase showed reticular pattern (red colour)

throughout the cytoplasm in both cells. No localisation of M2-PK was seen with nucleus or mitochondria in either of the cells.

8.3.4 Hypoxia (Figures 8.1 and 8.2)

Colo 357 cells were small in size with unremarkable cellular or nuclear morphology. Figure 8.2 shows Panc-1 cells with fragmented nuclei (bottom left) while two dividing cells were seen at the right side of the frame. M2-pyruvate kinase showed reticular pattern (red colour) throughout the cytoplasm in both cells. No localisation of M2-PK was seen with either nucleus or mitochondria.

8.4   Discussion

Mitochondrial and nuclear staining in addition to M2-pyruvate kinase staining was done in this study in order to see subcellular localisation of M2-pyruvate kinase to these organelles under the normal and altered culture conditions. The overall morphology of the cells appeared to be intact in different culture conditions in both the cell lines. Occasional dividing as well as apoptotic cells along with fragmented nuclei were seen, suggesting that cell proliferation and cell death process is occurring simultaneously, maintaining the cell population static, confirming my earlier findings (Chapter 5) on cell proliferation in different culture conditions.

M2-pyruvate kinase showed reticular pattern (red colour) throughout the cytoplasm in normal condition in both the cell lines. The pattern did not change on treatment of both the cell lines to acidic or glucose-deprived or hypoxic condition. These findings confirm the previous such finding which suggested M2-pyruvate kinase to be an intracellular membrane-bound protein (195) . This should be further verified in relation to other intracellular membrane-bound organelles like endoplasmic reticulum (ER) or liposome. This could be achieved by separating and measuring M2-PK from the cellular ER fraction. As the endoplasmic reticulum is the packaging organelle for binding of protein subunit to produce complex proteins in the cell, M2-PK switch-over between monomeric, dimeric and tetrameric form as observed in experiments in Chapter 6 under altered culture conditions may be occurring at the endoplasmic reticulum which could be considered a potential target for cancer treatment.

8.5   Conclusion

M2-pyruvate kinase is an intracellular membrane-bound protein with no localisation to either nucleus or mitochondria under acidic or glucose-deprived or hypoxic condition.
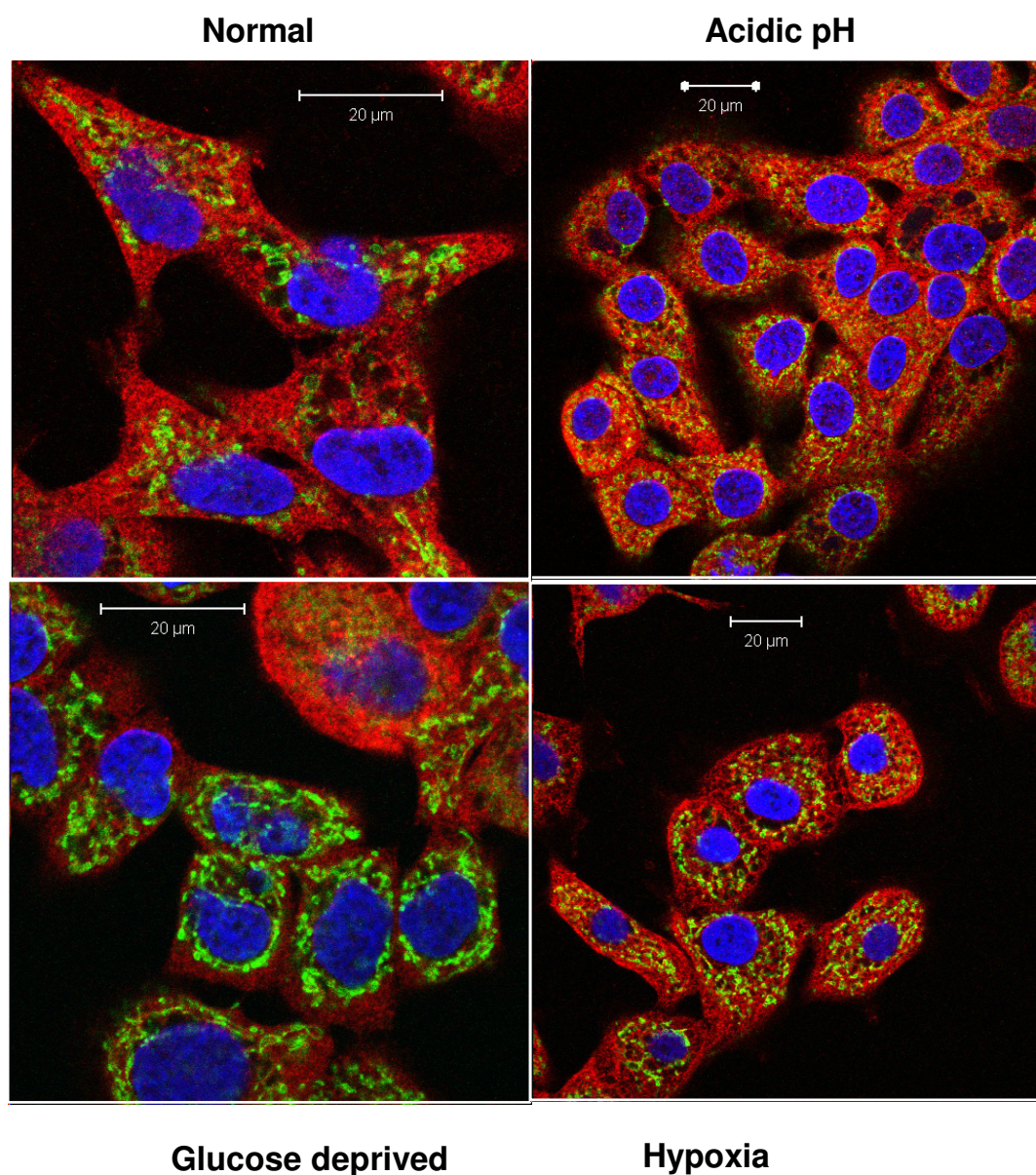
Normal

Acidic pH

Glucose deprived

Hypoxia

Figure 8.1: Immunocytochemical staining of M2-PK (red), mitochondria (green) and nucleus (blue) in Colo 357 cells (72 hrs exposure to altered culture condition). No localisation of M2-PK was seen with either nucleus or mitochondria.
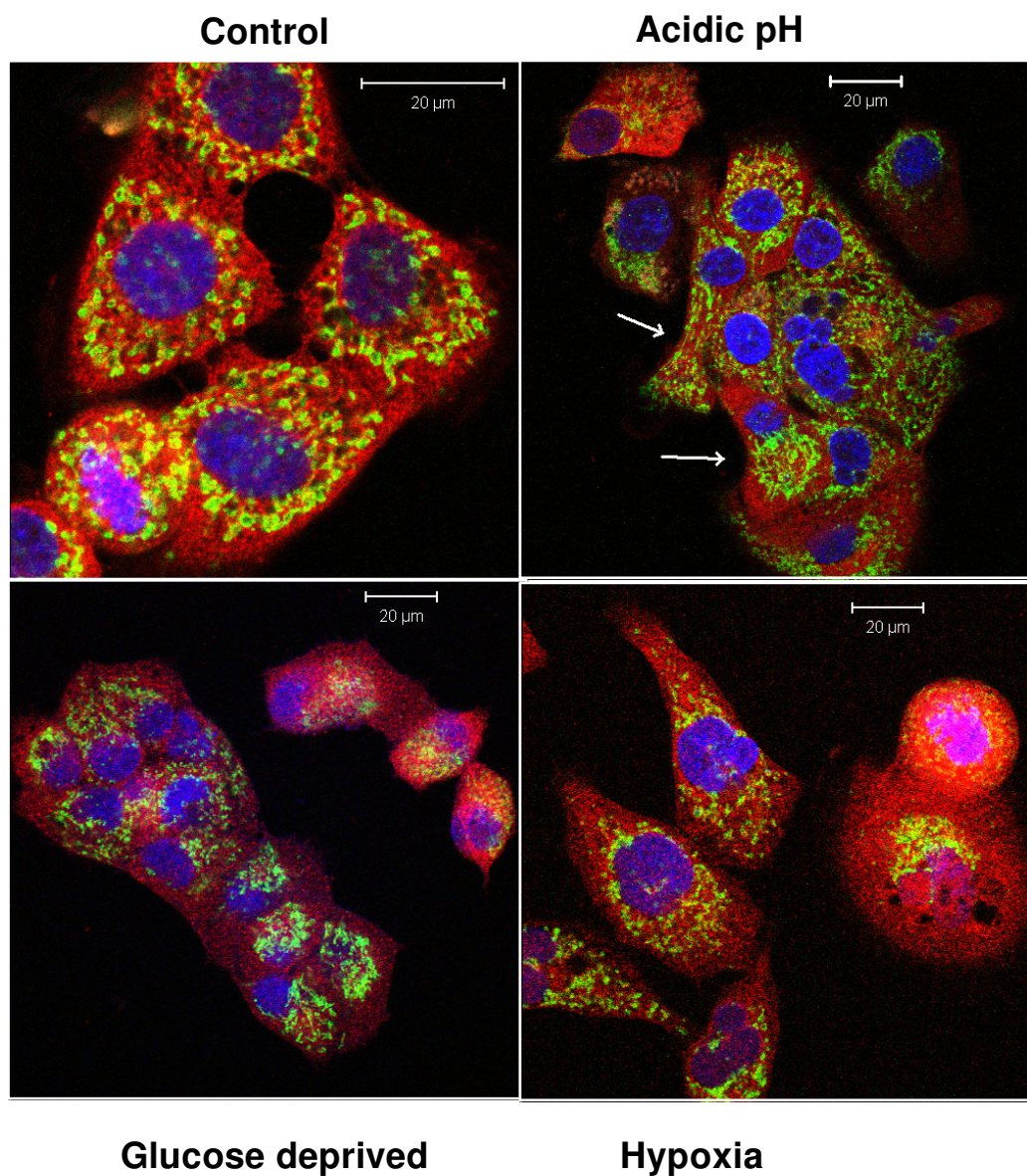
164

Control

Acidic pH

Glucose deprived

Hypoxia

Figure 8.2: Immunocytochemical staining of M2-PK (red), mitochondria (green) and nucleus (blue) in Panc-1 cells (72 hrs exposure to altered culture condition). A mitotic nucleus seen at the bottom left of the top left slide. Arrows indicate dividing cells. No localisation of M2-PK was seen with either nucleus or mitochondria.

9 CHAPTER 9 Resume, Conclusions and Future Research Implications

9.1   Resume

The results have been discussed in detail within each chapter (Chapters 5 – 8). This section addresses methodological considerations, overall conclusions and future experimental studies.

9.2   Methodological consideration

9.2.1   Clinical review

The clinical review included all the published studies and abstracts related to Tumour M2-PK. These were related to Tumour M2-PK measurement in plasma or faeces, diagnostic utility in GI cancer, screening and post-treatment surveillance. The clinical studies on pancreatic cancer and non-pancreatic GI cancers were limited. Most of the studies were case-controls with some studies using historical controls. Different cut-off values of Tumour M2-PK were used in different studies. However, this limitation was overcome by the calculation of the diagnostic odds ratio during the meta-analysis. Most of the clinical studies were of satisfactory quality with no significant bias in the evaluation of diagnostic test. There were no randomised controlled trials comparing Tumour M2-PK with other conventional cancer markers which otherwise would have increased the strength of our meta-analysis.

9.2.2   Experimental model

The ideal experimental model for this study would have been the one which could be easily controlled and modulated especially when M2-PK activity and protein expression was to be evaluated under different culture conditions. For understanding intracellular

events cultured cells have great benefits as they are readily amenable to single cell study and to the kinds of manipulations often necessary to grasp the basic mechanisms. However, a tissue culture system may not relate to a clinical scenario of a patient with cancer.

Cell culture model was ideally suited for these experiments and hence used in the current study. An animal model would have been an alternative for study of pancreatic cancer biology and has been used previously in our laboratory (48;49). However, the monoclonal antibody available for the Tumour M2-PK detection was highly specific for the human tissues and hence human cancer cell lines were used in this study. Pancreatic cancer cells were ideally suited for evaluating the metabolic response to altered tumour microenvironment as this cancer is known to have hypoxic, nutritionally deprived and acidic areas (44). In order to study the influence of the cancer phenotype on M2-pyruvate kinase levels and activity two different pancreatic cancer cell lines were chosen, one with ductal origin while another was of metastatic origin. The use of other known human pancreatic cancer cell lines would have increased the strength of this study and would have particularly allowed more useful conclusions relating to cell behaviour and M2-PK levels.

9.2.3   Culture conditions

All efforts were taken to simulate the different physiological conditions present *in vivo* in solid tumours. However, these conditions were considered separately in order to have full control on the experiments. The different permutation and combinations with which hypoxia, acidic pH or glucose-deprived conditions coexist *in vivo* in solid tumours was

difficult to simulate which could be a limiting factor in this study. Moreover, tumour microenvironment is influenced

in vivo by various factors like tumour neovascularisation, anaemia in patient or distance of tumour cells from main feeding vessel (196). Since many tumours which were initially sensitive to chemotherapy or radiotherapy eventually develop resistance to treatment, interest in tumour microenvironment has recently been rekindled (20). Therefore, acidic pH, glucose-deprived and hypoxic conditions, which are the hallmark of many solid tumours, were used to treat the pancreatic cancer cells in culture.

9.2.4 Biochemical assays for dimeric and total M2-pyruvate kinase

The main hallmark of the study was the use of ELISA as a quantitative method to measure Tumour M2-PK (dimeric form). This has not previously been reported in the cell culture model. The commercially available ELISA kit for measurement of Tumour M2-PK in plasma of cancer patients was used for quantification and was modified to measure the cell culture homogenate. The cell homogenate was used undiluted unlike the plasma samples as the amount of intracellular Tumour M2-PK was unmeasurable when the recommended dilution (1:100) was used. Hence the reproducibility in the form of inter-assay coefficient of variation (CV) was affected in this study (see Chapter 5, section 5.3.4). The main limitation of this study was the absence of an alternative method of quantifying Tumour M2-PK for comparison of the results of this study. Additionally there was no direct method available to measure the tetrameric form of M2-PK. Therefore easy and reproducible method to measure Tumour M2-PK and a direct method to quantify tetrameric M2-PK in tissue culture are needed.

9.2.5   Apoptosis detection methods

The pancreatic cancer cells were exposed to acidic pH, glucose-deprived or hypoxic condition. Trypan blue exclusion test was used initially to assess cell viability. A majority of the cells were viable on exposure to these conditions for up to 96 hrs in culture. Therefore, it would have been useful to quantify how much and what is the mode of cell death in cultured cells exposed to different conditions. Active Caspase 3 measurement by immunoblotting in cell homogenates prepared in RIPA or M2-PK homogenisation buffer failed to reveal any band. Active Caspase 3, 7 and 8 bioluminescence was measured which did not show any difference between control and altered culture conditions. Bax and Bcl-2, the apoptotic and anti-apoptotic markers of mitochondrial apoptosis pathway were also measured. No difference between Bax or Bcl-2 expression was observed between treated and untreated cells. A possible reason for difficulty in detecting these apoptotic markers could have been the difference in time interval between their appearance and measurement in cell culture. Loss of stability of these markers during culture processing also could not be ruled out. Therefore, fluorescein isothiocyanate (FITC) conjugated Annexin V-Propium Iodide (PI) staining, which is a general apoptotis-necrosis marker and was used for adherent cells to see early or delayed apoptosis and necrosis. Annexin PI staining of pancreatic cancer cells in this study showed that the mode of cell death was predominantly necrosis rather than apoptosis. Therefore, Flouroscein Activated Cell Sorting (FACS) analysis was used to quantitate necrosis as well as apoptosis which confirmed predominance of necrosis in normal as well as treated cells.

9.2.6 Tetramer-dimer conversion of M2-PK

The tetrameric form has a high affinity to its substrate phosphoenolepyruvate (PEP), whereas the dimeric form is characterised by a low PEP affinity (20). This means that at physiological PEP concentrations, the tetrameric form has high PK activity whereas the dimeric form has low. The tetramer:dimer ratio is not a stationary value, but rather oscillates between the tetrameric and dimeric forms of M2-PK and this oscillation is regulated by the intracellular fructose 1,6-biphosphate concentrations (197). This study hypothesised the tumour microenvironment to be a regulatory factor in tetramer-dimer switch. Direct measurement of tetramer and dimer ratio was challenging as an antibody directly against the tetrameric M2-PK was not available. Separation of the tetrameric and dimeric forms of M2-PK is possible by gelpermeation or by free-flow isoelectric focusing. In the isoelectric focusing, the entire glycolytic enzyme complex focuses at a common isoelectric point. In the case of M2-PK only the tetrameric form is associated with other glycolytic enzymes within the glycolytic enzyme complex. The dimeric form focuses outside the complex at a more alkaline pH value. Similarly, after separation of the tetrameric and dimeric form of M2-PK by gelpermeation the amount of the M2-PK protein in the eluted fractions of the tetrameric form and the dimeric form can be identified by immunoblotting with the DF4 antibody. However, the measurement of tetrameric and dimeric fraction by these methods is semiquantitative. Determination of the tetramer:dimer ratio by immunoblotting alone is not possible because the protein gets denatured, breaking the tetrameric and dimeric subunits into monomers. Antibody to detect the tetrameric form of M2-PK was not available. In the absence of locally available technical expertise, gelpermeation chromatography was not used for tetramer:dimer M2-

PK ratio measurement in this study. Instead, tetramer-dimer conversion was indirectly demonstrated by measuring total M2-PK by immunoblotting, dimeric M2-PK by ELISA and total pyruvate kinase activity by photometric analysis. The main strength of these methods is that these are validated, reproducible and local expertise was available. However with exception to ELISA, they do not directly quantify tetrameric or dimeric form of M2-PK. Therefore a direct method of measuring tetramer:dimer ratio in tissue culture is needed.

9.3   Overall Conclusion

– Tumour M2-PK can be reliably measured in cell lines using ELISA and the levels are inversely proportional to cell density. The higher levels of Tumour M2-PK in the cancer cell line with higher metastatic potential would suggest a correlation between growth and spread of cancer which should be further revalidated in other human pancreatic cancer cell lines.

– Suppression of growth of pancreatic cancer cell lines by the altered tumour microenvironment is associated with variations in levels of dimeric M2-PK without change in total M2-PK protein expression and a tetrameric-dimeric switch on glucose deprivation of tumour cells and a dimeric-tetrameric shift on exposure to hypoxia.

– The current experimental model does not support the tetrameric-dimeric switch-over of M2-pyruvate kinase as being a possible metabolic adaptation of pancreatic cancer cells to altered microenvironment.

– M2-pyruvate kinase activity in tumour cells is localised to intracellular membrane-bound cytoplasmic structures with no translocation to mitochondria or nucleus on exposure to altered microenvironment.

9.4   Future Research Implications

Although Tumour M2-PK is a cancer marker, this study has provided a platform for evaluating its biological role in understanding the commonest challenge faced by many cancer clinicians in treating pancreatic cancer i.e. resistance to therapy. This study has observed an altered metabolic response to adverse physiologic microenvironment in tumour cells. This association needs to be further consolidated by a robust experimental design involving blocking M2-PK or interfering with tetramer-dimer shift. The possible models that we propose are:

- Direct inhibitor of M2-pyruvate kinase activity as described recently (170;189)

- PK-M2 antisense oligonucleotide vector-transfected cell model as used recently (47)

- M2-PK tetramer:dimer ratio modulator – Fructose 1,6 biphosphate as described before.

If the association of M2-PK with pancreatic cancer tolerance to apoptosis is established by using the above-stated experimental models, targeting M2-PK could be a potential armamentum in cancer therapy.

APPENDICES

APPENDIX I

Presentations and Publications Out of Thesis

Presentations

 - Response of the dimeric form of M2-pyruvate kinase to altered microenvironment in pancreatic cancer cells.

8th World Congress of IHPBA 27 February–2 March 2008, Mumbai, India (Data from Chapter 6).

Background: Hypoxic and nutritionally deprived areas are thought to confer resistance to pancreatic cancer cells to apoptosis. Tumour M2-pyruvate kinase, a tumour associated isoenzyme of pyruvate kinase, is elevated in patients with pancreatic cancer.

Aim: This study aimed to observe the effect of altered tumour microenvironment on levels of Tumour M2-pyruvate kinase in relation to resistance of pancreatic cancer cell lines to apoptosis.

Materials and Methods: The dimeric form of M2-pyruvate kinase was measured in cell homogenate supernatant of Panc-1 (ductal cancer origin) and Colo 357 (metastatic lymph node origin) human pancreatic cancer cell lines, exposed to acidic pH (6.5), hypoxic (1% O_2) and glucose-deprived culture conditions for 24, 48, 72 and 96 hrs, using sandwich type ELISA (ScheBo® Tumour M2-PK™) based on monoclonal antibodies specific for it. Total M2-pyruvate kinase protein expression was measured semiquantatively by western blotting.

Apoptosis in these conditions was measured by FITC Annexin V and Propium Iodide FACS analysis and Active Caspase 3 and 8 assays.

Results: Tumour M2-PK level was significantly enhanced in Colo 357 cells ($p<0.05$) at acidic pH compared to normal, hypoxic or glucose-deprived culture condition without any change in total M2-PK protein expression. No significant difference was seen between normal and altered microenvironment conditions in terms of cell viability and apoptosis in both cell lines.

Conclusion: High levels of Tumour M2-pyruvate kinase levels in metastatic cancer cell lines exposed to acidic environment may indicate a survival strategy of cancer cells in altered tumour microenvironment.

- Levels of M2-Pyruvate kinase in tumour cells are influenced by low pH, nutrient deprivation and hypoxia.

Y. Kumar, S. Yang, B. Fuller, S. Mazurek, B.R. Davidson, 39th European Pancreatic Club meeting, 5 July 2007, Newcastle, UK **(Data from chapter 6).**

1) University Department of Surgery, Royal Free and University College Medical
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Introduction: Hypoxic and nutritionally deprived areas are thought to contribute to resistance of tumour cells to apoptosis in pancreatic cancers. Tumour M2-pyruvate kinase, a tumour associated isoenzyme of pyruvate kinase, is elevated in patients with pancreatic cancer. This study aims to measure Tumour M2-pyruvate kinase (TuM2-PK) in different human pancreatic cancer cell lines following exposure to acidic pH, hypoxia and glucose-deprivation.

Materials and Methods:

TuM2-PK expression was measured in cell lysate supernatant of HMEF, MCF7, Panc-1 and Colo 357 human cell lines using sandwich type ELISA kits (ScheBo® Tumour M2-PK™) based on two monoclonal antibodies specific for it. Panc-1 and Colo 357 were grown in acidic pH (6.5), hypoxic (1% O_2) and glucose-free medium (DMEM). TuM2-PK was expressed as milli U/10^6 cells.

Mean TuM2-PK values between different cell lines were compared using Two-way ANOVA and unpaired T test. Correlation between TuM2-PK levels and cell densities was made using Pearson's coefficient.

Results: M2-PK expression was significantly higher in Colo 357 cells compared to MCF 7 (p<0.05) and Panc-1 cells (p<0.001). Correlation between TuM2-PK levels and different [MCF7 (r=-0.382, p= 0.06), Panc-1 (r= - 0.096, p= 0.065) and Colo 357 (r= -0.528, p< 0.01) cell densities was negative. Tumour M2-PK expression was significantly enhanced in Colo 357 cells (p<0.05) at acidic pH or glucose-deprived condition compared to normal culture condition. Levels were also elevated in hypoxic condition.

Conclusion: TuM2-PK can be measured in different cell lines using ELISA. *In vitro* expression of TuM2-PK correlates with cell density. Acidic, glucose-deprived and hypoxic conditions in tumour cells enhance M2-PK expression.

- Tumour M2-Pyruvate kinase expression in pancreatic cancer cell lines.

Y. Kumar, N. Kirmani, S.Dijk, B. Fuller, S. Mazurek, B. Davidson.

Oral and poster presentation at Society of Academic Research Surgery meeting, 10–12 January 2007, Cambridge **(Data from chapter 5).**

Introduction: Hypoxic and nutritionally deprived areas are thought to contribute to resistance of tumour cells to apoptosis in pancreatic cancers. Tumour M2-pyruvate kinase, a tumour-associated isoenzyme of pyruvate kinase, is elevated in patients with pancreatic cancer. This study aims to measure Tumour M2-pyruvate kinase (TuM2-PK) in different human pancreatic cancer cell lines following exposure to acidic pH and glucose-deprivation.

Materials and Methods: TuM2-PK expression was measured in cell lysate supernatant of HMEF, MCF7, Panc-1 and Colo 357 human cell lines using sandwich type ELISA kits (ScheBo® Tumour M2-PK™) based on two monoclonal antibodies specific for it. Panc-1 and Colo 357 were grown in acidic pH (6.5) and glucose-free medium (DMEM). TuM2-PK was expressed as milli I.U/10^6 cells.

Mean TuM2-PK values between different cell lines were compared using two-way ANOVA and unpaired T test. Correlation between TuM2-PK levels and cell densities was made using Pearson's coefficient.

Results: M2-PK expression was significantly higher in Colo 357 cells compared to MCF 7 ($p<0.05$) and Panc-1 cells ($p<0.001$). Correlation between TuM2-PK levels and different [MCF7 ($r=-0.382$, $p= 0.06$), Panc-1 ($r=- 0.096$, $p= 0.065$) and Colo 357 ($r=-0.528$, $p< 0.01$) cell densities was negative. At acidic pH, Tumour M2-PK expression was

significantly enhanced in Colo 357 cells (p<0.05) at acidic pH or glucose-deprived or hypoxic condition in the culture medium compared to normal conditions.

Conclusion: TuM2-PK can be measured in different cell lines using ELISA. *In vitro* expression of TuM2-PK correlates with cell density. Acidic, glucose-deprived and hypoxic conditions around tumour cells enhance M2-PK expression.

British Journal of Surgery 2007/ 94 (S3): 4 (Published abstract).

Publications

- Kumar, Y., Gurusamy, K.S., Davidson, B.R., **Tumour M2-Puruvate Kinase: A marker of exocrine pancreatic cancer-A meta-analysis,** Pancreas 2007 Aug/35 (2): 114-9. **(Paper from chapter 2)**

- Kumar, Y., Tapuria, N., Kirmani, N., Davidson, B.R.**, Tumour M2-pyruvate kinase: A GI cancer marker,** Eur J Gastroenterol Hepatol 2007 Mar/19(3):265-76. **(Paper from chapter 1)**

APPENDIX-II

*Copy of a result **printout** from the plate-reader.*

Raw Data	Average	Corr. Av.	Conc.	Actual
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Standards

	Absorbance1	Absorbance2	Average	corr. Av.			
STD1	0.456	0.423	0.440	0.011		4.38	5
STD2	0.664	0.507	0.586	0.157		18.53	15
STD3	1.216	1.291	1.254	0.825		44.96	40
STD4	3.010	2.951	2.981	2.552		82.14	100

Positive Control

					Conc.	
	Absorbance1	Absorbance2	Average	corr. Av.	[U/ml]	
PC	0.629	0.671	0.650	0.221	22.27	**Control okay!**

Blanks

	Absorbance1	Absorbance2	Average
Blank	0.426	0.432	0.429

Samples

					Conc.
	Absorbance1	Absorbance2	Average	corr. Av.	[U/ml]
S1	0.674	0.544	0.609	0.180	19.96
S2	0.676	0.647	0.662	0.233	22.88
S3	0.730	0.696	0.713	0.284	25.46
S4	0.574	0.603	0.589	0.160	18.72
S5	0.671	0.652	0.662	0.233	22.88
S6	0.668	0.645	0.657	0.228	22.62
S7	0.431	0.429	0.430	0.001	1.25
S8	0.480	0.492	0.486	0.057	10.81
S9	0.950	0.896	0.923	0.494	34.21
S10	2.500	2.466	2.483	2.054	73.16
S11	0.495	0.564	0.530	0.101	14.63
S12	1.916	1.991	1.954	1.525	62.41
S13	0.488	0.455	0.472	0.043	9.24
S14	0.675	0.693	0.684	0.255	24.04
S15	0.515	0.574	0.545	0.116	15.76
S16	1.270	1.343	1.307	0.878	46.48
S17	0.793	0.767	0.780	0.351	28.51
S18	1.089	1.192	1.141	0.712	41.56

S19	2.617	2.744	2.681	2.252	76.84
S20	1.580	1.568	1.574	1.145	53.57
S21	2.756	2.803	2.780	2.351	78.62
S22			#DIV/0!	#DIV/0!	#DIV/0!
S23	2.613	2.588	2.601	2.172	75.37
S24			#DIV/0!	#DIV/0!	#DIV/0!
S25	2.039	2.083	2.061	1.632	64.72
S26	2.695	2.688	2.692	2.263	77.04
S27	1.704	1.808	1.756	1.327	57.95
S28	1.868	1.927	1.898	1.469	61.17
S29	2.421	2.528	2.475	2.046	73.00
S30	1.826	1.858	1.842	1.413	59.93
S31	0.886	0.903	0.895	0.466	33.14
S32	2.327	2.335	2.331	1.902	70.22
S33	1.414	1.392	1.403	0.974	49.14
S34			#DIV/0!	#DIV/0!	#DIV/0!
S35	0.928	0.844	0.886	0.457	32.82
S36	1.089	1.064	1.077	0.648	39.52
S37	1.394	1.887	1.641	1.212	55.21
S38	2.819	2.891	2.855	2.426	79.96
S39	0.950	1.033	0.992	0.563	36.66
S40	2.181	2.232	2.207	1.778	67.73
S41	1.465	1.434	1.450	1.021	50.38
S42	1.657	1.575	1.616	1.187	54.61

underlying formulae

Average = Average of Absorbance1 and Absorbance2

corrected Average = Average - Average of Blanks

Conc. : according to Regression Analysis and Standard Curve
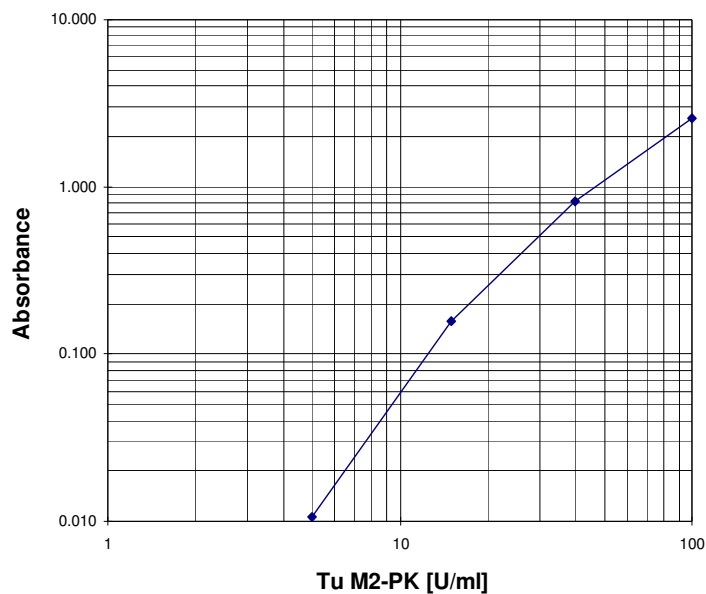
Standard Curve

using regression analysis

	STD-Conc.	corr. Av.	log(Conc.)	Log (corr. Av.)
	[U/ml]			
STD1	5	0.011	0.699	-1.979
STD2	15	0.157	1.176	-0.805
STD3	40	0.825	1.602	-0.084
STD4	100	2.552	2.000	0.407

log(conc)=m*log(OD)+b

Statistics

m	0.533	1.698	B
Std. F.	0.058	0.063	
r^2	0.977	0.104	
F	85.101	2.000	Df
ss(reg)	0.917	0.022	ss(resid)

List of Abbreviations

TuM2-PK: Tumour M2-pyruvate kinase

DMEM: Dulbecco's modified eagle's medium

EMEM: Earl's Minimum Essential Medium

FBS: Foetal bovine serum

PBS: Phosphated buffered saline

EDTA: Ethylenediaminetetraacetic acid

MES: 2-(N-Morpholino)ethanesulfonic acid

HEPES: 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

KH_2PO_4: Potassium phosphate monobasic

KCl: Potassium chloride

$MgCl_2$: Magnesium chloride

NADH: β-Nicotinamide adenine dinucleotide, reduced dipotassium salt

ADP: Adenosine diphosphate

LDH: Lactate dehydrogenase

PEP: Phosphoenolpyruvate

FITC: Fluorescein isothiocyanate

ATP: Adenosine triphosphate

MOPS: 3-(N-morpholino) propane sulfonic acid

PVDF: Polyvinylidene difluoride

COX: Cytochro

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