PROTEIN EXPRESSION IN COLORECTAL CANCER

Thesis submitted in accordance

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This MD thesis is dedicated to my father and step-mother, Mark and Annabel; my mother Sandra; and my sisters Rachel and Jennifer, with thanks for their support.

ABSTRACT

Introduction: Colorectal cancer is the second most common UK cancer. Biomarkers which predict survival may be valuable for targeting adjuvant therapy and can provide insights into tumour biology. Small and early cancers are being diagnosed more commonly in the UK population due to the introduction of population-based colorectal cancer screening in 2005. Analysis of resected small (≤20mm across) tumours in Liverpool has established that flat and depressed morphology can predict advanced stage at presentation. Proteomic analysis of small cancers was conducted with the aim of generating biomarkers which correspond to morphology, stage and patient survival.

Patients and Methods: Laser capture microdissection was used to procure enriched matched benign and malignant colorectal epithelial cell populations. Laser captured proteins were extracted into lysis buffer, normalised against a reference standard, separated using 2D SDS-PAGE and visualized with silver staining. Comparison was made between the tumour gels, (n=10) and matched normal colonic gels, (n=9) by two different observers and gel analysis software, Progenesis SameSpots. Differentially expressed proteins were identified using tandem mass spectrometry and included redox proteins peroxiredoxin 2, peroxiredoxin 6 and SH3 binding glutamic acid-rich protein-like 3; and cytoskeletal protein cofilin1. Also identified were the anti-apoptotic protein heat shock protein 27 and inflammatory protein S100A8, which had been previously identified in 2D gel analysis of undissected colorectal cancer in our Institution (n=12 gels) and previously validated in a small cohort of paraffin-embedded colorectal cancers (n=98). In this study, HSP27 was further

evaluated in a large cohort of paraffin-embedded colorectal cancer tissue (n=404). S100A8 and related proteins S100A9 and Smad4 were similarly evaluated in a large cohort (n=313).

Results: High HSP27 levels were strongly associated with poor cancer-specific survival in rectal cancer (n=205, *P*=0.0063) but not colon cancer; (n=199, *P*=0.7385). Multivariate Cox regression confirmed nodal metastases (*P*=0.0001) and HSP27 expression (*P*=0.0233) as independent markers of survival in rectal cancer. HSP27 levels remained unchanged in the majority of cases 65/80 (81%) between diagnostic biopsies and matched surgical samples, regardless of whether patients had undergone preoperative radiotherapy.

S100A8 expression co-localised with a subset of S100A9-positive monocytes. S100A9 was co-expressed with CD14 in tumour-associated monocytes, but not with CD68 in tissue macrophages. Smad4 was expressed in the tumour cytoplasm of 262/304 (14%) tumours. Loss of Smad4 expression correlated with a reduction in the stromal S100A8-positive, but not S100A9-positive cell count, (*P*=0.034, Mann-Whitney U test) and was associated with a poorer overall survival in patients with stage I-II disease, but not stage III disease. Antibodies to cofilin1 and cofilin-phospho(ser3) were assessed in colorectal cancer cell and tissue lysate and found to be specific on 1D and 2D western blot.

Conclusion: Elevated HSP27 is an independent marker of poor prognosis in rectal cancer whose expression is not altered by neo-adjuvant radiotherapy. Smad4-negative tumours are associated with fewer infiltrating S100A8 positive stromal monocytes. In node-negative tumours, loss of Smad4 expression in associated with a poorer prognosis. These findings provide a sound platform for further investigation of both S100A8 and HSP27 proteins in colorectal cancer.

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ABBREVIATIONS

2DE	Two-dimensional electrophoresis
5FU	5-fluorouracil
µg	Microgram
µl	Microlitre
µM	Micromolar
^{o}C	degrees Celsius
ACG	American College of Gastroenterologists
ACN	Acetonitrile
AJCC	American Joint Committee on Cancer
AMH	Anti-mullerian hormone
APC	Adenomatous polyposis coli
APS	ammonium persulphate
ATM	Ataxia-telangiectasia mutated gene
AUC	Area under curve
BCA	Bicinchoinic acid
BCL2	B-cell lymphoma 2
BMP	Bone morphogenic protein

BRCA	Breast cancer associated
BSG	British Society of Gastroenterologists
C	Cytosine
CA19-9	Carbohydrate antigen 19-9
CA242	Carbohydrate antigen 242
CCD	Charged couple device
Cdc2	Cell division cycle 2
CDK	Cyclin dependant kinase
CEA	Carcinoembryonic antigen
CHAPS	3-(3-cholamidopropyldimethylammonio-1-propane) sulphate
CHRPE	Congenital hypertrophy of retinal pigment epithelium
CI	Confidence interval
CID	Collision induced decomposition
CIMP	CpG-island methylator phenotype
CIN	Chromosomal instability
CRC	Colorectal cancer
CRM	Circumferential resection margin
CT	Computed tomography
DCBE	Double contrast barium enema
DCC	Deleted in colorectal cancer

DIGE	Difference in-gel electrophoresis
DNA	Deoxyribonucleic acid
Dpi	dots per inch
DSH	Dishevelled
dTMP	Deoxythymidine monophosphate
DTT	Dithiothreitol
dUMP	Deoxyuridine monophosphate
EDTA	Ethylendiaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTM	European Group on Tumour Markers
EMR	Endoscopic mucosal resection
ESI	Electrospray ionization
EVI	Extramural vascular invasion
FA	folinic acid
FACS	Flourescence activated cell sorting
FAP	Familial adenomatous polyposis
FGF2	Fibroblast growth factor 2
fmol	femtomole (10^{-15}M)
FOBT	Faecal occult blood test

FTICR	Fourier transform ion cyclotron
G	Guanine
GDF	Growth and differentiation factors
GDP	Guanidine diphosphate
GI	Gastrointestinal
GRX	Glutaredoxins
GSK3β	Glycogen synthase kinase 3- beta
GTP	Guanidine triphosphate
H	Hydrogen
H&E	Haematoxylin and eosin
HCl	Hydrochloric acid
HNPCC	Hereditary Non-polypoid Colorectal Cancer
HR	Hazard ratio
HSP27	Heat shock protein 27
HSE	Heat shock element
HSF1	Heat shock factor 1
ICAT	Isotope coded affinity tags
id	Internal diameter
IEF	Isoelectric focusing
IFL	irinotecan/fluorouracil/leucovorin chemotherapy

IHC	Immunohistochemistry
IPG	Immobilised pH gradient
IRA	Ileorectal anastomosis
iTRAQ	Isobaric tags for relative and absolute quantification
JRSC	Japanese Research Society on Colon Cancer
kDa	KiloDaltons
L	Deuterium
LC	Liquid chromatography
LC-MS	Liquid chromatography plus mass spectrometry
LCM	Laser Capture microdissection
M	Molar
ml	Mililitre
MALDI	Matrix assisted laser desorption ionization
Mdm2	Murine double minute 2
MGMT	0-6-methylguanine DNA methyltransferase
min	Minute
mM	Milimolar
MMP2	Metalloproteinase 2

MMR	Mis-match repair
ms	Milisecond
MS	Mass spectrometry
MSI	Microsatellite instability
MSI-H	High level microsatellite instability
MSI-L	Low level microsatellite instability
MS/MS	Tandem mass spectrometry
mW	Miliwatt
NBS1	Nijmegen breakage syndrome 1
NCBI	National Center for Biotechnology Information
NICE	National institute for clinical excellence
NCI	National cancer institute
ng	Nanogram
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMF	Peptide mass fingerprinting
Prx	Peroxiredoxin
PTEN	Phosphate and tensin homolog gene

RAGE	Receptor for advanced glycation end products
RB	Retinoblastoma
Rcf	Relative centrifugal force
RNA	Ribose nucleic acid
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RR	Relative risk
s	Second
SDS	Sodium dodecyl sulphate
SH3BGRPL3	SH3 binding glutamic acid rich protein like-3
SIL	Stable isotope labelling
SPARC	Secreted protein, acidic and rich in cysteines
TAM	Tissue associated macrophage
TCF	T- cell factor
TEM	Transanal endoscopic microsurgery
TEMED	N,N,N',N' – tetramethylethylenediamine
TFA	Trifluoroacetic acid
TGFβ	Transforming Growth Factor-beta
TIFF	Tagged image file format
TIMP	Tissue inhibitor of matrix metalloproteinase

TIP-B1	Tumour necrosis factor inhibitory protein – B1
TMA	Tissue microarray
TNFα	Tumour necrosis factor-alpha
TNM	Tumour/Nodes/Metastases
Tof	Time of flight
TPA	Tissue polypeptide antigen
TPS	Tissue polypeptide specific antigen
Tris	tris(hydroxymethyl)aminomethane
TS	Thymidylate synthase
US	Ultrasound
V	Volt
VEGF	Vascular endothelial growth factor
w/v	weight per volume

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1. INTRODUCTION

1.1 COLORECTAL ADENOCARCINOMA: INCIDENCE AND SURVIVAL

Colorectal cancer is the second most common cancer in the UK. Worldwide over a million new cases of colorectal cancer are diagnosed every year, accounting for more than 9% of all new cancer cases. In the UK there are approximately 16,000 deaths from colorectal cancer per year, of which 5800 (36%) are cancer of the rectum. The incidence of colorectal cancer in the UK is approximately 59 cases per 100,000 persons. It is strongly related to age with 83% of cases arising in people who are 60 years or older. Rectal cancer is more common in men with 58% diagnosed in males compared to 42% in females. The incidence of colon cancer is more equal, with 51% diagnosed in men and 49% women. The lifetime risk of the disease is estimated at 1 in 18 for men and 1 in 20 for women. Females demonstrate an improved five-year survival compared to men. In the early 1970s, five-year relative survival for male colon cancer was 22% (23% for women) and this rose to 47% (48% for women) in the late 1990s. Over the same time period, the five-year survival rates for male rectal cancer rose from 25% to 47% and from 27% to 51% for female rectal cancer.(1) Despite these improvements, five-year survival in the UK remains poor in comparison to the rest of Europe, with age-standardised rates of 45% compared to France and Germany with 50-55%.(2) The USA has consistently higher five-year survival rates for cancer of the colon and rectum compared to European countries. Data from nine areas in the USA in the period 1990-1994 showed 5-year

survival rates of 59-66%.(2) These results are attributed to the early detection of colorectal cancer through screening programs funded by private health insurance companies across the USA. (3)

1.2 COLORECTAL ADENOCARCINOMA: PROGNOSIS

The association between pathological stage and prognosis was first documented by Cuthbert Dukes in his work on rectal cancer in 1950.(4) Other later classification systems, such as the TNM and Astler-Coller staging are refinements of the principles established by Dukes, namely that depth of tumour invasion and presence of lymph node or distant metastases is inversely correlated with patient survival,(5-7) (Figure 1.1). In 2000 the American Joint Committee on Cancer Prognostic Factors Consensus Conference listed several histopathological parameters in addition to stage which were associated in prognosis in colorectal cancer which should be documented in pathology reporting. These were residual tumour at the radial or excision margins, differentiation grade into high versus low, tumour border configuration, venous and lymphatic invasion and perineural invasion. Insufficient evidence at that time was available for the routine use of DNA content, microvessel density and tumour tissue markers such as p53, k-ras, DCC, MSI and p21 for routine clinical prognostic grading.(8, 9)

1.2.1 STAGING

Staging of colorectal cancer is based on the three criteria; invasion through the bowel wall (T), lymph node metastases (N) and distant metastases (M). The components of the TNM score from the 7th edition of the American Joint Committee on Cancer staging for colorectal cancer are listed below.(10)

Primary tumour (T)	**Lymph node metastases (N)**	**Distant Metastases (M)**
TX-Primary tumor cannot be assessed	**NX** Regional lymph nodes cannot be assessed	**M0** No distant metastasis
T0-No evidence of primary tumour	**N0** No regional lymph node metastasis	**M1** Distant metastasis
Tis-Carcinoma in situ: intraepithelial or invasion of lamina propria	**N1** Metastasis in 1–3 regional lymph nodes	**M1a** Metastasis confined to one organ (for example, liver, lung, ovary, nonregional node)
T1-Tumour invades submucosa	**N1a** Metastasis in one regional lymph node	**M1b** Metastases in more than one organ or the peritoneum
T2-Tumour invades muscularis propria	**N1b** Metastasis in 2–3 regional lymph nodes	
T3-Tumour invades through the muscularis propria into pericolorectal tissues	**N1c** Tumor deposit(s) in the subserosa, mesentery, or nonperitonealized pericolic or perirectal tissues without regional nodal metastasis	
T4a-Tumour penetrates to the surface of the visceral peritoneum	**N2** Metastasis in 4 or more regional lymph nodes	
T4b-Tumor directly invades or is adherent to other organs or structures	**N2a** Metastasis in 4–6 regional lymph nodes	
	N2b Metastasis in 7 or more regional lymph nodes	

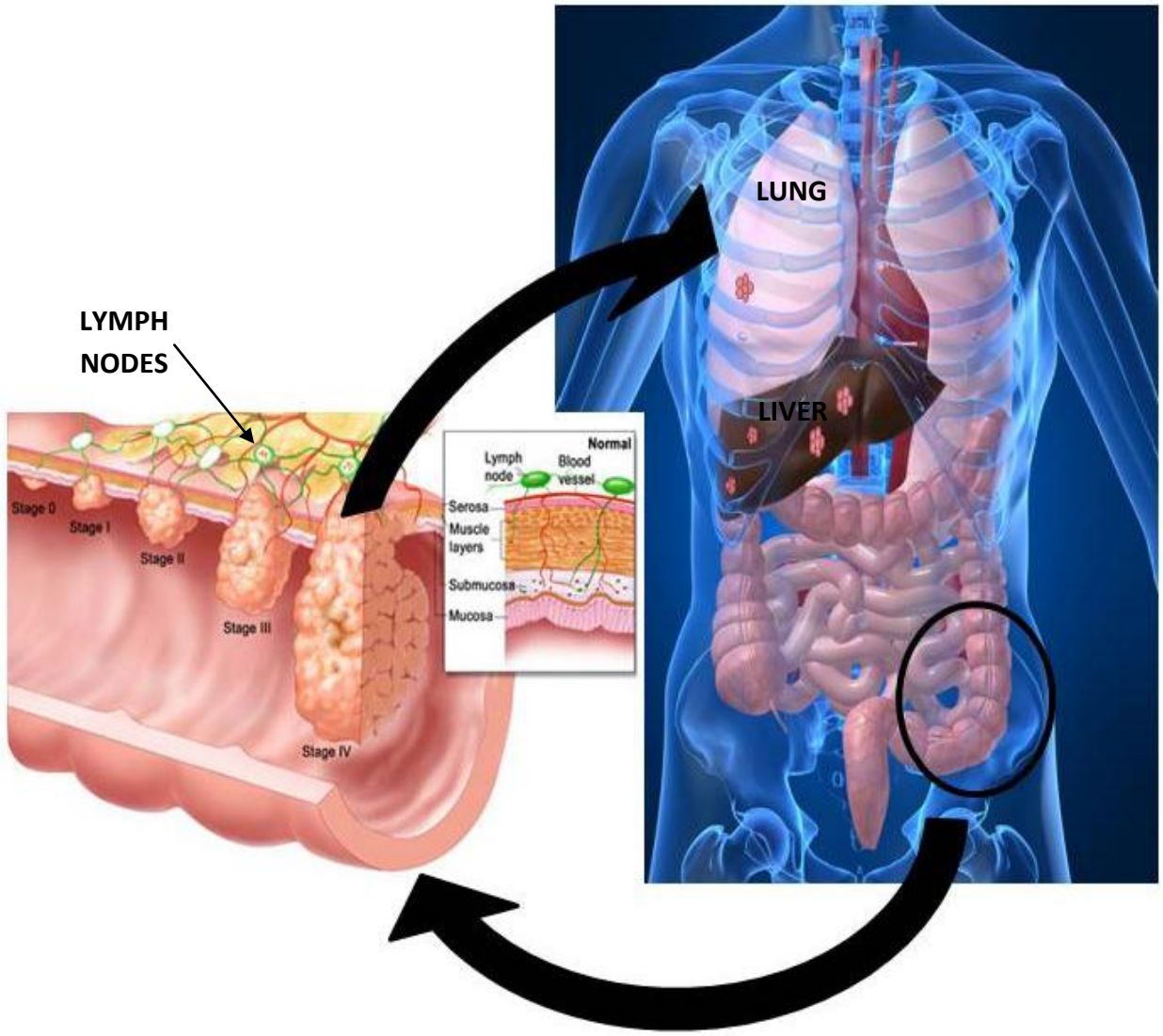


Figure 1.1: Staging of colorectal cancer. Depicts tumour infiltration through bowel wall (T-stage), Lymph node metastases (N-stage) and distant metastases (M-stage). Images adapted from The Carver Colon Cancer Surgical Centre (http://www.carverclinic.com/index.php) and Chicago's Northside Colon & Rectal Clinic (http://cnscrc.com/colon_and_rectal_screening).

Colorectal cancer is a curable disease. In the early stages of the disease, adequate resection of a tumour with no lymph node metastases can result in a five-year survival of 77% for T3-T4 lesion and 93.2% for T1-T2 lesions, (Table 1.1). The presence of lymph nodes metastases is the strongest prognostic factor for colorectal cancer. Five-year survival for patients with 1-3 positive lymph nodes (N1 disease) is 35-60%, whereas $\geq$ 4 positive lymph nodes is associated with a 25-27% survival.(11) Currently, 23.6% of patients with colorectal cancer present with nodal metastases and 9.2% present with distant metastases in the UK.(1) A further 40-50% of patients will develop distant metastases following resection of the primary colorectal cancer. The median survival in this group of patients is 8 months without treatment and five-year survival is 0-7%. Patients with liver or lung metastases amenable to surgical resection have a five-year survival of approximately 25% at five-years.(12)

Dukes' Stage	Frequency	Astler-Coller stage	TNM stage	5-year survival
A	8.7%	I	T1-2, N0, M0	80-95%
B	24.2%	IIA	T3, N0, M0	72-75%
		IIB	T4, N0, M0	65-66%
C1	23.6%	IIIA	T1-2, N1, M0	55-60%
		IIIB	T3-4, N1, M0	35-42%
C2		IIIC	Any T, N2, M0	25-27%
D	9.2%	IV	Any T, Any N, M1	0-7%
Unknown	34.3%	-	-	35.4%

Table 1.1: 5-year survival by stage[1,11,12]

1.2.2 EXCISION MARGINS

The radial margin of a colorectal resection specimen represents the adventitial soft tissue margin of a nonperitonealized surface. A corresponding adventitial margin also exists for the ascending colon, descending colon, and rectosigmoid colon, all of which are only partially peritonealized. In those segments of the colon that are encased completely by a peritonealized (serosal) surface (the cecum, transverse colon, and sigmoid colon), the only radial margin is the mesenteric resection margin. The radial margin has been defined fully and studied specifically in rectal cancer, multivariate analysis has suggested that tumor involvement of this margin may be the single most critical factor in predicting local recurrence of rectal carcinoma. One such meta-analysis of 17,500 rectal cancer resections demonstrated that involvement of the circumferential resection margin (CRM) is a powerful predictor of both development of distant metastases (HR = 2.8; 95% CI, 1.9 to 4.3) and survival (HR = 1.7; 95% CI, 1.3 to 2.3). After neoadjuvant therapy (both radiotherapy and radiochemotherapy), the predictive value of the CRM for local recurrence is significantly higher than when no preoperative therapy has been applied (hazard ratio [HR] = 6.3 v 2.0, respectively; $P < 0.05$).(13) Involvement of the retroperitoneal resection margin in right-sided colon cancer has also been shown as a poor prognostic marker. In this group of patients margin involvement appears to correlate with the presence of distant metastases and advancing stage at presentation.(14)

1.2.3 HISTOLOGICAL GRADE

Histological grade has been shown repeatedly to be of independent prognostic significance by multivariate analysis, as long as the grade assigned is reproducible and reflects the most poorly differentiated area of tumour.(15) One multivariate analysis in n=368 showed that differentiation

grade was the only other variable apart from Dukes' stage and tumour site which remained independently significant on multivariate analysis with a hazard ratio 0.76 (<0.02) for moderate to well differentiated and 0.78 (p<0.01) for poor to moderately differentiated.(16)

1.2.4 EXTRAMURAL VASCULAR INVASION AND LYMPHATIC INVASION

Extramural vascular invasion (EVI) by tumour has been demonstrated to have an independent adverse impact on outcome by multivariate analysis in many studies. A recent review of 5947 resected colorectal cancers in the Yorkshire area showed that 17.8% of tumours are EVI+ and that the relative five year survival is 0.41(0.37 0.44), *P*=0.01.(17) Lymphatic invasion was also associated with poorer overall survival in a study of 462 patients (colon cancer: 57% vs. 84%, *P*=0.0001; rectal cancer: 38% vs. 71%, *P*=0.004). Patients with lymphatic vessel invasion also had a significantly increased incidence of positive nodes (59% vs. 25%, *P*=0.0004).(18)

1.2.5 PERINEURAL INVASION

Perineural invasion is closely correlated with extramural vascular invasion, however a number of studies have suggested that it is independently predictive of survival on multivariate analysis. One such study found that overall survival was 25% in the presence of neural invasion and 64% without neural invasion (p<.01).(19)

1.2.6 TUMOUR BORDER CONFIGURATION

For colorectal carcinoma, the growth pattern of the tumour at the advancing edge (tumour border) has been shown to have prognostic significance that is independent of stage and may predict liver metastasis. Specifically, an "irregular, infiltrating pattern of growth" as opposed to a "pushing

border" has been demonstrated to be an independent adverse prognostic factor by several univariate and multivariate analyses.(20) Diagnostic criteria recommended for detection of an infiltrative border are as follows; inability to define limits of invasive border of tumour and/or an inability to resolve host tissue from malignant tissue, dissection of tumour through the full thickness of the muscularis propria without stromal response and/or dissection of mesenteric adipose tissue by small glands or irregular clusters or cords of cells.(8)

1.3 MERSEYSIDE AND THE NORTH WEST

The incidence of colorectal cancer in the North West is above the English average in males (56 versus 51 cases per 100,000 population) and very similar to the UK average in females (34 cases per 100,000 population). The mortality in the North West region however is proportionally greater than the English average in both sexes and is the highest of all the regions in England. In the UK as a whole the North West region fares better than Scotland, Ireland and Wales; all of which have a higher incidence and mortality rate for colorectal cancer.(21) I retrospectively analysed 662 patients who underwent surgical resection for colorectal cancer in the Royal Liverpool University Hospital between 1999 and 2004. Overall survival data are shown in Figure 1.2. The proportion of patients with nodal metastases is high compared to the UK average, (40% versus 23.6%) whereas the proportion of patients presenting with distant metastases is less, (8% versus 9.2%).[1] The

Liverpool group of patients, however only include those who underwent resection of their primary tumour.

A univariate and multivariate analysis was conducted of the data using a stepwise forward selection approach. We confirmed that in our Liverpool cohort of n=662 the usual factors, age, T-stage, N-stage, differentiation grade, resection margin status and vascular invasion remained independently significant. In this group, perineural invasion and border configuration did not retain independent significance.

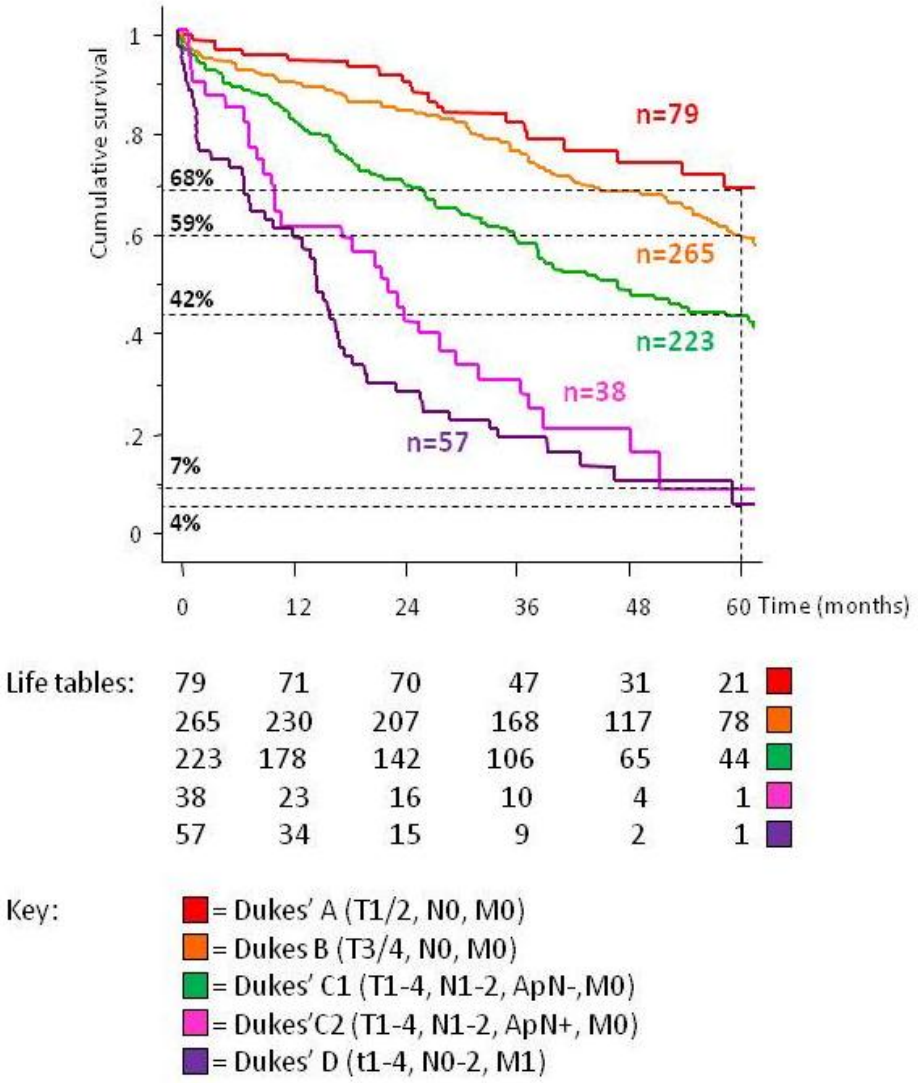


Figure 1.2: Overall survival according to Dukes' stage in n=662 patients with resectable colorectal cancer operated between 1999 and 2004.

Variable	Group	Cases n= 662 (%)	Univariate			Multivariate		
			HR(95%CI)	χ^2	p-value	HR(95%CI)	χ^2	p-value
Age (years)	71 years (34-99)	-	1.029 (1.017-1.041)	23.613	**0.001**	1.037 (1.025-1.050)	34.964	**0.001**
Diff Grade	Well	9 (1)	-	21.259	**0.001**	-	8.098	**0.017**
	Moderate	564 (85)	0.973 (0.311-3.04)	0.002	0.963	0.657 (0.207-2.082)	0.511	0.475
	Poor	44 (7)	2.313 (0.208-7.560)	1.926	0.165	1.123 (0.337-3.738)	0.036	0.850
	Unrecorded	45 (7)						
Resection margins	Involved	563 (85)	-			-	-	
	Clear	98 (15)	0.449 (0.343-0.588)	33.931	**0.001**	0.660 (0.482-0.902)	6.771	**0.009**
	Unrecorded	1 (0)						
Vascular invasion	Yes	189 (17)				-	-	
	No	439 (66)	0.376 (0.297-0.476)	66.328	**0.001**	0.518 (0.408-0.668)	25.626	**0.001**
	Unrecorded	44 (7)						
Perineural invasion	Yes	54 (8)	-			-	-	
	No	564 (85)	0.479 (0.387-0.679)	16.993	**0.001**	0.824 (0.566-1.200)	1.018	0.313
	Unrecorded	44 (7)						
Border	Pushing	69 (11)	-			-	-	
	Infiltrating	544 (82)	1.666 (1.088-2.550)	5.509	**0.019**	1.213 (0.784-1.876)	0.755	0.385
	Unrecorded	49 (7)						
Infiltrate	Sparse	584 (88)	-			-	-	
	Dense	24 (4)	0.527 (0.249-1.117)	2.792	0.095	-	-	-
	Unrecorded	54 (8)						
T-stage	T1	25(4)	-	51.773	**0.001**	-	11.699	**0.009**
	T2	78 (12)	1.009 (0.449-12.27)	0.001	0.982	1.073 (0.454-2.536)	0.026	0.872
	T3	403 (61)	1.832 (0.903-3.716)	2.817	0.933	1.433 (0.667-3.078)	0.852	0.359
	T4	156 (23)	12.642 (1.797-7.59)	12.642	**0.004**	2.157(0.976-4.764)	3.612	0.057
N-stage	N0	354 (53)	-	62.054	**0.001**	-	27.281	**0.001**
	N1	187 (28)	1.848 (1.433-2.384)	22.356	**0.001**	1.619 (1.224-2.242)	11.390	**0.007**
	N2	121 (19)	2.914(2.219-3.838)	59.082	**0.001**	2.337 (1.884-3.242)	25.812	**0.001**

Table 1.2: Univariate and multivariate analysis of overall survival in n=662 resected colorectal cancers from the Royal Liverpool University Hospital between 1999-2003

1.4 GENETIC PREDISPOSITION TO COLORECTAL CANCER

Hereditary cancer syndromes account for approximately 10% of colorectal cancer cases. The most common condition is Lynch syndrome or hereditary nonpolyposis colorectal cancer (HNPCC) with 4-5% of the total; 1% is attributed to familial adenomatous polyposis (FAP) and less than 1% of cases are due to hamartomatous polyposis syndromes such as Peutz-Jegher, familial juvenile polyposis and Cowden's syndrome. Evidence suggests that the risk of developing colorectal cancer increases with presence of a first-degree relative with the disease in the absence of an identifiable cancer syndrome.(22) A list of conditions associated with an increased risk of colorectal cancer is shown in Table 1.3.(23)

Hereditary condition	**Genes implicated**
Hereditary Non-polyposis Colorectal Cancer	hMLH1, hMSH2,hMSH6
Familial Adenomatous Polyposis	APC
Peuz-Jeghers syndrome	STK11
Familial juvenile polyposis	PTEN, SMAD4, BMPR1A

Table 1.3: Inherited predisposition to colorectal cancer (23)

1.4.1 HEREDITARY NON-POLYPOSIS COLORECTAL CANCER, HNPCCC

HNPCC sufferers are reported to have a 70% risk of developing colorectal cancer by 70 years.(24) The tumours associated with the condition have the following characteristics;

1. Lower average age of onset of colorectal cancer than in the general population (45 years in Lynch syndrome versus 63 years in the general population)

2. Proximal (right-sided) colonic cancer predilection (70%–85% of colorectal cancers in Lynch syndrome are proximal to the splenic flexure)

3. Accelerated carcinogenesis (tiny adenomas can develop carcinomas within 2–3 years in Lynch syndrome versus 8–10 years in the general population)

4. High risk of additional colorectal cancers (25%–30% of patients who have surgery for a Lynch syndrome–associated colorectal cancer have a second primary colorectal cancer within 10 years of surgical resection if they received a less than subtotal colectomy)

5. Increased risk of malignant disease at certain extracolonic sites; endometrium (40%–60% lifetime risk for female mutation carriers), ovary (12%–15% lifetime risk for female mutation carriers), stomach, small bowel, hepatobiliary tract, pancreas, upper uroepithelial tract (transitional cell carcinoma of the ureter and renal pelvis), brain (in the Turcot syndrome variant of Lynch syndrome).

The pathology of colorectal cancer is more often poorly differentiated, with an excess of mucoid and signet-cell features, a Crohns-like reaction and an excess of infiltrating lymphocytes within the tumour. Survival from this form of colorectal cancer is better than for sporadic cancers of the same stage.(25) HNPCC is caused by mutations in the mismatch repair genes, most commonly *MLH1*,

MSH2 or *MSH6*, that segregates in the patient's family. Individuals at risk in HNPCC kindred are heterozygous for mutations in the mismatch repair genes and so their normal cells do not have an elevated mutation rate. The cells lose the ability to correct errors in replication only when a further somatic mutation occurs in the functioning copy. Certain areas of the genome are vulnerable to replication error once the mismatch repair mechanism is damaged, in particular, poly-oligo tracts and base pair repeats known as microsatellites. Microsatellite instability, MSI, is seen in 90% of colorectal cancers in HNPCC patients and in 15% of sporadic tumours, described in greater detail in section 1.5.2.

The Revised Bethesda guidelines (2004) are used for detecting those colorectal tumours which should be tested for microsatellite instability.(26) Detection of MSI in tumour tissue should prompt further investigations in order to establish whether the patient carries a germline mutation consistent with HNPCC. Sporadic tumours which display MSI have a more favourable prognosis than non-MSI tumours, which can guide patient management and follow up. The criteria are as follows;

1. Patients aged less than 50 years with a diagnosis of colorectal cancer.
2. Patients with synchronous or metachronous colorectal or other syndrome-associated tumours, regardless of age.
3. Patients aged less than 60 years with colorectal cancer with histology suggestive of microsatellite instability (presence of tumour infiltrating lymphocytes, Crohns disease–like lymphocytic reaction, mucinous or signet-ring differentiation, or medullary growth pattern).
4. Patients with at least one first-degree relative with a diagnosis of colorectal cancer or a syndrome-associated tumour under age 50 years.

5. Colorectal cancer or syndrome-associated tumour diagnosed at any age in two first- or second-degree relatives.

1.4.2 FAMILIAL ADENOMATOUS POLYPOSIS, FAP

Familial adenomatous polyposis (FAP) has an incidence at birth of about 1 in 8,300. It manifests equally in both sexes, and accounts for less than 1% of colorectal cancer (CRC) cases. The disease is characterised by multiple polyps (>100) in the colon and rectum which have a 100% lifetime risk of malignancy, usually before the age of 40 years.(27) This autosomal dominant condition is caused by mutation in the Adenomatous Polyposis Coli (APC) tumour suppressor gene on chromosome 5q21. Prophylactic colectomy is recommended before the age of 25 years, British Society of gastroenterology (BSG) guidelines.(28) The conventional prophylactic operation has been subtotal colectomy and ileorectal anastomosis (IRA). This necessitates surveillance screening of the rectal stump with rigid or flexible sigmoidoscopy carried out at least every 12 months. There is a cumulative risk of rectal cancer of 4%, 5.6% 7.9% and 25% at 5, 10, 15 and 20 years.(29) Approximately 60% of FAP sufferers now undergo proctocolectomy and ileal pouch-anal anastomosis.(30) FAP may present with some extraintestinal manifestations such as osteomas, dental abnormalities (unerupted teeth, congenital absence of one or more teeth, supernumerary teeth, dentigerous cysts and odontomas), congenital hypertrophy of the retinal pigment epithelium (CHRPE), desmoid tumors, and extracolonic cancers (thyroid, liver, bile ducts and central nervous system).(27)

1.4.3 HAMARTOMATOUS POLYPOSIS SYNDROMES

This spectrum of syndromes include juvenile polyposis, Peutz-Jeghers syndrome, hereditary mixed polyposis syndrome, the phosphatase and tensin homolog gene (PTEN) hamartoma tumor syndromes (Cowden and Bannayan-Riley-Ruvalcaba syndromes), which are autosomal-dominantly inherited and Cronkhite-Canada syndrome, which is acquired.(31) They are characterised by multiple hamartomatous polyps in the small bowel, colon and rectum. Peutz-Jegher syndrome is also associated with pigmentation of the peri-oral region, hand and feet and is caused by mutations in the STK11 gene on chromosome 19p13. Familial Juvenile Polyposis is associated with mutations in the *PTEN*, Smad4 and BMPR1A genes.

1.5 DEVELOPMENT OF SPORADIC COLORECTAL CANCER

The vast majority of colorectal cancers, 90%, develop sporadically following a series of genetic mutations in the epithelial cells of bowel mucosa. Two distinct pathways of development have been identified, chromosomal instability and microsatellite instability.

1.5.1 CHROMOSOMAL INSTABILITY: THE ADENOMA-CARCINOMA SEQUENCE

The adenoma-carcinoma sequence of colorectal cancer development was first proposed by Morson *et al.* who described the similarities in the age and sex of patient, distribution and size of adenomas and the subsequent development of colorectal cancer.(32) An estimated 5% of benign adenomas undergo malignant transformation; though the risk of transformation increases with increasing size,

grade of dysplasia and villous architecture. In 1988 Fearon and Vogelstein published a seminal paper that elucidated the sequence of genetic mutations, which underpins the transformation of a benign adenoma to a malignant lesion.(33) The candidate genes responsible for the various stages were consequently identified as APC, *K-ras*, Deleted in colorectal cancer gene (DCC) and p53. Mutations in these genes were described as occurring sequentially as the phenotype of the lesion changed from normal mucosa, through small adenoma to adenocarcinoma (Figure 1.3). Rates of mutation of these key genes in benign and malignant lesions are shown in the Table 1.4.

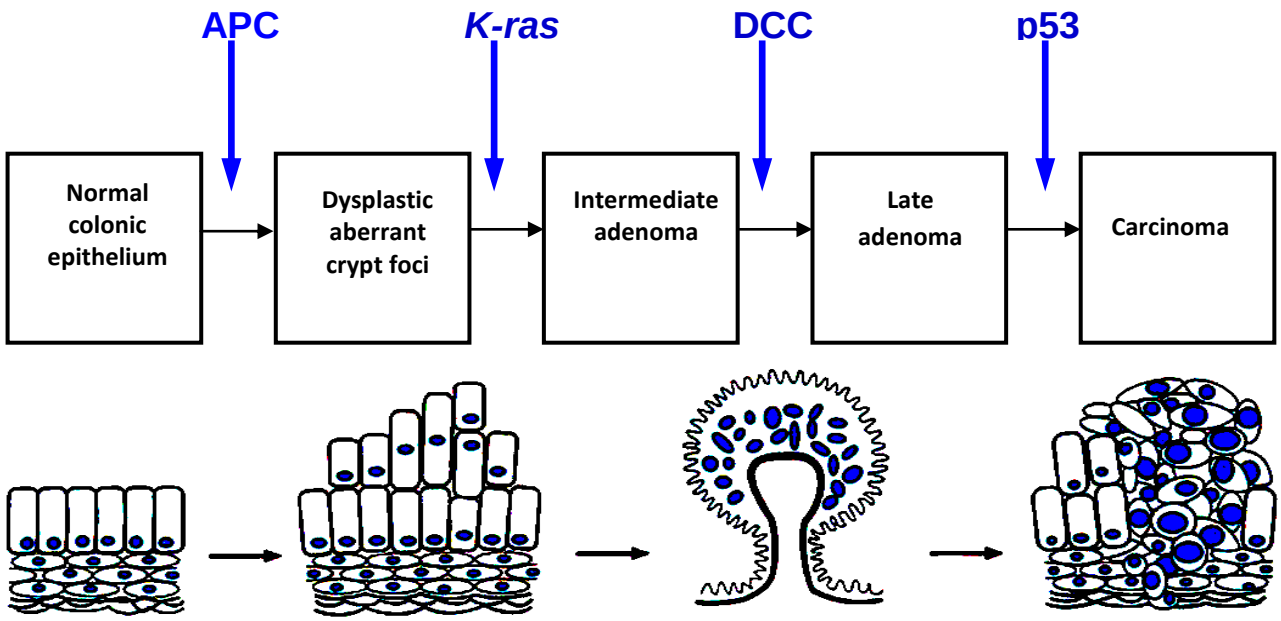


Figure 1.3: The adenoma-carcinoma sequence. The transition from normal colonic epithelium through adenoma to carcinoma. The increasing loss of cellular differentiation and capacity to metastasise correspond to the accumulation of genetic mutations described by Vogelstein. Image adapted from Fearon ER, Vogelstein B. Cell, Vol. 61, 759-767. (34)

	% frequency	
Mutation	**Adenoma**	**Adenocarcinoma**
APC	59-82	52-60
K-ras	12-34 (<1cm) 44-59 (>1cm)	35-41
p53	2-26	51-74

Table 1.4: Mutation rates of key genes in the adenoma-carcinoma sequence. A number of adenomas and carcinomas were tested for mutations in APC, k-ras and p53. Higher rates of p53 mutation in carcinoma compared to adenoma support the theory that mutation of this gene is a late event in the adenoma-carcinoma sequence.

An estimated 60-80% of colorectal cancers are thought to demonstrate chromosomal instability; however the conventional sequential model of mutation accumulation has been questioned. Studies have demonstrating that only 6.6% of all colorectal tumours were found to contain mutations in APC, *K-ras* and p53, with 38.7% of tumours containing mutations in only one of these genes.(35) Further work has indicated that this original model may be an over-simplification of colorectal carcinogenesis. As new genetic mutations have been reported it is clear that there may be several integrated routes towards the development of colorectal cancer,(36) shown later in Figure 1.4. Some of the common genetic pathways are discussed below.(37)

1.5.1.1 THE WNT PATHWAY

In the normally functioning colonic epithelial cell, β-catenin is held in a complex in the cytoplasm which includes the proteins APC and axin2.[35] This complex targets β-catenin for phosphorylation by Glycogen synthase kinase 3-beta (GSK3β). Phosphorylated β-catenin becomes multi-ubiquitinated

and subsequently degraded in proteasomes. Wnt proteins bind to cell-surface receptors of the Frizzled family, causing the receptors to activate the membrane-bound Dishevelled (DSH) family of proteins, inhibiting degradation of β-catenin and ultimately resulting in migration of the protein into the nucleus. Activation of DSH inhibits the axin/GSK-3/APC complex preventing proteolytic degradation of the β-catenin intracellular signalling molecule. A pool of cytoplasmic β-catenin stabilizes, and some β-catenin is able to enter the nucleus and interact with the T-cell factor (TCF) family of transcription factors to promote specific target genes promoting cell proliferation. Mutations in components of the 'β-catenin destruction complex' lead to aberrant stabilization of β-catenin and thus to constitutive, Wnt-independent activation of TCF/β-catenin signalling.(38)

The most frequent Wnt pathway mutation is in the APC gene which commonly produces a truncated form of the protein which is incapable of complexing with β-catenin. APC is an enigmatic protein which has been implicated in numerous other cellular processes, such as cell migration, chromosomal stability, cell cycle regulation and cell adhesion. APC is connected to microtubules, and it plays a role in the correct establishment of the mitotic spindle. Disturbance of APC function by mutations could lead to chromosomal instability (CIN).(39) Aneuploidy (the abnormal number of chromosomes both quantitatively and qualitatively) is a sign of CIN and is a common characteristic of colon cancer cells, occurring in 85% of cancers. A role for APC in regulating the cell cycle has also been described, both directly and via components of the retinoblastoma, RB pathway.(40)

β-Catenin is mutated in up to 10% of all sporadic colon carcinomas. These mutations result in stabilization of β-catenin and activation of Wnt signalling. Germline mutations of axin2 have also been described and sporadic mutations occur in 25% of microsatellite-unstable colorectal tumours.

Recent evidence suggests that colorectal cancer cells frequently express Wnt proteins and thus stimulate the Wnt pathway by autocrine mechanisms.(38)

1.5.1.2 RAS/RAF PATHWAY

K-ras is a member of a group of oncogenes (the others being *H-* and *N-ras*) located on chromosome 12p12. The *ras* proteins are membrane bound guanine nucleotide binding proteins that transduce growth-receptor signals across the cell membrane to effector molecules. In the resting state they are bound to GDP. Signalling by the *ras* proteins is activated by GTP binding and inactivated by intrinsic GTPase activity which automatically terminates the signal. Mutated *K-ras* is unable to hydrolyse GTP resulting in abnormal prolongation of growth signal. This results in increased and unregulated cell proliferation and growth factor induced differentiation.(41)

The first identified downstream effectors of *ras* were the RAF serine/threonine kinases. The RAF family is composed of three members, ARAF, BRAF and RAF1, which exhibit a high degree of homology within three conserved regions. Each kinase has a *ras*-binding domain and cysteine-rich domain that mediate interaction with GTP-bound *ras*. On *ras* association, *ras* is recruited to the plasma membrane and is phosphorylated. Gene disruption studies in mice have shown that BRAF interacts with the MEK/ERK pathway causing activation. The higher propensity of BRAF towards MEK/ERK activation is thought to be a reason that only BRAF mutations, and not comparable mutations in the other RAF proteins, have been observed to be associated with malignancy.(42) BRAF mutations have been reported in 4-9% of sporadic colorectal tumours and are associated with a significantly higher proportion of MSI tumours.(43, 44) Research into BRAF mutations in MSI tumours has identified that the presence of a BRAF mutation indicates the tumour is not related to

HNPCC and that germline testing of MLH1 in that individual is not warranted. It has been suggested as a rapid and inexpensive method of selecting patients for HNPCC testing.(45)

1.5.1.3 18Q

Chromosomal loss within the region of chromosome 18q has been shown to be associated with poor prognosis in stage II and III colorectal cancer.(46, 47) This genomic region contains a number of genes which have been studied for their possible contribution to colorectal cancer, including, DCC (Deleted in Colorectal Carcinoma),(48) Smad2(49, 50) and Smad4.(50, 51) The Smad proteins form complexes which migrate into the nucleus causing gene transcription after Transforming Growth Factor-Beta (TGF-β) ligand signalling.(52) The TGF-β superfamily of ligands include: Bone morphogenetic proteins (BMPs), Growth and differentiation factors (GDFs), Anti-müllerian hormone (AMH), Activin, Nodal and TGFβ. Signalling begins with the binding of a TGF-β superfamily ligand to a TGF-β type II receptor. The type II receptor is a serine/threonine receptor kinase, which catalyses the phosphorylation of the Type I receptor. Receptor-regulated Smad proteins (R-Smad) are recruited to the activated membrane-bound receptor-ligand complex and are in turn phosphorylated on their c-terminus leading to R-Smad activation. R-Smads include Smad2 and Smad3 from the TGF-ß/Activin/Nodal branch, and Smad1, Smad5 and Smad8 from the BMP branch of TGF-ß signalling. Cytoplasmic Smad4 forms heteromeric complexes with activated R-Smads which migrate into the nucleus and interact with transcriptional activators.

There is considerable evidence implicating mutation of the Smad4 gene in the development and progression of colorectal cancer. Germline mutations of the Smad4 gene are associated with human familial juvenile polyposis,(53, 54) an autosomal dominant disorder characterized by a

predisposition to Smad4-negative gastrointestinal polyps and cancer at a young age. Consistent with a contributory role for Smad4 in this process, Smad4 (+/-) knock-out mice also develop Smad4-negative gastrointestinal polyps.(55, 56) In human colorectal cancer, the frequency of mutational inactivation of Smad4 increases with advancing stage.(57) Smad4 mutations in adenoma or primary invasive carcinoma without distant metastasis occur in approximately 10% of cases.(58) In contrast, for patients who have primary invasive carcinoma with distant metastasis, the frequency of Smad4 mutations is 35%, suggesting a strong link between Smad4 loss and disease progression. Our group reported a connection between the numbers of S100A8 and S100A9-positive monocytes in the stroma of pancreatic adenocarcinomas and the Smad4 expression in the tumour.(59) A similar report in colorectal cancer demonstrated a strong relationship between stromal infiltration and Smad4-loss and concluded that the combination of these features correlated with poor survival.(60)

1.5.1.4 P53

p53 is a tumour suppressor gene with numerous vital functions including prevention of inappropriate cell division, repair of DNA damage, induction of apoptosis and inhibition of angiogenesis.(61) In unstressed cells, p53 levels are kept low through a continuous degradation of p53. The murine double minute 2 protein (Mdm2) binds to p53 preventing its action and transports it from the nucleus to the cytosol. Mdm2 acts as ubiquitin ligase and covalently attaches ubiquitin to p53 and thus marks p53 for degradation by the proteasome.(62)

p53 becomes activated in response to a myriad of stress types, which include but are not limited to DNA damage (induced by ultraviolet light, irradiation, or chemical agents), oxidative stress, osmotic shock, ribonucleotide depletion and deregulated oncogene expression. This activation is marked by

two events; firstly the half-life of p53 increases markedly leading to a rapid accumulation of the protein in stressed cells. Secondly, phosphorylation of the N-terminal domain of p53 causes a conformational change, activating the protein as a transcriptional regulator. Activated p53 directly stimulates p21Waf1/Cip1 expression which in turn inhibits cyclin dependent kinases (CDKs).(63) CDKs are key regulators of the cell cycle which ensure that DNA replication (S phase) follows on from the resting phase G1. Down regulation of CDKs inhibits both the G1 to S and the G2 to mitosis transitions in the cycle, effectively causing cell cycle arrest.

A proportion of cells in which p53 is activated undergo apoptosis rather than cell cycle arrest. There are a number of mechanisms by which p53 has this affect. p53 is thought to cause translocation of various apoptosis-inducing proteins from the mitochondria including members of the BCL-2 family, Bax, Noxa and PUMA.(64) p53 has also been implicated in the membrane death receptor induced pathway of apoptosis. DR5/KILLER and FAS are two of the death receptors observed to be up-regulated by p53. Activation of PIDD, a death domain containing protein, by p53, also induces apoptosis and is likely to function through the death receptor pathway.(65)

p53 participates in DNA damage repair. Ataxia-telangiectasia mutated gene product (ATM) is a general sensor to DNA damage and phosphorylates p53, breast cancer protein (BRCA), Nijmegen breakage syndrome 1 (NBS1) and Fanconi-Anemia–related tumor suppressor (FA) activate the DNA repair process. BRCA and p53 work together in nucleotide excision repair of DNA adducts.(66) p53 also participates in chromosomal recombination and chromosomal segregation.(67)

Mutation in p53 prevents the normal function of the gene which is to prevent replication of cells with damaged DNA. p53 mutations more often occur late in carcinogenesis, frequently preceding

metastasis. It is perhaps unsurprising that these tumours have a worse prognosis than tumours with functioning p53.(68)

1.5.2 MICROSATELLITE INSTABILITY, MSI

Microsatellites are short, tandem repeats of nucleotide sequences which make up 3% of DNA and are found throughout the genome. Due to their repetitive nature they are prone to errors in replication. In normal cells, the mis-match repair (MMR) system is responsible for the correction of errors which result from DNA polymerase slippage during DNA replication. MMR is accomplished in eukaryotes by a number of highly conserved proteins including heterodimers of MSH2/MSH3 or MSH2/MSH6 that are required for the recognition of mismatches or small insertions or deletions, and a heterodimeric complex of MLH1/PMS2 which links mismatch recognition to the activation of cleavage and repair. In MMR-deficient cells, microsatellite sequences are highly susceptible to insertion and deletion mutations due to increased error rates of replicative DNA polymerases at these loci.(69) Hence, the microsatelite instability (MSI) phenotype represents a surrogate marker for the detection of MMR deficiency.(70) The National Cancer Institute recommend the testing of five microsatellite markers for determination of MSI, (BAT-25, BAT-26, D2S123, D5S346 and D17S250).(71) Two or more positive markers which demonstrate instability is classed as MSI-high and germline screening for HNPCC is indicated. Approximately 15% of sporadic cancers show high levels of MSI (MSI-H). Most of these tumours are MMR deficient due to epigenetic silencing of MLH1. It appears that BRAF mutations are associated with the sporadic form of MSI-H, but not the hereditary form, (HNPCC).(72) Epigenetic silencing occurs when the promoter region of genes are methylated, preventing them for being transcribed. CpG-islands are DNA sequences where

cytosine, (C) and guanine, (G) lie adjacent to each other. These islands are very susceptible to methylation and are often found in promoter regions. Patients who display this trait are referred to as having the CpG-island methylator phenotype, CIMP+.(73)

Tumours have been described with low levels of MSI, termed MSI-L.(74) These tumours are thought to have a separate pathway of development to MSI-H tumours involving either (i) increased generation of methylG:T mismatches due to loss of expression of 0-6-Methylguanine DNA Methyltransferase (MGMT) that would stress the DNA mismatch repair machinery and (ii) partial methylation and loss of expression of the DNA mismatch repair gene MLH1. Tumours with MGMT methylation are often associated with *K-ras* mutation. Increasing interest in serrated adenomas as precursor lesions for these MSI-L tumours is developing.(37, 75)

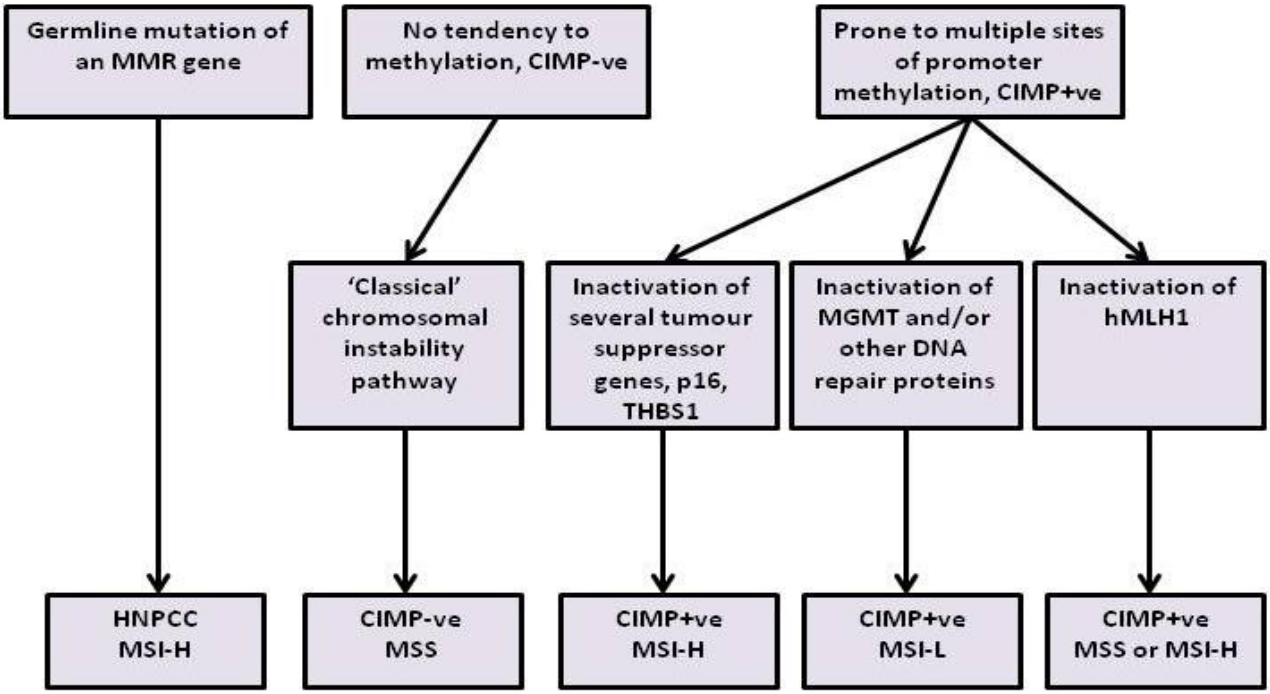


Figure 1.4: Putative pathways in the development of colorectal cancer. Multiple pathways of development are shown including germline mutation, chromosomal instability (CIMP-negative) and microsatelite instability (CIMP-positive) pathways. Image adapted from Jass JR. Surg Oncol. 2007 Dec;16 Suppl 1:S7-9. Epub 2007 Nov 26. (72)

1.6 DIAGNOSIS: POPULATION-BASED COLORECTAL CANCER SCREENING

Diagnosis of colorectal cancer to date has relied on presentation of the patient to the general practitioner or local Accident and Emergency department with symptoms or signs suggestive of a colonic neoplasm. These features include, change in bowel habit (frequently to looser stool), blood in the stool, weight loss, iron deficiency anaemia or an abdominal mass.(76) The natural history of colorectal cancer makes it an ideal candidate for population-based screening. The disease is widespread in the population, readily detected at a pre-neoplastic stage, (adenomatous polyps) and is easily treated. Population-based screening for colorectal cancer commenced in June 2006 in the UK and is anticipated to reduce cancer-specific mortality by 15%. The current protocol in the UK uses the Faecal Occult Blood Test, FOBT, for those aged 60-69 years. Several different modalities can be used for screening; the evidence-base for these is discussed below. Screening is set to have a major impact on the management of colorectal cancer.(77) The proportion of patients presenting with small and early colorectal cancers in the UK is predicted to double promoting a shift towards local excision and other minimally-invasive surgical techniques. The efficacy of the various methods used for screening will be vital for the early detection of flat and depressed tumours.(78)

1.6.1 COLONOSCOPY

Colonoscopy is recognized as the 'gold-standard' for detection of cancers and polyps. It is the only modality with the capacity to detect and remove adenomatous polyps from the whole colon in one session. Most alternative colorectal cancer screening modalities such as FOBT, flexible sigmoidoscopy and barium enema are utilised as a tool for the selection of patients for colonoscopy

and ultimately polypectomy and/or surgery.(77) It is perhaps surprising that there are no randomised controlled trials that demonstrate the effectiveness of colonoscopy in reducing mortality from colorectal cancer. One small, randomised trial from Norway showed a 75% reduction in the number of colorectal cancers that developed in the screened group after 10 years follow up but no survival information was available.(79) The efficacy of colonoscopy can be extrapolated from case-control studies of sigmoidoscopy; these demonstrated a risk reduction of 80% for death from colorectal cancer in those with a history of flexible sigmoidoscopy examination.(80, 81) Evidence from the large cross-sectional study of the US National Polyp Database reported reductions in the expected number of colorectal cancers following colonoscopy and polypectomy of 76 – 90% compared to reference populations.(82, 83) There is evidence for relative risk reduction for colorectal cancer of 70% following negative colonoscopy; this reduces to 55% at 5 years and 28% at ten years.(84) The detection rate for invasive cancer at colonoscopy in cross-sectional studies is reported as 0.5-1% with 6-12% having high-risk lesions (size ≥10mm, villous architecture, poorly differentiated histology or invasive cancer).(85) Almost 50% of patients with a proximal neoplasm have a sentinel lesion in the distal colon. In one such study 64% of all cancers and polyps ≥10mm were located within reach of a flexible sigmoidoscope and 53% were in the sigmoid colon or rectum.(86) In the younger age group, 40-49 years, the detection rate for significant polyps falls to 3.5% and screening below the age of 50 years is not recommended in the USA.(87)

No perforations have been reported as a result of colonoscopy in the three large US trials although 10 patients (0.3%) had post-polypectomy bleeding.(86) In the UK FOBT trial the overall

complication rate following colonoscopy was 0.5%; 5 perforations, 1 major bleed and 1 snare entrapment in 1474 screened.(88) Missed lesions have been reported after colonoscopy. Miss-rates have been reported up to 6% for polyps <10mm after back-to-back colonoscopy. One such study found that 2% of asymptomatic patients undergoing screening had a missed polyp. Almost one-quarter of these missed lesions were within 10cm of the anal verge, the majority of the remainder were located on a fold in the colon.(89) Ten-yearly colonoscopy for the over 50-year age group is the favoured colorectal cancer screening policy by the American College of Gastroenterologists.(3, 90) There is not sufficient evidence at present to determine whether this a cost effective approach in the UK or other European countries.

1.6.2 FLEXIBLE SIGMOIDOSCOPY

Three large randomised trials of once-only flexible sigmoidoscopy have been performed in the UK(91), Italy(92) and Norway(93) and reported on neoplasia detection rates. The UK trial has now reported and found that 727 were died of certified colorectal cancer out of 170038 participants, (538/112939 in the control group versus 189/57099 in the flexible sigmoidoscopy group). In intention-to-treat analyses, colorectal cancer incidence in the intervention group was reduced by 23% (hazard ratio 0.77, 95% CI 0.70-0.84) and mortality by 31% (0.69, 0.59-0.82).(94) Two case-control studies from the US have shown a reduction in colorectal cancer mortality from a combination of flexible and rigid sigmoidoscopy of 60-80%.(80, 81) Cancer detection rates for flexible sigmoidoscopy have been reported as 0.3-0.7%. The incidence of distal cancers following sigmoidoscopy is decreased to 4% of expected in the first year of follow-up and remains 18% of expected after 4 years.(95) The removal of significant distal adenomas during the initial

examination is an attraction of the flexible sigmoidoscopy approach. In direct comparison with FOBT, flexible sigmoidoscopy has been shown to detect approximately three times as many of these lesions.(96, 97)

There are disadvantages of using flexible sigmoidoscopy for screening. Referral rates for colonoscopy varied from 5-19% depending on the protocol used, but were higher than the 1.2-2.1% referral rates seen in FOBT trials, (unrehydrated tests). Two perforations after flexible sigmoidoscopy were seen in total during the three trials, a complication rate of 3 per 100,000 examinations. Compliance with flexible sigmoidoscopy screening is lower than that for FOBT screening. In the UK 55% of the general population expressed an interest in flexible sigmoidoscopy after letter invitation, of these 71% attended for the examination, an overall compliance rate of 39%.(91) This is lower than the reported 54% compliance rate recorded in the first round of the UK FOBT trial.

1.6.3 DOUBLE-CONTRAST BARIUM ENEMA, DCBE

No randomised controlled trials are available on either single or double contrast barium enema for colorectal cancer screening of asymptomatic patients. The sensitivity of DCBE for cancers has been reported as 80-100%, whereas detection of polyps has been found to be poor.(98) Studies of DCBE in the average-risk population are rare. One study, of patients with a positive FOBT, compared DCBE to colonoscopy and found that barium enema detected 100% of cancers but only 27% of polyps ≥10mm. Polyps in the rectum were particularly poorly diagnosed with none of 7 polyps <10mm and 2 of 3 polyps ≥10mm detected.(99) One retrospective cross-sectional study of DCBE in the average-risk population had a detection rate of 0.7% for adenocarcinoma and 4.3% for polyps

≥10mm.(100) The low detection rate for polyps makes DCBE an unattractive option for screening and it is rapidly being superseded by CT colonography.

1.6.4 CT COLONOGRAPHY

CT colonography is a new diagnostic tool for lesions in the colon and very few studies have used this technology for screening of asymptomatic patients. The initial results of one cross-sectional study of 1110 asymptomatic adults found the rate of invasive cancer to be 0.3% and of large polyps (≥10mm) to be 3.9%.(101) Conventional colonoscopy was performed in 40 of these patients and the polyp was identified in 38 patients (95% concordance). The detection rate for polyps in this study was vastly improved compared to DCBE but not as high as optical colonoscopy (3.9% vs. 5.4%). No complications of CT colonography were reported and many authors in the US predict this examination becoming available via private health insurance schemes as a minimally invasive alternative to ten-yearly colonoscopy.

1.6.5 GUAIAC FAECAL OCCULT BLOOD TESTING, G-FOBT

The guaiac-based test for faecal blood was first developed in the 1970s as a screening test for colorectal cancer when endoscopic technology was in its infancy. G-FOBT makes use of the pseudoperoxidase activity of haemoglobin. Guaiac turns blue after oxidation by oxidants or peroxidases in the presence of an oxygen donor such as hydrogen peroxide. For the guaiac test to function, the haemoglobin must be degraded in the GI tract into haem and globin. As a result of the mechanism of action guaiac-based tests were initially thought to be more sensitive for causes of upper GI or proximal bleeding, however, they have been found to be better at detecting larger,

more distal lesions. The sensitivity of G-FOBT for distal (rectosigmoid) polyps has been reported as 86% compared to 26% for proximal (ascending or transverse colon) polyps.(102)

G-FOBT with follow-up colonoscopy is the only method of colorectal cancer screening which has been shown to reduce mortality from the disease in several randomised controlled trials. Three major randomised controlled trials from Minnesota, USA(103, 104), Nottingham, UK(105, 106) and Funen, Denmark(107) have shown a consistent reduction in colorectal cancer specific mortality of 15-21%. The Minnesota trial had the longest follow up period of 18 years; they also rehydrated the FOB tests with a drop of distilled water prior to processing in order to increase the test sensitivity. These differences may explain the higher rate of colonoscopy compared to the Nottingham trial, 38% versus 4%.(108) The detection rate for invasive cancer using G-FOBT with follow-up colonoscopy is reported as 0.2%, with a 1.8% chance of detecting any high-risk lesion. The main disadvantage of using G-FOBT is its relatively low sensitivity for polyps and cancer, (30% and 50% respectively). Unrehydrated G-FOBT detected half of colorectal cancers in the average-risk population. This may have been affected by compliance rates in the UK and Danish pilot studies, which, at 57-67% were lower than anticipated.(105-107)

1.6.6 FAECAL DNA TESTING

Faecal DNA testing relies on small amounts of altered DNA being exfoliated into the stool from colonic adenomas or carcinomas.(109) DNA is stable in stool and can be isolated and specific human DNA sequences extracted and amplified.(110) The main disadvantage of this technique is the relatively low frequency in which single genes are mutated in adenomas, (60% APC, 50% *K-ras).* This has been overcome by forming a panel of genes which may include APC, *K-ras*, p53 and BAT-

26. Faecal DNA screening in the average risk population has been shown to be more sensitive than G-FOBT with the same specificity.(111) The detection rate for advanced neoplasia, (carcinoma or adenoma≥10mm) was 18% for faecal DNA and 11% for G-FOBT which was disappointingly low in both cases. Currently, the cost of faecal DNA testing prohibits widespread use on a population basis.(112) Refinements to the marker panel which increase sensitivity and efforts to reduce costs may see faecal DNA testing ultimately supersede FOBT.

1.7 MANAGEMENT OF COLORECTAL CANCER: THE RATIONALE FOR TREATMENT

1.7.1 SURGERY

The mainstay of treatment for colorectal cancer is complete surgical excision of the lesion including the arterial supply, venous drainage and lymph node group for the involved segment of bowel. Approximately 80% of patients who present with colorectal cancer undergo resection of the primary lesion. Adequate surgical resection is most vital in anterior resection or abdomino-peroneal resection for rectal cancer in which the aim is to preserve the mesorectal envelope and thus minimising the risk of a positive resection margin. The specimen is graded from 1-3 with 3 corresponding to a completely intact specimen; a grade 3 resection is proven to be associated with lower recurrence rates. (113) Resection of the tumour is deemed to be complete on histopathological evaluation of the specimen if the tumour is >1mm from the surgical excision

margins. The National Cancer Institute also recommends that 12 or more lymph nodes should be examined in each specimen in order to accurately stage and treat colorectal cancers.(114)

1.7.2 ADJUVANT CHEMOTHERAPY

Adjuvant chemotherapy has been recommended by the National institute for Clinical Excellence, NICE for all patients with lymph node metastases.(115) A number of large prospective randomised trials have now beyond doubt recognised the value of 5-fluorouracil (5FU) based chemotherapy in the adjuvant treatment of stage-III (Dukes C) colon cancer. (116-118) The mechanism of action of 5FU is via incorporation in RNA as fluorouridine triphosphate and inhibition of thymidylate synthetase by fluorodeoxyuridine monophosphate, often in conjunction with reduced folates for maximum efficacy to interfere with DNA synthesis. Most studies have demonstrated a reduction of 22-33% in risk of death for this group. There is a large body of evidence that suggests the first line use of a combination therapy of irinotecan and 5FU/folinic acid (FA) may provide a more efficacious treatment regime.(119) However, NICE guidelines, produced in 2002, suggest irinotecan be reserved for use as a single-agent, if 5FU treatment fails. The QUASAR trial reported a very small benefit of adjuvant treatment for stage-II patients overall, (4-5%).(120) Patients with poor prognostic indicators on histology, such as poor differentiation, T4 or perforated lesion, may be offered chemotherapy at the discretion of the treating clinician. In patients with unresectable disease, 5FU has been shown to improve survival over supportive care alone, (median survival 11.7 versus 8 months and 1-year survival of 50 versus 34%).(121) Oxaliplatin has been recommended as a first-line treatment in the very few cases where inoperable liver metastases could potentially be

made operable with chemotherapy. Potentially 13.5% of these patients could be rendered resectable with chemotherapy and those resected patients achieved a 39% 5-year survival.(122)

1.7.3 ADJUVANT RADIOTHERAPY

Cells exposed to ionizing radiation acquire multiple sites of bulky DNA lesions and double strand breaks. The accumulation of damage, specifically double strand breaks or adducts stalling the replication forks are stimulation signals for a global response to DNA damage. The common features of global response are induction of multiple genes, cell cycle arrest, and inhibition of cell division culminating in apoptosis. Radiotherapy is only used in the management of rectal cancer as the mobility of the colon within the abdomen makes consistent targeting of the lesion difficult. Small bowel is very sensitive to radiotherapy and irradiation of the abdomen can result in severe radiation enteritis.(123) Evidence from the CR07 trial suggests that recurrence rates for resectable rectal cancer are reduced by approximately 15% with the routine administration of short-course radiotherapy prior to surgery.(124) Some rectal tumours are bulky and appear to infiltrate the resection margin on pre-operative imaging. Pre-operative chemoradiation therapy for 6 weeks to ‘down-stage’ the tumour doubles the rate of sphincter-sparing operations and lowers the rates of local recurrence, acute toxicity, and long-term toxicity. No difference in disease-free or overall survival was observed on comparison of pre or post-operative chemoradiotherapy.(125)

1.7.4 NEW ADJUVANT TREATMENT MODALITIES

Monoclonal antibodies directed against epidermal growth factor receptor, EGFR (cetuximab) and vascular endothelial growth factor, VEGF (bevacizumab) are new adjuvant therapies. In an initial Phase II study of 121 patients whose tumours expressed EGFR and were refractory to irinotecan,

treatment with the combination of cetuximab and irinotecan resulted in a response rate of 17%. (126) In a randomized, Phase III trial of 815 previously untreated patients with metastatic disease, the addition of bevacizumab to irinotecan plus fluorouracil/leucovorin (IFL) led to a statistically significant improvement in response rate (44.8% versus 34.8%, *P* = 0.004) and a 4.7-month prolongation in median overall survival (20.3 months versus 15.6 months, *p* = 0.001).(127) Sub-group analysis of metastastic colorectal cancer treated with cetuximab has revealed that response to the drug is poor in those patients with *K-ras* mutations in the tumour. Mutant *K-ras* activates the MARP*/ras* cell proliferation pathway downstream of the EGFR, which means that targeting the receptor with cetuximab is ineffective.(128) Tumour-specific adjuvant chemotherapy treatment is increasingly possible with the new generations of monoclonal antibodies.

1.8 EARLY COLORECTAL CANCER: MANAGEMENT OPTIONS

As more pre-cancerous adenomatous polyps and early cancers are detected with screening the scope for local resection of tumours will increase. The impact of tumour morphology on these treatment options is still controversial with some studies suggesting local resection should be avoided in depressed tumours, whereas other studies reporting safe and effective treatment in this group.(129) The accurate staging of all tumours prior to local treatment is imperative and signs that predict which early tumours are likely to have infiltrated deep into the bowel wall, or have lymph node metastases are vital. Biomarkers which predict recurrence of tumour after local resection will be unvaluable in the management of early and small tumours.

1.8.1 TRANSANAL ENDOSCOPIC MICROSURGERY (TEM)

TEM is a minimally invasive surgical technique originally designed by Buess *et al.* in the 1980s.(130) The anal sphincter is dilated with a large-diameter, (4cm) operating sigmoidoscope to accommodate optics, suction, and ports for dissecting instruments. Endoscopic surgical instruments are advanced into the rectum after insufflation. The magnified three-dimensional image allows an optimal view of the tumour and facilitates precise dissection. The plane of dissection extends through the muscularis propria into the surrounding adipose tissue; the remaining defect is typically closed using a continuous suture. TEM is most commonly used to resect large, benign rectal adenomas. In this group, microscopic involvement of the resection margin is significantly associated with recurrence; the overall recurrence rate is reported as 2 - 10.5% at 24 months.(131) No guidelines have been established for following up this patient group, common practise is rigid or flexible sigmoidoscopy at 4 months then 6-12 monthly thereafter. Three-monthly examinations are suggested for patients with positive margins. TEM has been used to resect rectal cancers, either with curative intent (for T1 tumours) or palliative intent in more advanced lesions. The recurrence rate in the UK has been reported as 20% pT1 (five-year survival of 77%), 25% pT2 (five-year survival 74%) and 59% pT3 (five-year survival 35%).(132) No consensus on follow up for these patients has been established. Most authors have recommended salvage open surgery for patients with high-risk lesions, (villous architecture, positive resection margins or poorly differentiated tumour on histology), initially treated with curative intent.

1.8.2 ENDOSCOPIC MUCOSAL RESECTION (EMR) AND SNARE-POLYPECTOMY

EMR was first reported in 1988 by Tada *et al.* as a method of excising large, sessile polyps not amenable to conventional snare-polypectomy.(133) The technique describes the injection of fluid, (typically 1:40,000 adrenaline solution and methylene blue) into the submucosa around a polyp causing the lesion to 'lift' away from the muscularis propria. The lesion is then resected with a conventional polypectomy snare, piecemeal if necessary. Most authors advocate EMR for benign polyps. Lesions that are indurated, ulcerated, and/or exceedingly friable at endoscopy, as well as those that fail to 'lift' in response to a submucosal injection of fluid may contain invasive malignancy and should be avoided.(134) A recurrence rate of 17-21% with a median follow-up of 12-24 months has been reported following resection of benign sessile polyps in the colon and rectum. The rate of invasive adenocarcinoma in these studies was reported as 12-18%.(134, 135) The guidelines issued by the British Society of Gastroenterologists for the follow-up of patients following conventional snare polypectomy are well established.(136) These polyps generally have a narrow stalk which, when snared, allows for easier, safer removal with a greater chance of complete excision. The British Gastroenterology Society recommends that those who have undergone removal of ≥5 adenomas or ≥3 adenomas (at least one of which is ≥10mm) are categorised as high-risk and should be offered follow-up colonoscopy in 12 months.(136) The cumulative risk, for these patients, of developing invasive cancer is estimated as 24% at 20 years without polypectomy.(137) Patients who have undergone removal of 1-2 small adenomas, (<10mm) are classed as low-risk and given the option of one surveillance colonoscopy in five years, or no follow up. Intermediate risk patients, 3-4 adenomas or one adenoma >10mm should be offered a further examination in three years.(136)

1.9 FLAT AND DEPRESSED-TYPE COLORECTAL CANCER

Flat and depressed colorectal cancers have been reported in Japan since Muto in 1985.(138, 139) A series of case reports documented patients with lymph node or distant metastases from colorectal cancer in which the primary lesion was a diminutive depressed colorectal cancer, often 10-20mm in diameter. A common feature of these reported lesions was the small size, infiltrative growth pattern and the presence of early, aggressive metastases. These rare tumours were thought to be a phenomenon confined to the Japanese population; however colonoscopic studies began to identify depressed tumours in Western populations. The presence of flat and depressed cancers in European populations is now firmly established with a succession of papers from the UK, France, Germany and Sweden.(140-146)

1.9.1 MORPHOLOGICAL CLASSIFICATION OF COLORECTAL LESIONS

In 1997, Kudo devised the Japanese Research Society Classification (JRSC) for the morphology of colorectal adenomas which he based on the established classification of early gastric cancer. Four basic groups were described: Type I (polypoid) in which the adenoma protruded into the lumen of the colon with a height > 2x the depth of the mucosa: Type IIa, (flat), the lesion is elevated but the height is < 2x the depth of the surrounding mucosa, Type IIb (flat) the lesion lies flush with the mucosa and Type IIc, (depressed), the lesion is sunken in relation to the surrounding mucosa. The full classification is shown in Figure 1.5.(147)

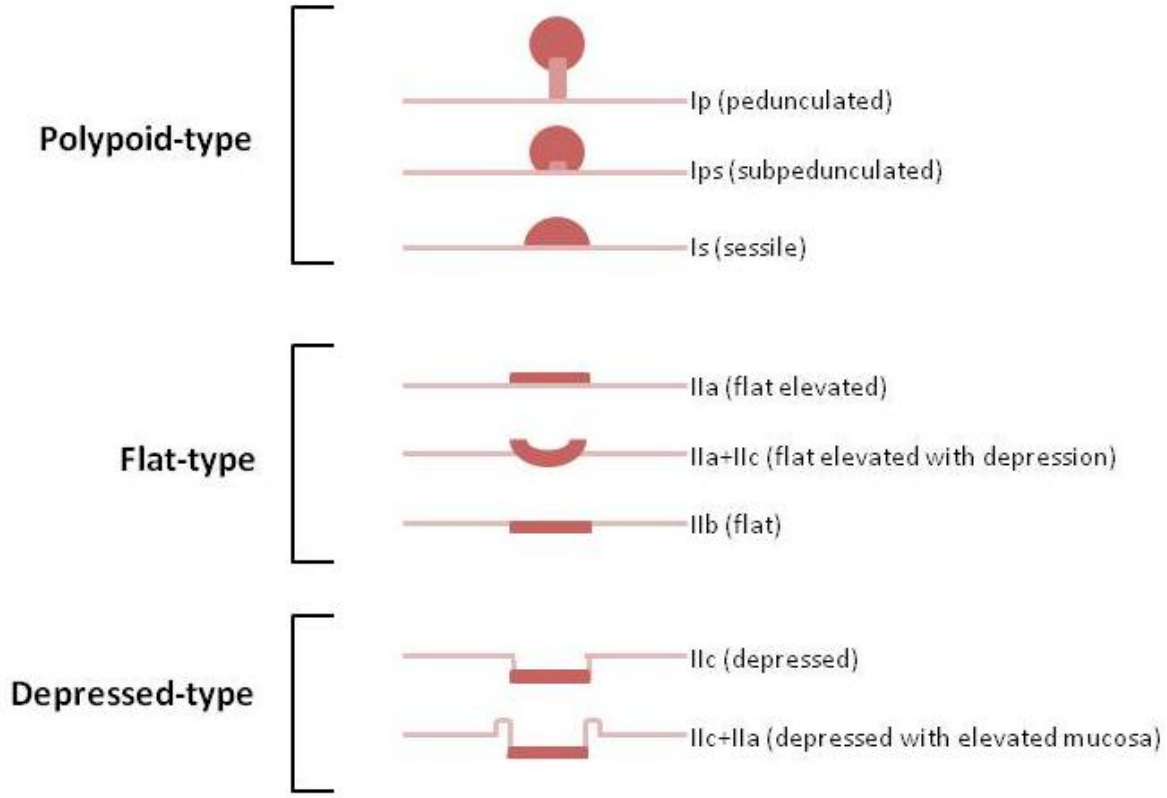


Figure 1.5: JRSC classification of colorectal cancer morphology. Polypoid, flat and depressed cancer types are depicted in relation to level of normal colonic mucosa. Image adapted from Kudo, S *et al*. World J Surg 21, 694-701 (1997).

1.9.2 FLAT AND DEPRESSED LESIONS: INCIDENCE

Flat and depressed tumours remain rare. Detection rates at routine colonoscopy are similar in Japanese and Western populations, shown in Table 1.5.(138, 142, 143, 146, 148-154) The most recent UK study from St Marks' Hospital found that 71% of T1 lesions detected at colonoscopy were flat or depressed with 10% of all detected cancers of flat or depressed morphology.(153) Establishing the incidence of flat and depressed tumours in resected specimens retrospectively is

hampered by the difficulties in applying the JRSC classification to large cancers. Large cancers often show a mixed pattern of growth as they progress with areas of ulceration and distortion of surrounding tissue.(155) Ishihara (2000) and Nasir (2003) retrospectively compared a number of surgically resected polypoid and non-polypoid tumours of all sizes. In both papers there were difficulties in classifying tumours with mixed growth patterns or surface ulceration, leading to a number of exclusions. This may go some way to explain why a greater variation in incidence of flat cancers is reported in resected colorectal cancer. Small or diminutive tumours have been studied with more consistent results. Kubota (1996) reported an incidence of 1% diminutive, (≤10mm), flat or depressed colorectal cancer seen in resected colon which approaches that of colonoscopic studies.[156] Ishihara (2000) analyzed small colorectal cancers, (≤20mm), and demonstrated an overall incidence of 1.3% with 15/23 (65%) of small cancers having flat or depressed morphology(156) (Table 1.6). In a retrospective study of small, flat and depressed colorectal cancers, (≤20mm) that I undertook with colleagues in the Royal Liverpool University Hospital, we reported an incidence of 2.2% (39/1763) which concurs well with the Japanese studies.(157) This suggests that the aetiology and natural history of these lesions is similar between East and West. The study is described in detail in section 1.9.4.

Table 1.5: Colonoscopically detected flat colorectal cancer/adenomas

Main Author	Year	Country	No of patients	No of flat Carcinomas (%)	Size (mm)
Muto (138)	1985	Japan	-	0	1-10
Iiashi (158)	1991	Japan	3872	0	<5
Mitooka (149)	1995	Japan	1152	5* (0.13%)	12-27
Kudo (150)	1995	Japan	17939	53 (0.30%)	5-21
Smith (151)	1999	UK	2198	9 (0.41%)	9-30
Fujii (140)	2000	UK	1000	4 (0.40%)	9**
Tsuda (146)	2002	Sweden	1328	5 (0.43%)	16**
Togashi (152)	2003	Japan	10939	34 (0.31%)	< 20
Diebold (142)	2004	France	-	2	4-20
Suzuki (159)	2004	UK	1026	5 (0.49%)	8-15
Gorgun(160)	2009	USA	2003	23(0.8%)†	-

*depressed lesions only **median size †adenomas only

Table 1.6: Flat and depressed colorectal cancers in surgical resections

Main Author	Year	Country	No of specimens	No flat carcinomas/ total specimens (%)
Kubota (161)	1996	Japan	300	3/300 (1.0%)
Ishihara (156)	2000	Japan	1140	15/1140(1.3%)
Nasir A (162)	2003	USA	190	22/190 (11%)*
Tweedle EM (157)	**2005**	**UK**	**1763**	**39/1763 (2.2%)**

*All sized flat and depressed lesions included

1.9.3 FLAT AND DEPRESSED LESIONS: DETECTION

The detection of flat and depressed tumours can be challenging. Magnified colonoscopy with the use of dye spraying has been recognized as the most sensitive method of detection.(163) Acceptance of the adenoma-carcinoma sequence as the main pathway of development for sporadic colorectal cancer has reinforced the need to identify and remove adenomas. Increasing awareness of flat and depressed morphologies has lead to a corresponding increase in detection and diagnosis.(164)

1.9.4 FLAT AND DEPRESSED TUMOURS: LIVERPOOL EXPERIENCE

1.9.4.1 MATERIALS AND METHODS

From 1st January 1995 to 31st December 2004, 1763 colonic resections were performed for adenocarcinoma in the Department of Colorectal Surgery, Royal Liverpool and Broadgreen University Hospitals. I conducted a retrospective review of all the histology reports. Tumours were selected when their maximum diameter was reported to be 20mm or less, regardless of the accompanying macroscopic description. Tumours associated with Familial Adenomatous Polyposis, (FAP), Hereditary Nonpolyposis Colorectal Carcinoma, (HNPCC) or Inflammatory Bowel Disease, (IBD) were excluded. Patients treated with pre-operative radiotherapy were excluded.

The original histology slides of all 61 patients were reviewed by the same experienced consultant histopathologist to avoid observer variation.(165) All lesions invaded the submucosa and were at least pT1 lesions classified according to the Vienna Classification.(166) The morphology of the tumours was classified according to the Japanese Research Society for Cancer of the Colon (JRSC).

Using these criteria, flat colorectal cancer was defined as a tumour in which the carcinomatous component is not more than twice the thickness of the surrounding non-neoplastic mucosa, all type II lesions. Fisher's exact test was used for statistical analysis. Values of $p<0.05$ were considered significant.

1.9.4.2 RESULTS

Sixty-one patients fulfilled the criteria. M: F 1:1. Age 69, (42 - 90 years). The lymph node yield, obtained from the original histology report, was 9 (0 -22). Sixty-four percent (39/61) of our cohort were flat and depressed tumours compared to thirty-three percent (20/61) polypoid tumours. Two tumours were unclassifiable due to a lack of preserved tissue. The site of the tumours was caecum 8 (13.1%), ascending colon 6 (9.8%), transverse colon 5 (8.2%), sigmoid colon 23 (36.7%) and rectum 19 (31.2%). The ratio of tumours found in the right hemicolon compared to the left hemicolon is 7:13 (35%) for polypoid tumours and 11:28 (28%) for flat and depressed tumours. The number of tumours with residual adenomatous components on histology was 5/20 (25%) in the polypoid group and 1/39 (3%) in the flat and depressed group. Seven patients had ten synchronous adenomas, all of which were polypoid. The low numbers of reported flat lesions in the background mucosa most likely reflects lack of awareness of this entity at the time of reporting.

The prevalence of small, flat and depressed colorectal cancers in our population of resected tumours was 2.2% (39/1763), an incidence of 4 resectable cancers per year. Sixty-four percent (39/61) of our cohort were flat and depressed tumours compared to thirty-three percent (20/61) polypoid tumours. Two tumours were unclassifiable due to a lack of preserved tissue. Table 1.7 shows the distribution of tumours according to the JRSC classification. All small cancers were staged

using both TNM and Dukes staging. The results are shown in Tables 1.8 and 1.9 respectively. The distribution of tumours according to T stage is shown in Figure 1.6. The proportion of flat versus polypoid tumours at each T stage was analyzed using Fisher's exact test. Statistical differences were found in the proportion of flat or depressed tumours presenting at T1, (*P*=0.033) and T3, (*P*=0.016). T1 lesions were most likely to be polypoid, T3 lesions were most likely to be flat or depressed. The rate of metastases was high in both groups, (30% polypoid versus 39% flat and depressed). Flat and depressed tumours showed a trend towards a greater proportion of distant metastases (0% polypoid v. 8% flat and depressed), this did not reach significance. See published paper Tweedle *et al.*(157)

Table 1.7: Classification of lesions according to JRSC. Table shows proportion of small lesions which fell into each morphological group.

JRSC classification	Nos of small cancers(<20mm)	Total
Polypoid		20
• Peduculated (Ip)	2	
• Subpedunculated (Ips)	4	
• sessile (Is)	14	
Flat		17
• Flat elevated (IIa)	3	
• Flat elevated with depression(IIa+IIc)	10	
• Flat (IIb)	4	
Depressed		22
• Depressed (IIc)	9	
• Depressed with rolled edge (IIc + IIa)	13	

TMN Staging	Polypoid	Flat and Depressed	All small cancers	p-value
T1	**6 (30%)**	**3 (8%)**	**9 (15%)**	**0.033***
N+	1 (17%)	1 (33%)	2 (22%)	
M+	0	0	0	
T2	**4 (20%)**	**6 (15%)**	**10 (17%)**	0.457
N+	0	1 (17%)	1 (10%)	
M+	0	0	0	
T3	**4 (20%)**	**19 (49%)**	**23 (39%)**	**0.016***
N+	2 (50%)	7 (37%)	9 (39%)	
M+	0	2 (11%)	2 (9%)	
T4	**6 (30%)**	**11 (28%)**	**17 (29%)**	0.676
N+	3 (50%)	6 (55%)	9 (53%)	
M+	0	1 (9%)	1 (6%)	
Total	**20**	**39**	**59**	

Table 1.8: Small cancers according to TNM staging. Proportion of polypoid versus flat and depressed tumours by depth of invasion (T-stage); shows a greater percentage of T1 lesions in polypoid group, and greater percentage of T3 lesion in flat/depressed group. For each T-stage, the percentage of tumours with nodal metastases (N-stage) and distant metastases (M-stage) is shown.

* Fisher's Exact Test

	A	B	C	D	Total
All small cancers	15(25%)	23*(39%)	18 (31%)	3 (5%)	59
Polypoid	9 (45%)	5 (25%)	6 (30%)	0	20
Flat and Depressed	6 (15%)	18 (46%)	12 (31%)	3 (8%)	39

Table 1.9: Small cancers according to Dukes Staging. Demonstrating fewer Duke's A and more Duke's D cancers in the flat/depressed group. *2 Dukes B tumours were not classifiable according to morphology due to lack of paraffin-embedded tissue.

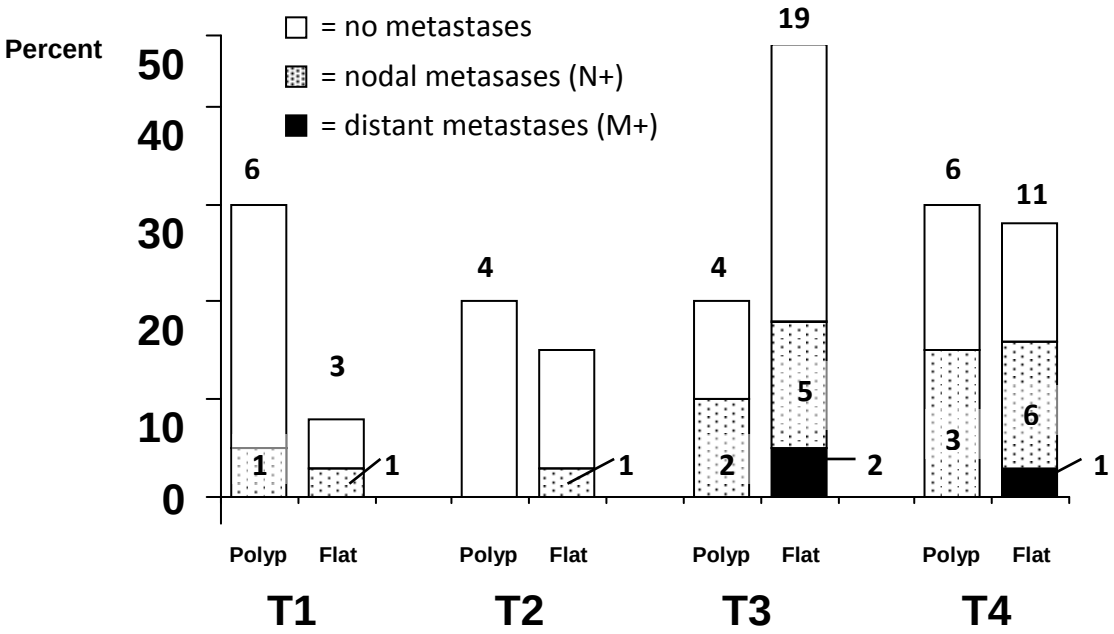


Figure 1.6: T-stage of small colorectal cancers according to morphology. Shows numbers of tumours in each group and proportion with nodal or distant metastases.

1.9.5 FLAT AND DEPRESSED LESIONS: MOLECULAR BIOLOGY

The pathway of development of flat and depressed colorectal cancers is the subject of debate. A higher frequency of high grade dysplasia and submucosal invasion in flat adenomas compared to polypoid adenomas has been demonstrated in a number of papers. This may suggest that flat adenomas are the precursor lesions of flat and depressed carcinomas.(167, 168) An alternative theory proposes that these cancers arise *de novo* from the colonic mucosa. The low or absent rates of residual adenomatous component in flat and depressed cancers support this theory.(148, 169, 170)

Many key components of both the chromosomal instability and microsatellite instability pathways have been assessed in flat and depressed tumours. Reports are conflicting; however differences in the genetic and epigenetic events which influence carcinogenesis that have been consistently detected are as discussed below.

There is some evidence that APC mutations occur less frequently in flat and depressed adenomas than polypoid adenomas with rates as low as 7% in some papers.(171) However, other studies have found higher rates of APC mutations which approach those seen in polypoid adenomas.(172) β-catenin, of the Wnt pathway and has been reported to have a higher level of mutation (24%) in flat adenomas with a central depression compared to polypoid lesions.(173) The *K-ras* signalling pathway has been investigated in flat and depressed tumours since the 1990's. Most studies have reported lower rates of mutation in early flat and depressed cancers than in polypoid cancers.(174-176) One study reported a higher rate of *K-ras* mutation in flat adenomas.(176) Recent investigations into the BRAF gene, part of the *K-ras* signalling pathway has found higher rates of mutation of this gene in flat and depressed tumours.(177) One study reported that BRAF mutations were only detected in lesions with a flat or depressed morphology.(178) Interestingly, mutations in the BRAF gene have been found to correlate closely with the presence of microsatellite instability, MSI. This contrasts with evidence from flat and depressed tumour studies which have reported low levels of MSI. p53 mutations are a late event in the chromosomal instability pathway and as such are very infrequently detected in adenomas and early cancers. No difference in rates of p53 mutations have been reported in flat and depressed lesions compared to polypoid lesions.(179, 180)

Methylation of CpG islands in the genome, epigenetic silencing, is one pathway by which genes can be functionally 'switched-off' without mutation of the genetic sequence. It is associated with the development of microsatellite instability, MSI as DNA repair genes are often affected. Two studies have assessed methylation of the promoter regions of MGMT, CDKN2A (p16) and MLH1 genes. Lower rates of methylation in flat and depressed adenomas and early cancers have been reported in both studies; this indicates that epigenetic alterations are not a feature of flat tumour development.(176, 177) In support of these findings, the panel of five microsatellite markers have been assessed in two separate studies of flat and depressed adenomas and found to occur very infrequently compared to polypoid tumours.(181, 182)

The genetic findings lend credence to the theory that these morphologically different tumours have a separate pathway of development. No proteomic studies have yet been published in relation to flat and depressed tumours. My research, in part, has attempted to address whether protein expression between different morphological groups of tumour can be characterised.

1.10 OVERVIEW OF PROTEOMICS

The genetic basis of many human cancers has been described and it is apparent that analysis of genetic material alone is insufficient to understand all of the complex cellular processes involved in tumorigenesis in many cases. Cellular proteins, (the proteome) are the expressed products of genes, (the genome), and are responsible for nearly all cellular functions. There are a number of levels at which the quantity and activity of a given protein can be regulated, including rates of gene transcription, translation into protein and post-translational modifications. The result of these complex levels of regulation means that the proteome can consists of several hundred thousand different proteins and may be a more sensitive indicator of disease. Analysis of cellular proteins, (proteomics) can offer a unique insight into the molecular mechanisms of cancer development and enable identification of tumours markers or novel therapeutic targets.(183) The advances in biotechnology mean that proteomic techniques can be used to quantify and identify proteins in cell culture, tissue and body fluids, (such as plasma or urine). Proteomics encompasses the study of proteins in health and disease, characterisation of post-translation modifications and protein-protein interactions. Investigation of this intricate environment means that there are several essential steps which must be taken in order to produce a result. These include sample storage and preparation, purification, separation, visualisation and identification of proteins.(184)

1.10.1 SAMPLE STORAGE

The method of sample preparation used is very dependent on the subject under analysis. Cells in culture can be very simply prepared by lysing sufficient quantities of cells in lysis buffer. Analysis of

tissue is more complex as fresh tissue must be fixed quickly to avoid degradation. Formalin is a commonly-used fixative and DNA is relatively stable in formalin-fixed material. RNA and protein is subject to cross-linkage formation by formalin which reduces the quality of material which can be procured.(185, 186) Snap-freezing in liquid nitrogen is currently the preferred method of storage for fresh tissue. New alcohol-based fixative agents may prove useful if they are shown to preserve the quality of RNA and protein in the tissue.

1.10.2 PURIFICATION

The gold-standard of proteomics is the analysis of cells in the context of their native tissue.(187) The complexity of tissue means that the cells of interest only constitute a small proportion of the sample. Three main approaches have been taken to address the issue; cultured cell-lines, global survey and microdissection. The use of cell-lines cultured from fresh tissue is common; it is particularly useful for investigating the effects of interventions such as drugs on cells. The disadvantages in terms of cancer research are that the cells are removed from their microenvironment and this influences their behaviour and protein profile. The global survey approach utilises the assumption that RNA levels accurately reflect gene expression within a tissue. The disadvantages are that the cell population of interest is present in different proportions in whole tissue samples. Microdissection involves the separation of one or more specific cell populations directly from tissue for analysis. Various methods have been described; all involve sectioning of tissue and selection of the cells of interest under direct vision. Initial approaches involved manual dissection of tissue using a microscope and needle. More recently, two laser assisted techniques have emerged as the methods of choice.(188) Laser-beam microdissection

involves excising selected areas of tissue with a laser, then retrieving the material by pressure catapulting or needle transfer into extraction buffer. Laser-capture microdissection, LCM was developed at the National Institute of Health, USA. In this technique the tissue section is placed in contact with a cap coated with a heat-sensitive ethylene vinyl acetate film. On firing of the laser at the cells of interest, a small area of the film (7.5-30μm in diameter) melts and fuses the cells to the cap. The cap can then be lifted, removing the selected cells from the tissue. The cap is placed in extraction buffer which is used to lyse the cells and solubilise the protein or RNA for analysis, figure 1.7.(188) The disadvantages of this technique are that staining and sectioning of the tissue to render it suitable for laser capture can degrade the protein or RNA content.(189) Strong H&E staining of sections in our laboratory was found to reduce the quality and quantity of protein extracted after laser capture. Other stains have been tried, such as methyl green which appear to have little effect on the extracted protein. One disadvantage of all microdissection techniques is that a level of expertise is required to examine the tissue and identify the cell population of interest. Moreover the process is labour intensive and time-consuming. Despite these problems, microdissection is the only utilised technique which enables comparisons of different cell population from the same tissue.

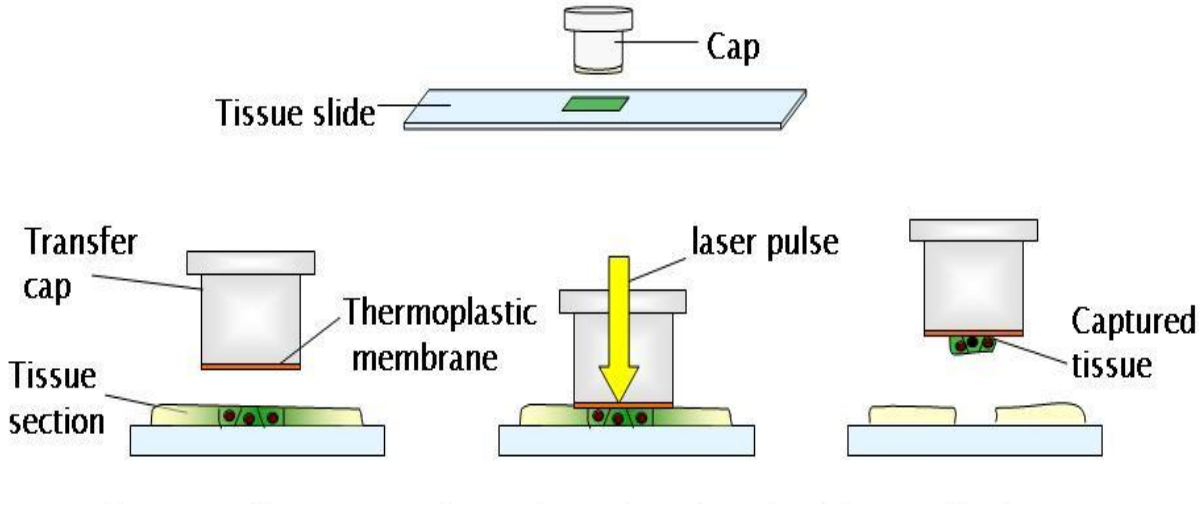


Figure 1.1: Schematic representation of laser-capture microdissection **(from Mr Ali Shekouh)**

Fluorescence-activated cell sorting (FACS) is a type of flow cytometry devised and trademarked by Becton, Dickinson and Company. It provides a method for sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. A cellular suspension is generated from blood or tissue and the cells of interest labelled with an immunofluorescent antibody tag. In the flow cytometer the suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is broken into individual droplets, each one containing a cell. A laser is used to measure the fluorescent character of each cell as it passes; simultaneously an electrical charge is applied to the droplet. The droplets then fall through an electrostatic deflection field that diverts them into containers based upon their charge.(190) Flow cytometry has been in use since the 1960's, but the technology to capture in addition to quantifying populations of living cells have seen it recently

applied in the proteomic analysis of circulating human leukocytes.(191) It is likely that in the future this technology will be applied to effectively separate cancer cell populations from tissue for proteomic analysis.

1.10.3 SEPARATION AND VISUALISATION

There may be several hundred thousand different proteins in any given sample for proteomic analysis. None of the methods for fractionation and separation of proteins are sensitive enough to allow detection of the entire proteome. There are two main methods of protein separation, gel-based and non gel-based techniques.

1.10.3.1 GEL-BASED

Separation of proteins in two dimensions on a SDS-polyacrylamide gel is a technique which was first described in the 1960's.(192) Proteins are first separated by charge and pH in the first dimension, then by molecular weight in the second dimension. Commonly a gel strip with a fixed pH gradient is used to separate proteins in the first dimension. Once a voltage is applied the protein migrates along the strip towards the anode until it reaches a pH where the charge is lost (isoelectric point). Varying the pH gradient can allow different sets of proteins to be visualised. Common variations include non-linear gradients, with an expanded section from pH 7-9, or reduced pH range. Following protein separation, gels are stained so that the individual protein spots can be visualised. A relatively small quantity of protein, (100-200µg) can produce a good quality gel with up to 10,000 spots visible. The most common protein visualization methods utilised are Coomassie blue staining, silver staining and increasingly, fluorescent staining.(193) Coomassie blue stains in a linear manner

and is easily reproducible, however, it is only sensitive for protein levels greater than 2mg per 2D gel (15x20cm). Silver staining is exponential, meaning that the rate of development of staining increases as more staining develops. Silver ions bind to protein within the gel and are reduced, (usually by formaldehyde or gluteraldehyde) to visible silver.(194) Deposits of non-ionic silver further catalyse the reduction of silver ions in the vicinity. This method is very sensitive (0.1ng per spot) however, it has a number of disadvantages. The dynamic range is only 10-20 fold and the complexity of the staining procedure makes reproducibility difficult. Some of the reduction agents, (such as gluteraldehyde) used for silver staining can cause structural changes in the proteins making subsequent identification with mass spectrometry impossible. Nevertheless, silver staining is a sensitive and reproducible method of gel staining, and despite its drawbacks, is frequently utilised.

Difference gel electrophoresis (DIGE) is a staining method in which two protein samples are separately labelled with different fluorescent dyes and then co-electrophoresed on the same 2DE gel. There are two fluorescent dyes in common use, propyl-Cy3-NHS and methyl-Cy5-NHS; they have no net charge and have molecular masses which differ by only 2Da. Two labelling techiques are available (minimal and saturation labelling) depending on which amino acids residue is targeted; lysine (average of 30 residues per 50 kDa protein) or cysteine (average of 1-2 residues per 50kDa protein). Minimal labelling targets 1%–2% of all lysines, which results in only a fraction of each protein species carrying a single dye molecule, while the rest are unlabelled. This creates spot shift in the lableled versus unlabelled protein population in the molecular mass dimension. This shift is negligible for proteins >30 kDa; however, proteins <30 kDa are generally shifted by up to one spot diameter. In practice, the minimal labelling dyes are used when one has >100 µg

protein/sample. Saturation labelling targets 100% of cysteine residues and labels the majority of protein in each sample, therefore this technique does not result in spot shift. It is typically used for low abundance, precious samples.(195)

DIGE is sensitive, detecting <0.5 fmol protein per spot with minimal labelling and <25 amol per spot with saturation labelling. Fluorescent dyes have a detection range of about 30-fold under constant illumination, which is similar to silver staining.(196) This can be increased to 20,000-fold but requires the use of a scientific-grade, cooled charge-coupled device (CCD) camera. The main disadvantages with the use of DIGE are that a maximum of three samples can be compared per gel. In addition gels typical have a limited lifespan in storage, three months at -70 C for minimally-labelled gels or one month at -70 C for those labelled with saturation dyes.(197)

The advantages of the gel-based method are that a large amount of data can be generated from each gel as the isoelectric point and molecular weight of thousands of proteins can be calculated. Post-translational modifications of proteins can often be identified as horizontal or vertical clusters of proteins.(198) The disadvantage of gel-based techniques is that it is capable of demonstrating only 10-20% of the entire proteome. It has a particularly low sensitivity for certain types of protein including membrane-bound proteins, highly acidic or basic proteins, low molecular weight and low abundance proteins. Often the low abundance proteins are those of interest in terms of protein markers of disease.(199) Non-gel based protein separation techniques have been developed to attempt to address some of these problems.

1.10.3.2 NON-GEL BASED TECHNIQUES

There are two commonly used non-gel based techniques that allow relative quantification of proteins in samples; these are isotope-coded affinity tags (ICAT) and isobaric tags for relative and absolute quantification (iTRAQ). Both techniques use stable isotope labelling (SIL) to 'tag' peptides and allow identification and relative quantification of proteins. ICAT uses two isotope tags; one contains hydrogen (H), the other deuterium (L) with a mass difference of 8Da. The isotopes bind to the amino acid cysteine which is a component in an estimated 6 out of 7 of peptides. Two protein samples are separately labelled with H and L, the samples are then combined and subject to trypsin digestion.(200) The mixed, labelled peptides are eluted through a column containing a stationary phase which binds the peptides to the column matrix. The pH of the elution buffer, (mobile phase) is gradually increased so that the peptides are washed from the column individually in a process known as liquid chromatography (LC). Mass spectrometry is then used to identify ICAT pairs of peptides. A search of the database containing peptide fragmentation data results in the identification of the peptides and hence the original protein.

iTRAQ is based upon tagging the N-terminus of peptides generated from protein digests.(201) Up to eight protein solutions are subject to trypsin digestion, each peptide solution is then labelled with a different tag. The tags are all neutral with the same molecular mass so they do not interfere with the liquid chromatography fractionation. Fragmentation of the tag attached to the peptides in the mass spectrometer generates a low molecular mass reporter ion that is unique to the tag used to label each of the digests. The relative intensity of these reporter ions enables relative quantification of the peptides in each digest and hence the original proteins.(202) iTRAQ is rapidly superseding

ICAT for proteomic analysis. It is more sensitive, allows eight samples to be compared and because the tags bind to the N-terminus rather than cysteine residues all proteins are potentially represented in the analysis.(203)

1.10.4 PROTEIN IDENTIFICATION

Peptide mass fingerprinting (PMF) is an analytical technique for protein identification that was developed by John Yates and colleagues.(204) In short, the unknown protein of interest is cleaved into peptides. Proteolysis using trypsin is most commonly used for this purpose as it has a high specificity and efficiency. The collection of peptides resulting from this cleavage comprises a unique identifier of the unknown protein. The absolute masses of the (still unknown) peptides are accurately measured with a mass spectrometer such as matrix-assisted laser absorption ionisation-time of flight (MALDI-ToF) or electrospray ionization-time of flight (ESI-ToF). Many proteins can be identified simply by knowing the mass of each constituent peptide; however the best chance of identification comes by providing the amino acid sequence of one of the peptides.

1.10.4.1 ION PRODUCTION, (MALDI AND ESI)

MALDI is the most commonly used technique to ionise the peptide molecules within a sample. The sample is loaded onto and organic matrix and laser energy is transferred to the sample causing ionisation. ESI is a newer technique where the sample is pushed through a very small, charged and usually metal, capillary. The buffer in the sample is more volatile than the peptides dissolved in it. As the sample is forced out of the capillary it forms an aerosol, a mist of small droplets about 10µm across. As the buffer evaporates, the peptide molecules are forced closer together, repel each other and break up the droplets further. The process repeats until the peptide is free of buffer and

is a lone ion. These lone ions then move to the mass analyzer component of the mass spectrometer. As technology in the field of proteomics has advanced, liquid chromatography columns have been coupled directly to ESI mass spectrometers allowing the identification of peptides as they are eluted from the mixture, a process known as liquid chromatography-mass spectrometry (LC-MS).(205)

1.10.4.2 ION SEPARATION AND ANALYSIS

Once the peptides have been ionised using MALDI or ESI, they pass into the analyser which uses ToF to calculate the mass. Essentially, the peptides are accelerated in a vacuum tube towards a detector. The amount of time it takes for the ion to reach the detector is proportional to the mass of the peptide. Most analysers now include delayed extraction or reflection tubes which essentially 'focus' the cloud of peptides in the field and have a longer flight-time. One method of detecting the mass of peptides is called the Fourier transform ion cyclotron, (FTICR). This relies on exciting the peptides in a magnetic field then detecting the sine wave signature released; this sine wave can then be converted into a mass. The advantages of this technique are that the peptides do not have to hit the detector, just pass close enough for the wave to be detected. Additionally, it is useful for complex mixtures of peptides as the specific resolution (narrow peak width) allows the signals of two ions of similar mass to charge ratio to be detected as distinct ions.(206) The ToF analyser is still the most commonly ion detection method used in MS.

Tandem mass spectrometry (MS/MS) is the now the preferred technology for many applications in which mass spectrometry plays a part. Hybrid MS instruments consist of two ToF mass analyzers in series. After passing through the first detector the ions are subject to collision-induced

decomposition (CID) in a second chamber by subjecting them to energetic collisions with inert gas molecules. These collisions further fragment the peptides which are then passed through the second ToF detector. In this way fingerprints of component peptides can be produced to aid identification of the original protein.(207)

1.11 PROTEIN MARKERS IN COLORECTAL CANCER

Vast arrays of proteins have been identified as over-expressed or under-expressed in colorectal cancer tissue from proteomic analysis. Analyses of differential protein expression between normal colonic mucosa and colonic adenocarcinoma have identified a list of commonly identified proteins,(208, 209) listed in Table 1.10. Few of these markers have been verified as prognostic indicators of disease, and fewer yet are used clinically. A serum marker of colorectal cancer is the gold standard in terms of diagnosis and screening for the disease. Unfortunately, there is no protein marker which is sensitive enough for the task. Serum markers can be used to determine response to treatment and for surveillance. A number of promising serum and tissue biomarkers have been identified by the European Group on Tumour Markers (EGTM); however the only one currently recommended for clinical use is carcinoembryonic antigen (CEA).(210, 211) Tissue-based markers have been investigated for potential prognostic and predictive value. The potential prognostic and predictive value of the most widely studied tissue markers in colorectal cancer are discussed below.

Up-regulated proteins		Down-regulated proteins
Annexin IV		NCF2
MTA-1		PMM2
SSX5 protein		Serpin 1
Dynein heavy chain		CNRC
Cytochrome P450		Annexin V
CPT1		APC
Keratin 10		VAV3 protein
Keratin 8		RSP 4
Keratin 19		SPARC like protein 1
Vimentin		PDI
β-actin		GN6ST
REL1		Cathepsin D
HSP60		Calreticulin
Mortalin		SM31
Cytochrome P450 enzymes		PDA6 ApoA1 precursor
HSP70		ATP synthase b chain
S100A9		Albumin
S100A8		Liver fatty acid-binding protein
S100A11		Actin-binding protein/smooth muscle
S100A6		protein 22-a
Adenosyl homocysteinase		Cyclooxygenase 2
Leukocyte elastase inhibitor, claude B		Puromycin-sensitive aminopeptidase
Macrophage capping protein	Cathepsin fragment	NADH-ubiquinone oxidoreductase
Biliverdin reductase A	Proteasome subunit α type 6	Succinate dehydrogenase subunit A
Annexin 1 fragment	Triosephosphate isomerase	Aldehyde dehydrogenase, cytosolic,
α-tubulin	14-3-3 proteins	class I
Elongation factor 1-d	GST-P	Selenium-binding protein
Tropomyosin α 1	P13693 translationally controlled tumour protein	Creatin kinase B chain
Tropomyosin α 4 chain	Nucleoside diphosphate kinase A	Placental thrombin inhibitor
Actin fragment	Calgranulin B; S100 A9	Vimentin
Annexin 3, 4 and 5		Desmin
Microtuble-associated protein RP/EB		Tubulin β 5 chain
Pyridoxal kinase		Carbonic anhydrase I
		Myosin regulatory light chain 2

Table 1.10: Proteins with altered regulation in colorectal cancer detected by proteomics (208, 209)

1.11.1 CARCINOEMBRYONIC ANTIGEN (CEA)

The main use of serum CEA in colorectal cancer is in surveillance following curative resection for primary cancer. It is not recommended for screening or diagnosis because of its low sensitivity for cancer. A number of independent meta-analyses have compared outcome in patients undergoing intensive follow-up versus minimal or no follow-up. Although the frequency and modalities of screening used in the individual studies varied, all meta-analyses concluded that use of an intensive follow-up regime resulted in a modest but statistically significant improvement in outcome compared with a minimal follow-up strategy.(212-214) The Cochrane Review concluded that there was an overall survival benefit at five years of follow-up for patients undergoing more intensive surveillance (OR = 0.67, 95% confidence interval, 0.53–0.84).(215) Two of these meta-analyses investigated the specific contribution of CEA to the improved outcome. In the first of these, Bruinvels et al. concluded that intensive follow-up was associated with an improved outcome, but only if regular CEA determinations were carried out.(216) Similarly, Figueredo *et al.* (2003) found that trials using serial CEA measurements had a significant impact on survival, whereas those not using CEA failed to impact on outcome.(214) Recent reports suggest that a postchemoradiotherapy CEA level <5 ng/ml is a favorable prognostic factor for rectal cancer and is associated with increased rates of earlier disease staging and complete tumor regression.(217) Determination of CEA and carbohydrate antigen 19-9 (CA19-9) in peritoneal fluid at resection may also predict peritoneal recurrence in the absence of detectable tumour cells.(218) The EGTM Panel guidelines state that for stages II and III colorectal cancer patients, CEA should be measured every 2–3 months for at least 3 years, not only for patients who are suitable candidates for liver resection, but also for patients who are candidates for receiving systemic therapy.(210)

1.11.2 CARBOHYDRATE ANTIGEN 19-9 (CA19-9)

The CA19-9 assay was devised to detect sulfated mucins isolated from normal human colonic mucosa. It is an antibody which recognizes the sialylated Lewis (a) antigen, fucopentaose II. CA19-9 is the best serum marker for pancreatic adenocarcinoma; however it is less sensitive than CEA for the detection of colorectal cancer.(219) Elevated preoperative levels of CA19-9 have been found to correlate with adverse patient outcome.(220) The largest of these studies (n=495 patients), found the prognostic impact of CA19-9 to be independent of both Dukes' stage and CEA concentration.(221) Filella and colleagues compared CEA and CA19-9 in the follow-up of 370 patients with diagnosed colorectal cancer. While CEA was abnormal in 84% of patients with recurrence and provided a lead-time in 75%, CA19-9 levels were elevated in only 48% and gave a lead-time in only 25%.(222) Similarly, in monitoring the treatment of metastatic colorectal cancer, CA 19-9 results yielded no additional information to that provided by CEA. The use of CA19-9 for diagnosis and surveillance of colorectal cancer is not recommended by the EGTM panel.

1.11.3 CARBOHYDRATE ANTIGEN 242 (CA242)

As with CEA and CA19-9, serum CA242 cannot be used for the detection of early stage colorectal cancer. Carpelan-Holmstrom and colleagues have shown using multivariate analysis, that high preoperative levels of CA242 were a significant predictor of outcome when CEA was included in the analysis, but CEA only became an independent factor if CA242 was excluded.(223) Hall and colleagues compared CEA and CA242 in the surveillance of 149 patients who had undergone apparent curative resection for colorectal cancer. For the detection of recurrent disease, CEA alone had a sensitivity of 76% and a specificity of 86%. The corresponding sensitivity and specificity for

CA242 were 60% and 87%, respectively. Combination of the two markers increased the sensitivity to 88%, but reduced specificity to 78%.(224) The authors concluded that although CA242 alone is inferior to CEA, it may complement CEA in the follow-up after curative resection for colorectal cancer.

1.11.4 TISSUE POLYPEPTIDE ANTIGEN (TPA) AND TISSUE POLYPEPTIDE SPECIFIC ANTIGEN (TPS)

TPA is a complex of polypeptide fragments from cytokeratins 8, 18 and 19 and TPS is one epitope that contains fragments of cytokeratin 18. Both antigens have been subjected to limited evaluation in colorectal cancer. In a study of 202 newly diagnosed patients, Lindmark and colleagues found that high levels of CEA, C 19-9, CA242, TPA and TPS were all associated with aggressive disease.(225) In patients undergoing liver resection or radiofrequency ablation, TPA and TPS but not CEA, CA19-9 or thimidine kinase (TK), were important predictive markers of overall survival and disease-free survival.(226)

1.11.5 TISSUE INHIBITORS OF METALLOPROTEINASES (TIMP-1)

TIMP-1 is a multifunctional glycoprotein that inhibits metalloproteinase activity, stimulates cell growth and inhibits apoptosis.(227) Using a research enzyme-linked immunosorbent assay (ELISA), Holten-Andersen and colleagues reported that total serum levels of TIMP-1 were significantly higher in patients with both colonic (n=338) and rectal cancer (n=108) than in either healthy subjects (n=108) or patients with inflammatory bowel disease (n=50). At 95% specificity, TIMP-1 detected colonic cancer with a sensitivity of 65% and rectal cancer with a sensitivity of 42%. Combining CEA with TIMP-1 increased sensitivity for colonic cancer from 65% to 75% and rectal cancer from 42% to 54% (at 95% specificity).(228)

1.11.6 THYMIDYLATE SYNTHASE (TS)

TS is the rate limiting enzyme involved in the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). This reaction provides the only de novo source of thymidylate which is essential for DNA synthesis.(229) TS expression has been widely investigated in colorectal cancer tissue as both a prognostic and a therapy predictive marker. The rationale for using TS as a therapy predictive marker in colorectal cancer is that it acts as the key target for several cytotoxic agents used to treat this disease such as the fluoropyrimidines, 5-fluorouracil (5-FU) and 5-fluorodeoxyuridine and the antifolate agent, tomudex.(230) Although widely used to treat colorectal cancer, only about 20% of patients with advanced disease respond to 5-FU. A retrospective clinical trial suggests that high TS levels in colorectal cancer tissue are associated with either relative resistance to 5-FU or poor outcome following treatment.(231) Popat *et al.* performed a systematic review of the literature and meta-analysis. For patients with advanced colorectal cancer, 13 studies containing a total of 887 patients were identified. Of these, 12 were regarded as suitable for pooling of data. All of the patients in these trials were treated with TS inhibitors. Following a pooled-analysis, the overall hazard ratio (HR) associated with high levels of TS for overall survival was 1.74 (95% CI, 1.34–2.26). The HR improved if the assay was performed on the metastatic lesion to 2.39 (95% CI, 1.43–4.01).(232) The findings of this meta-analysis are promising in correlating high TS expression with poor outcome in patients with colorectal cancer, particularly metastatic disease. The disadvantages are that there was evidence of publication bias in the studies on patients with advanced disease and at present there is no standardised assay for the measurement of TS. The EGTM do not recommend the use of TS as a prognostic marker at present.

1.11.7 MICROSATELLITE INSTABILITY (MSI)

Testing for MSI has a number of potential applications in colorectal cancer including; use as a surrogate marker for hereditary non-polyposis colorectal cancer (HNPCC), determining prognosis and predicting response to adjuvant chemotherapy in patients with sporadic colorectal cancer. Popat et al carried out a systematic review and pooled analysis of published studies relating MSI to prognosis in patients with colorectal cancer. Following a pooled analysis, patients with MSI had a 15% better outcome compared to those without MSI.(233) The reason for the association between MSI and favourable prognosis may be related to a protective role provided by functionally active lymphocytes which infiltrate MSI-positive colorectal cancer.(234) The EGTM Panel recommended that MSI testing be validated in a clinical trial prior to its use as a general prognostic marker.(210)

1.11.8 P53

The p53 tumour suppressor gene encodes a transcription factor that regulates the expression of genes involved in apoptosis, angiogenesis, cell cycle and genome maintenance.(235) p53 has been widely investigated both as a prognostic and as a therapy predictive marker in colorectal cancer.(236) A multiplicity of methods was used to determine p53 abnormalities, including immunohistochemistry to detect p53 protein or DNA sequence analysis to detect gene mutations. Munro et al. carried out a systematic review of published studies that investigated the relationship between p53 abnormality and outcome in patients with colorectal cancer.(68) In total, 168 eligible studies comprising survival data on 18,766 patients were identified. The key findings were as follows:

• Patients with abnormal p53 whether detected by immunohistochemistry (IHC) or DNA sequence analysis had an increased risk of death. The relative risk (RR) with IHC was 1.32 (95% CI, 1.23–1.42) and with sequence analysis was 1.31 (95% CI, 1.19 1.90).

• p53 had no impact on outcome in patients treated with chemotherapy.

• Abnormal p53 correlated with failure of response to radiotherapy in patients with rectal cancer (RR, 1.49, 95% CI, 1.25–1.77).

Due to the modest impact on survival described, routine determination of p53 status is not recommended in colorectal cancer by the EGTM panel. However, new data which combines estimation of p53 and its downstream activator p21 may be useful in predicting outcome and response to 5FU chemotherapy.(237)

1.11.9 *K-RAS*

K-ras is one of the most frequently mutated c-oncogenes in human cancer. It functions as a guanine nucleotide binding protein involved in signal transduction.(41) Conflicting data exist on the relationship between the presence of mutant *K-ras* and prognosis in patients with colorectal cancer. A recent meta-analysis involving 4268 patients from 42 different institutions concluded that only a specific type of *K-ras* mutation predicted poor outcome (patients with a G–T transversion at codon 12). This specific mutation was detected in less than 10% of tumours and was prognostic in Duke's C but not in Duke's B patients.(238) The EGTM panel do not recommend determination of *K-ras* mutation for prediction of prognosis in patients with colorectal cancer. However, recent studies revealed that the presence of *K-ras* mutation predicts lack of response to EGFR inhibition in the treatment of metastatic colorectal cancer.(128)

Marker	Use for test	EGTM recommendations
Serum		
CEA	Determining prognosis	May be used in combination with standard prognostic factors
	Surveillance following curative resection	Should be used for stages II and III patients who may be candidates for liver resection or systemic treatment, should recurrence develop
	Monitoring therapy in advanced disease	Should be used, especially in patients with non-evaluable disease using standard criteria. Measure prior to start of treatment and at 2–3 monthly intervals during therapy; use in combination with imaging.
CA19-9	Determining prognosis	Not recommended
	Surveillance following curative resection	Not recommended
CA 242	Determining prognosis	Not recommended
TIMP-1	Determining prognosis	Not recommended
Tissue		
TS	Determining prognosis	Not recommended
	Response to chemotherapy	Not recommended
MSI	Determining prognosis	Not recommended
	Response to chemotherapy	Not recommended
DCC	Determining prognosis	Not recommended
K-ras	Determining prognosis	Not recommended
	Predicting response to EGFR inhibition	Should perform *Kras*-gene testing before prescribing EGFR inhibitors
p53	Determining prognosis	Not recommended

Table 1.11: EGTM recommendations for the use of common protein markers.

Duffy *et al*. 2007 (210)

1.12 AIMS AND OBJECTIVES

Proteomic analysis using 2D electrophoresis is well established for the identification of differentially expressed proteins in human cancers. The procurement of enough tissue from small colorectal tumours with low numbers of cancer cells in the specimen is a particular challenge which has been resolved with laser-capture microdissection. Prognostic markers in small colorectal cancers will be more crucial in coming years as the National Colorectal Cancer Screening Program detects a higher frequency of small and early cancers. We have demonstrated an association between increasing stage at presentation and flat and depressed morphology in small cancers.

In this study I compared dissected adenocarcinoma tissue according to morphology in small cancers with a view to identifying differential protein expression related to prognosis. Benign matched epithelial tissue was also dissected for comparison. The aims of the project were as follows;

i. To compare protein profiles of small colorectal tumours with matched normal colonic epithelium
ii. To compare protein expression profiles of small polypoid and depressed adenocarcinoma tissue
iii. To identify differentially expressed protein markers between the groups
iv. To verify the protein markers using immunohistochemistry in a large group of up to 404 microarrayed colorectal cancers (199 colon cancer and 205 rectal cancers)

2. MATERIAL AND METHODS

The methods utilised in this project were devised following similar work on pancreatic ductal adenocarcinoma by A Shekouh *et al.* in 2003 in the Division and Surgery and Oncology, University of Liverpool.(239)

2.1 TISSUE PREPARATION

Specimens of colon and rectum were obtained following surgery, with full ethical consent, and examined by a specialist pathologist. Areas of tissue, considered upon macroscopic viewing to contain malignant or non-malignant colon or rectum were selected. The samples were cryofixed in liquid isopentane (-160°C), cooled by liquid nitrogen and stored at -160°C in the Liverpool Tissue Bank, School of Cancer Studies, University of Liverpool. Microscopic examination of the specimens to verify the presence of malignant tumour was undertaken by a consultant histopathologist after 5µm frozen sections were cut onto slides and stained with haematoxylin and eosin (H&E). Each tissue sample was assigned an anonymised code on the Liverpool Tissue Bank, database. A microscopic description of the sample, details of the formal histopathology report of the specimen and clinical details of the patients were recorded under this code.

2.2 IDENTIFICATION OF SAMPLES

Samples for this study were identified by searching the Liverpool Tissue Bank database for tumours with a maximum diameter of 20mm. Fourteen frozen tumours samples, resected between 1995 – 2006 met the criteria. A consultant histopathologist reviewed the H&E slides of these tumours and classified them according to morphology using the Japanese Research Society Classification. Matched normal colonic tissue was available in all cases except one, 148-01T. The morphology and histopathological characteristics of the samples are documented in Table 2.1. H&E stained sections of each tumour are presented in Figure 2.1.

Liverpool Tissue Bank No	Morphology	Matched normal colon	T-stage	N-stage	Dukes' stage	Differentiation
104-06T	Depressed	✓	3	1	C	Moderate
194-00T	Depressed	✓	3	1	C	Moderate
084-06T	Depressed	✓	3	0	B	Moderate
149-01T	Depressed	✓	4	0	B	Moderate
264-05T	Depressed	✓	3	0	B	Well
097-99T	Depressed	✓	1	0	A	Moderate
075-00T	Polypoid	✓	4	1	C	Moderate/poor
233-00T	Polypoid	✓	1	0	A	Moderate
292-99T	Polypoid	✓	1	0	A	Moderate
259-01T	Polypoid	✓	1	0	A	Well
148-01T	Flat	×	3	1	C	Moderate
054-96T	Flat	✓	3	0	B	Moderate
108-03T	Flat	✓	2	0	A	Moderate
010-03T	Flat	✓	2	0	A	Moderate

Table 2.1: Characteristics of frozen Tissue samples

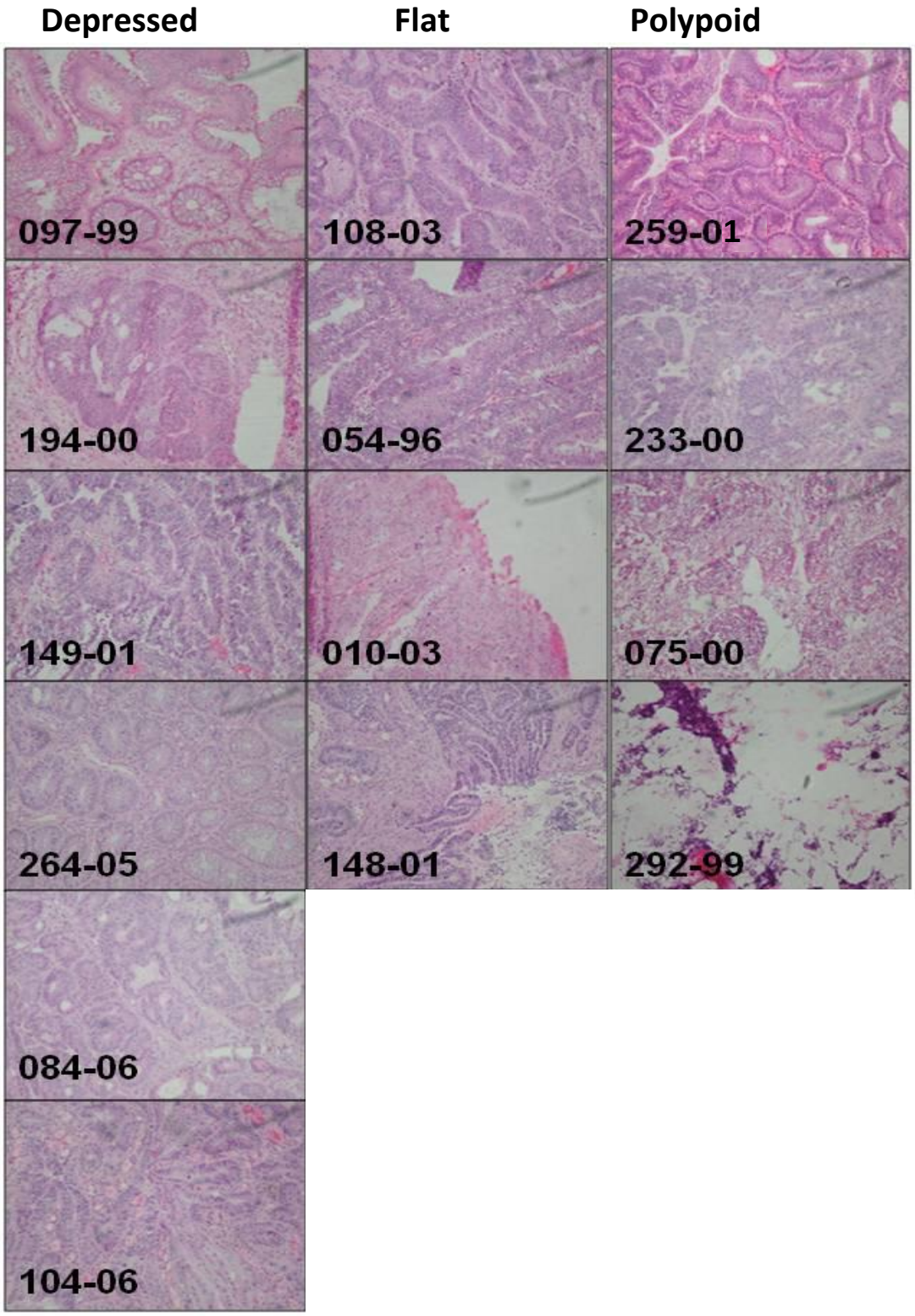


Figure 2.1: H&E stained frozen sections from tumour tissue according to morphology

2.3 SECTIONING AND STAINING OF SAMPLES

Sectioning of frozen tissue was performed using a Bright OTF 5000 cryostat, (Huntingdon, Cambridgeshire, UK), with the chamber temperature set to -20°C. Frozen sections 8µm thick were cut and immediately transferred to BDH frosted slides which had been pre-cleaned using detergent, washed with deionised water and oven-dried at 40°C. Sections were fixed immediately in 70% ethanol for 1 minute and rested in deionised water for no more than 10 minutes. Complete protease inhibitor cocktail tablets (Roche) were added to all the solutions used for fixing and staining, except xylene, (one tablet/80 mL solution). Two separate staining protocols were employed; H&E staining and Methyl green staining (see below). H&E stained sections were viewed using an inverting microscope (Leica CME microscope) and reference photographs taken with a Nixon Coolpix 4500. The reference slides and images were used to guide LCM, which was performed on methyl green stained sections only.

H & E staining protocol	
Ehrlich's haematoxylin (BDH)	30 seconds
Deionised water	wash
Acid alcohol 0.3%	5 seconds
Deionised water	wash
Scott's tap water (2% ammonium hydroxide)	5 seconds
Deionised water	wash
100% ethanol	15 seconds
Eosin (Shandon Inc.)	15 seconds
100% ethanol	30 seconds x3
Xylene	1 minute x2
Coverslips applied using DPX mountant (VWR Ltd.)	

Methyl green staining protocol	
Violet-free methyl green (2% in deionised water)	30 seconds
Deionised water	wash x 2
70% ethanol	30 seconds
95% ethanol	30 seconds
100% ethanol	30 seconds x2
Xylene	5 minutes x 2
Sections air-dried in fume hood for 30-60 minutes	

2.4 LASER CAPTURE MICRODISSECTION

Air-dried methyl green stained sections were microdissected using an Arcturus Pixcell II system (Arcturus, Mountain View, CA, USA) with a 7.5 µm laser beam. Arcturus Capsure^{TM} Macro LCM Caps were applied to the slides so that the thermoplastic film was in direct contact with the section. Images of the section at 10x and 100x were viewed on the attached monitor (Sony Triniton) and the laser beam targeted at the cells of interest. Power and pulse duration were adjusted to generate melting of the film at the desired point on firing the laser. The conditions were typically 60-80 mW and 3–4 ms respectively. Two cell types were captured;

- Malignant epithelial cells (n=12; 4 depressed, 4 polypoid and 3 flat type)
- Normal columnar epithelial cells (n=11, matched to tumour)

An estimated 80,000 cells from each specimen were laser captured using an average of 40,000 pulses. Approximately 7-10 hours of LCM was required to obtain this quantity. Microdissection caps

were inserted into 0.5 mL eppendorf tubes containing 50 µL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris base) and the cells solubilised by inversion of the tubes for 30 min on ice, followed by vortex-mixing for 30s and a brief pulse-centrifugation at 16,000 rcf. Five tubes, each containing 50 µL of lysis buffer were used for each specimen. The lysates from each tube were pooled once sufficient material had been collected and stored at -80ºC.

Two specimens, 194-00 and 097-99 were sectioned and subjected to laser capture, but insufficient material was acquired to proceed to SDS-PAGE due to the small volume of tumour in the sample. A third specimen 292-99 was found to be adipose tissue only on sectioning. All other specimens (n=11 tumours and n=10 normal colon) underwent successful accrual of material by laser capture, with a minimum of 40,000 pulses. One specimen, 233-00T, underwent laser capture of 70,000 pulses in order to produce enough material for a second 2D gel. The first 2D gel of the same specimen had inadequate separation in the first dimension and identification of protein spots on the right hand side of the gel was impaired. A summary of the results of laser capture and the corresponding SDS-PAGE gel produced are presented in Table 2.2. An example of the laser capture results obtained from the dissection of one small colorectal cancer with matching normal colonic tissue from the same patient is shown in Figure 2.2.

Liverpool Tissue Bank No	Gel no	Gel batch	Number of pulses at LCM	Morphology
104-06T	A	1	40,000	Depressed tumour
104-06N	B	1	40,000	Normal colon
075-00T	C	1	40,000	Polypoid tumour
075-00N	D	1	40,000	Normal colon
010-03T	E	1	40,000	Flat tumour
010-03N	F	1	40,000	Normal colon
084-06T	G	2	40,000	Depressed tumour
084-06N	H	2	40,000	Normal colon
233-00T	I	2 and 3	70,000	Polypoid tumour
233-00N	J	2 and 3	40,000	Normal colon
054-96T	K	2	40,000	Flat tumour
054-06N	L	2	40,000	Normal colon
148-01T	M	2	40,000	Flat tumour
264-05T	N	3	40,000	Depressed tumour
264-05N	O	3	40,000	Normal colon
259-01T	P	3	40,000	Polypoid tumour
259-01N	Q	3	40,000	Normal colon
108-03T	R	3	40,000	Flat tumour
108-03N	S	3	40,000	Normal colon
149-01T	T	3	40,000	Depressed tumour
149-01N	U	3	40,000	Normal colon
194-00T	-	Not run	15,000 (insufficient)	Depressed tumour
097-99T	-	Not run	10,000 (insufficient)	Depressed tumour
292-99T	-	Not run	0 (no tumour)	Polypoid tumour

Table 2.2: Summary of Laser Capture Microscopy procurement of material for proteomic analysis

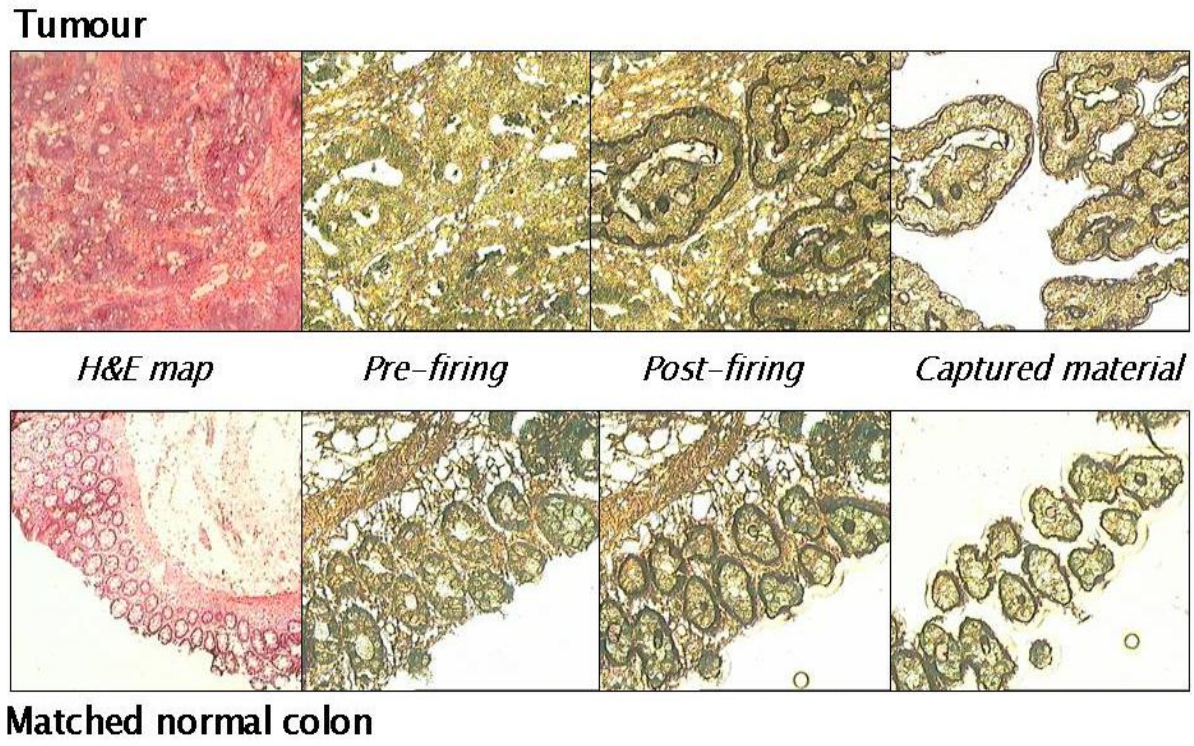


Figure 2.2: Laser capture microdissection of colonic tumour and normal colonic epithelium form the same patient

2.5 ESTIMATION OF SAMPLE PROTEIN CONCENTRATIONS

Conventional protein assays, such as the Bradford assay were incompatible with the LCM-derived samples due to the low concentrations of protein present, the small volume of lysate and the reducing agents present in the lysis buffer. A relative quantification was performed using a reference sample for comparison in order to ensure good quality gels with equal loading.

2.5.1 REFERENCE SAMPLE PREPARATION

A protein lysate was prepared from an undissected polypoid colorectal tumour specimen by the addition of 32 whole tumour sections to 2mL of lysis buffer. Viscosity was reduced by repeated passage through a 21 gauge needle. The lysate was centrifuged at 16,100 rcf for 60 minutes at -4ºC and the resulting supernatant aliquoted into 100µL lots and stored at -80ºC. Serial dilutions of the lysate were prepared by diluting 100µL of sample with an equal volume of lysis buffer to produce a 1:2 solution. This process was repeated until a range of concentrations from 1:2 to 1:16 was obtained. Five samples from undiluted to 1:16, each 100 µL in volume, were run on separate two-dimensional SDS-PAGE gels, as described below. The concentration of reference solution required to produce a high quality silver-stained gel image with an appropriate number of protein features (≈800 spots) was determined.

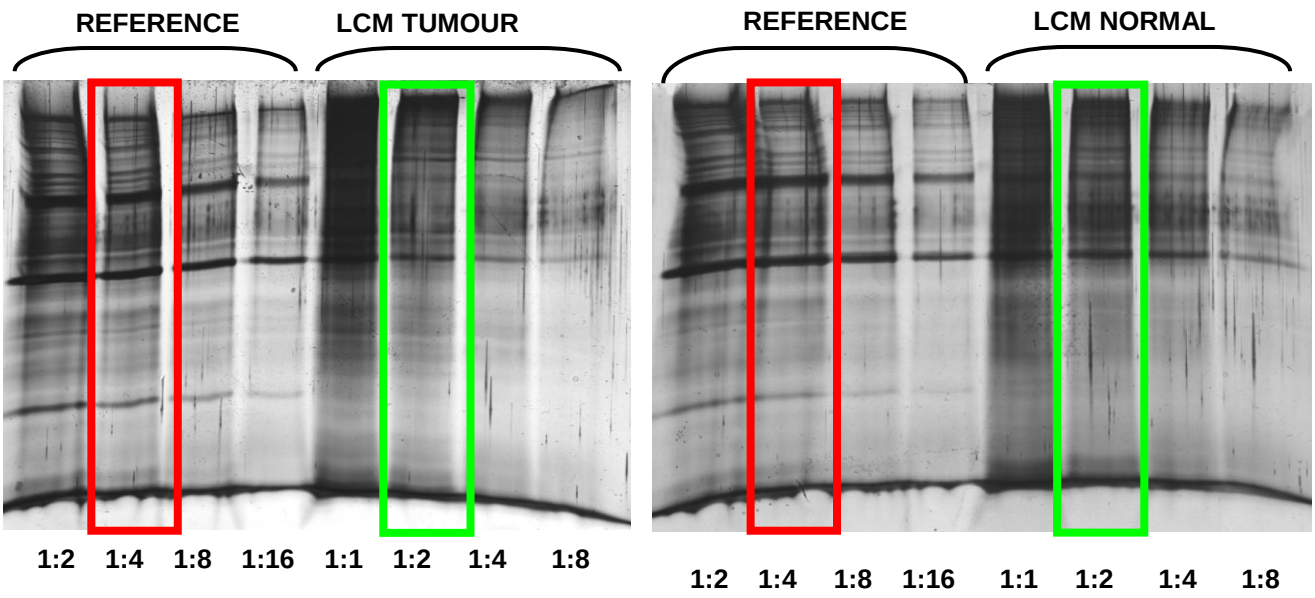
2.5.2 COMPARISON WITH LCM-DERIVED SAMPLES

The concentration of lysate found to produce the best quality 2D SDS-PAGE gel was then used as a reference against which relative sample protein concentrations of LCM procured material were estimated. During the two-dimensional phase, 100µL of the reference sample was used in comparison to 250µL of LCM-procured material. The 2.5 fold difference in sample volume was maintained during the one-dimension gel phase in order to facilitate a direct comparison. Two-fold dilution series, from undiluted to a 1:16 dilution, of the LCM-procured samples were prepared to a volume of 10µL per lane. A similar two-fold dilution series was prepared for the reference sample to a volume of 4µL per lane. Three µL of sample loading buffer (0.48M Tris- HCl, pH 6.8, 50% w/v glycerol, 10% w/v SDS, 10% DTT, trace bromophenol blue) was added to each sample. All samples

were made up to 15µL per lane with lysis buffer and heated at 100ºC for 10 min. Protein samples were run on small format Tris-glycine SDS-PAGE gel consisting of a 10% separating gel (10% w/v acrylamide, 0.375M Tris pH 8.8, 0.1% w/v SDS, 0.1% w/v ammonium persulfate) and 5% stacking gel (5% w/v acrylamide, 0.125M Tris pH 6.8, 0.1% w/v SDS, 0.1% w/v ammonium persulfate) at 90V for 15 minutes, then 150V until completion. Silver staining was carried out using the same protocol as the two-dimensional gels, (see silver staining section). The relative protein concentration of the LCM-procured samples was estimated by comparison to the reference samples. Dilution of the laser-captured samples was then performed where necessary in order to obtain the optimal concentration for two-dimensional electrophoresis.

1D small format SDS-PAGE gels were produced for relative protein quantification of all laser captured samples, (n=21), an example is shown in figure 2.3. On comparison of the protein band intensity between the reference standard and the laser captured material, all but two laser captured samples showed a match with the 1 in 2 dilution. In these instances half of the total sample volume, (125µL) was used for 2D SDS-PAGE. In one case, 010-01T the undiluted sample showed a match with the reference and therefore the entire 250µL sample was utilised. In the second case, (sample 233-00T) the staining was too weak at the 1:2 dilution and too strong at the 1:1 dilution to match the reference standard. In this case three-quarters of the sample volume was utilised, (188µL).

Figure 2.3: Relative quantification of laser captured material. Lanes outlined in red represent reference standard. Lanes outlined in green correspond to matched dilution of LCM material



2.6 IEF AND SECOND DIMENSION SDS-PAGE

2.6.1 IMMOBILISED PH GRADIENT (IPG) STRIP REHYDRATION

IPG rehydration solution was prepared by adding 2% v/v IPG buffer (containing carrier ampholytes, pH range 3–10 non-linear; Amersham Biosciences, Uppsala, Sweden) and 2.8% w/v DTT to a stock solution containing 9 M urea, 2% CHAPS and a trace of bromophenol blue. Protein samples for analysis were made up to a volume of 350µL using IPG rehydration solution. Each 350µL sample mix was used to passively rehydrate one IPG focusing strip, (180mm, pH range 3–10, non-linear; Amersham Biosciences) in an IPG DryStrip reswelling tray at room temperature overnight.

2.6.2 ISO-ELECTRIC FOCUSING (IEF)

Rehydrated strips were transferred to the Multiphor II system (Amersham Biosciences) for focusing. A linear gradient of 0–500V was initially applied to the strips over 1 minute, followed by 500-3500V linearly over 90 minutes and finally 3500V over 5 hour 40 minutes. The total running time was 7 hours 11 minutes, after which the strips were removed and stored in separate sealed tubes at -80ºC.

2.6.3 EQUILIBRATION AND SECOND DIMENSION SEPARATION

12% acrylamide gels were cast and left to polymerise for at least 5 hours. Gels were then covered with cling film and refrigerated at 4ºC for no longer than 48 hours prior to use.

Prior to the second dimension separation, strips were equilibrated in two successive buffers, each consisting of a stock solution comprising 1.5 M Tris-HCl (pH 8.8), 6 M urea, 34.5% v/v glycerol, 2% w/v SDS and a trace of bromophenol blue. To the first buffer was added 1% w/v DTT, while the second buffer incorporated 2.5% w/v iodoacetamide. Each rehydrated IPG strip was equilibrated for 15 minutes in 10mL of buffer 1, then a further 15 minutes in 10mL of buffer 2, with continuous agitation throughout. Strips were then rinsed in electrophoresis buffer (25mM Tris-base, 192mM glycine and 0.1% w/v SDS), applied to 12% acrylamide gels and sealed with melted agarose (0.5% w/v agarose in electrophoresis buffer containing a trace of bromophenol blue). Electrophoresis was carried out using an Ettan Dalt II apparatus (Amersham Biosciences) at 25ºC, with initial separation at a constant 5 W per gel for 30 min followed by 20 W per gel until the dye front had migrated approximately 18 cm. The total run time was typically 4 – 4.5 hours. Once electrophoresed, gels

were transferred to polypropylene containers and immersed in 250-300ml fixative solution (50% ethanol, 12% acetic acid) prior to staining.

2.7 STAINING OF POLYACRYLAMIDE GELS

2.7.1 COLLOIDAL COOMASSIE BLUE STAINING

Coomassie blue staining was performed according to Neuhoff *et al.* Gels were incubated for three hours in a 4:1 mixture of staining solution (0.1% w/v Coomassie brilliant blue G250 dye, 2% orthophosphoric acid, 10% w/v ammonium sulphate, 25% methanol). Gels were washed to remove excess background staining in a wash solution containing 10% acetic acid and 25% methanol solution for 4 hours. Gels were scanned as described in scanning and analysis section and stored in 25% methanol at 4°C.

2.7.2 SILVER STAINING

Two silver-staining protocols were used in order to determine the most sensitive and consistent method for detecting protein. Protocol 1 was described by Yan *et al.*(240) and protocol 2 was described by Blum *et al.*(241) Both protocols used formaldehyde as the reducing agent and were compatible with the subsequent use of MALDI-ToF for protein identification. Gels were removed from fixative solution (50% ethanol, 12% acetic acid) and incubated in a series of staining solutions as outlined below. Gentle agitation of the gels on a rotary platform was used throughout.

Protocol 1 (Yan *et al*; 2000)	
SENSITIZATION SOLUTION	30 MINUTES
(30% ethanol, 0.2% sodium thiosulfate, 0.83M sodium acetate)	
DISTILLED WATER	5 MINUTES X 3
SILVER STAINING SOLUTION	20 MINUTES
(0.25% w/v silver nitrate)	
DISTILLED WATER	20 SECONDSx2
DEVELOPING SOLUTION	6-10 MINUTES
(2.5% w/v sodium carbonate, 0.04% v/v formalin (37% formaldehyde).	
Development was carried out in glass containers until protein spots appeared.	
STOP SOLUTION	10 MINUTES
50mL of fixative solution (50% ethanol, 12 % acetic acid) was added to terminate development once background started to darken.	
DISTILLED WATER	5 MINUTES X 3
Protocol 2 (Blum *et al*; 1987)	
WASH	10 MINUTES x 3
(30% ethanol)	
SENSITIZING SOLUTION	1 MINUTES
(0.8 mM sodium thiosulfate)	
SILVER STAINING SOLUTION	40 MINUTES
(11.8mM silver nitrate, 0.02% formaldehyde)	
DISTILLED WATER	20 SECONDSx2
DEVELOPING SOLUTION	6-10 MINUTES
(566 mM Na2CO3, 0.02% formaldehyde, 0.02 mM sodium thiosulfate).	
Development was carried out in glass containers until protein spots appeared.	
STOP SOLUTION	10 MINUTES
50mL of fixative solution (50% ethanol, 12 % acetic acid) was added to terminate development once background started to darken.	
WASH (30% ethanol)	5 MINUTES x 3

2.7.3 GALLYAS PROTEIN STAINING OF SDS-PAGE GELS

This protocol, described by Sorensen *et al.* was based on procedures used previously for histochemical intensification of silver staining.(194) The Gallyas staining solution was prepared by mixing two stock solutions, A (5% w/v sodium carbonate) and B (0.2% ammonium nitrate, 0.2% silver nitrate, 1% tungstosilicic acid and 0.5% v/v formalin (37% formaldehyde)), in a 1:1 ratio whilst stirring continuously. Silver-stained gels were washed in distilled water for 3x10 minutes, and then sensitized with 2% sodium acetate for 2 x15 minutes. Sensitized gels were incubated in freshly prepared Gallyas solution to intensify silver staining. Development was stopped with 10% acetic acid once the background began to darken, typically 3-6 minutes.

2.8 SCANNING AND ANALYSIS OF GELS

Gel images were obtained using a GS-800 scanner, at 400 dpi resolution using the green channel for silver-stained gels and the red channel for Coomassie blue stained gels (Bio-Rad, Hercules, CA, USA). Data were acquired using PDQuest software (Bio-Rad, V6.2). QuantityOne software was used to crop the raw gel images to the size 176 x 176mm. The cropped images were saved in TIFF format at 16 bit and imported into Progenesis SameSpot software (Nonlinear Dynamics, Newcastle, UK) for analysis. Protein spot identification was performed by careful visual comparisons of the gels and by using SameSpot spot recognition software.

2.9 IDENTIFICATION OF PROTEINS BY LC-MS/MS

Silver-stained gels were matched with well-loaded Coomassie-stained gels of undissected tumour and normal colonic tissue lysate. Protein spots of interest were identified and referenced to the correct spot on the Coomassie stained gels (Figure 2.8). Spots were excised and trypsin digested essentially according to the method of Courchesne and Patterson.(242) Briefly, the excised gel spots were washed in 50% v/v acetonitrile/ 25 mM w/v ammonium bicarbonate, pH 7.8, and dried in a SpeedVac. The dried gel spots were rehydrated with 4–10µL digestion buffer (10µg/mL modified sequencing grade trypsin in 25mM NH_4HCO_3) and incubated overnight at 37°C. The resulting peptides were extracted by the addition of 4µL water followed by 7µL 30% v/v acetonitrile/0.1% v/v TFA with mixing and brief centrifugation. The supernatants were recovered and liquid chromatography with tandem mass spectrometry, LC-MS/MS was used for analysis. The tryptic digest was delivered into a QSTAR® Pulsar i hybrid mass spectrometer (Applied Biosystems) by automated in-line RP-LC (integrated LCPackings System, 5mm C18 µ-precolumn cartridge and 75µm x 15cm C18 column, Dionex, Camberley, UK) via a nano-electrospray source head and a 10mm id PicoTip (New Objective, Woburn, USA). A gradient from 5% ACN/0.05% TFA v/v to 48% ACN/0.05% TFA v/v in 60 min was applied at a demanded flow rate of 200nL/ min, and MS and MS/MS spectra were acquired automatically in positive ion mode using information-dependent acquisition (Analyst® software, Applied Biosystems). Data were submitted to MASCOT and the NCBI database was searched with the MS tolerance set to 1.2Da and the MS/MS tolerance to 0.6Da, with carboxamidomethyl as a variable modification.

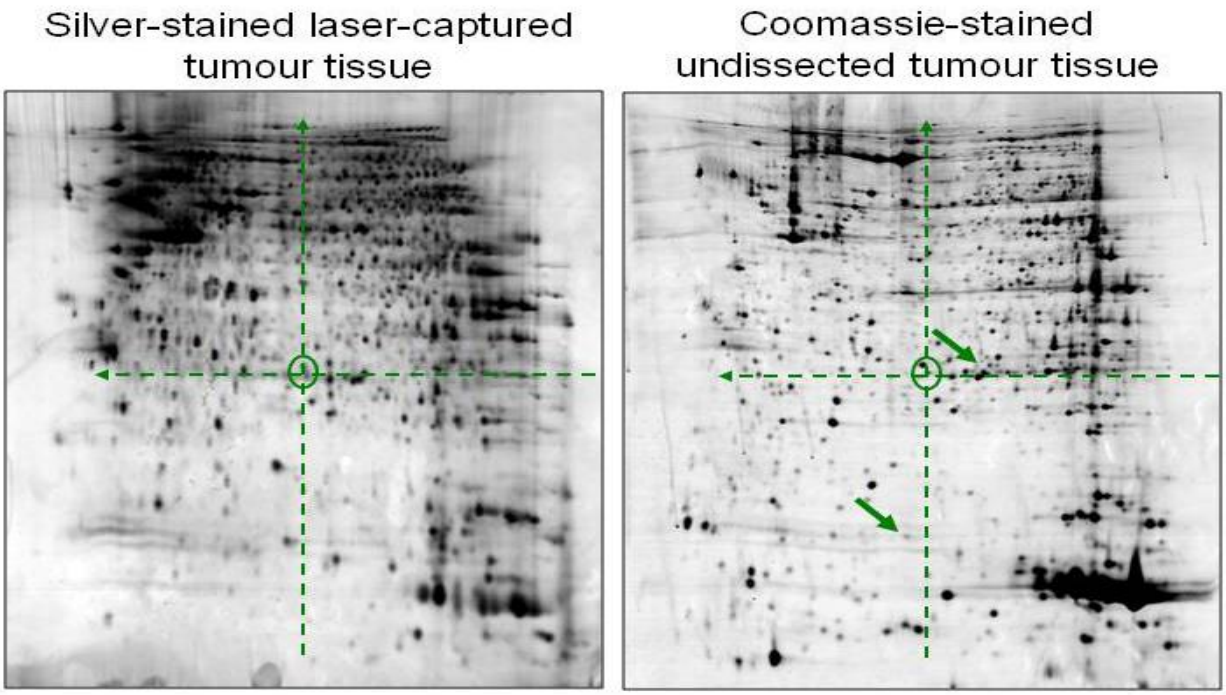


Figure 2.4: Matched laser-captured silver-stained 2D gel of tumour tissue lysate to undissected Coomassie stained 2D gel of same tumour tissue. Lines converge on protein spot of interest (circled) and were referenced to other protein spots (arrowed) in order to accurately locate.

2.10 CELL LINES AND WESTERN BLOTTING

2.10.1 CELL LINES AND CELL CULTURE

The human colonic adenocarcinoma cell line, SW480 and the human rectal adenocarcinoma cell line, SW837 were obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were maintained in L15 medium supplemented with 10% foetal calf serum, 2mM L-glutamine and 5Ag/mL streptomycin (Sigma, Poole, United Kingdom) at 37°C in a humidified atmosphere of 5% CO2 (SW480) or 0% CO2 (SW837).

2.10.2 1D WESTERN BLOT

Confluent cells were collected and resuspended in buffer containing 0.1M Tris-HCl (ph 6.8), 2% SDS supplemented with complete protease inhibitors (Roche Diagnostics, Indianapolis, USA) and sonicated on ice. Protein was quantified using the Bicinchoninic Acid (BCA) method, and an identical mass of protein was loaded in each lane of a small format SDS-Tris HCl gel (15% separating and 4% stacking gels) and subject to electrophoresis at a constant voltage of 150V (Pierce Biotechnology, Rockford, USA). Proteins were electrophoretically transferred to Hybond nitrocellulose membranes at 100V for 60 minutes (Amersham Biosciences, Buckinghamshire, UK), and membranes were blocked in 5% milk in PBS for 60 minutes before application of primary antibodies. Membranes were washed thrice in PBS containing 0.1% Tween20 for 20 minutes followed by incubation with a horseradish peroxidase–conjugated secondary antibody. After extensive washing in PBS containing 0.1% Tween20, protein-antibody complexes conjugated with peroxidase were visualized with Western Lighting chemiluminescence reagent (Perkin-Elmer Life Sciences, Boston, MA).

2.10.3 2D WESTERN BLOT

Confluent cells were collected and resuspended in lysis buffer as for 1D western blot analysis. Whole tumour lysate was prepared as per section 2.5.1. Protein was quantified using the BCA method, and an identical mass (100µg) of protein was loaded onto each gel with two identical gels run for every sample. Gels were prepared for 2D electrophoresis with the exception that the second dimension run was stopped at 16cm. Proteins were electrophoretically transferred to Hybond nitrocellulose membranes at 400V for 2.5 hours (Amersham Biosciences, Buckinghamshire,

UK), then blocked in 5% milk in PBS for 60 minutes. Membranes were incubated with primary antibody for either 1 hour at room temperature or overnight at 4ºC. Protein transfer was verified using ponceau stain. Membranes were washed thrice in PBS containing 0.1% Tween20 for 20 minutes followed by incubation with a horseradish peroxidase–conjugated secondary antibody. After extensive washing in PBS containing 0.1% Tween20, protein-antibody complexes conjugated with peroxidase were visualized with Western Lighting chemiluminescence reagent (Perkin-Elmer Life Sciences, Boston, MA).

Antibody	Indication	Dilution	Manufacturer
Anti-SH3 binding glutamic acid rich protein like 3	Western blotting	1:10	Gifted from Dr Q Zhang
Anti-peroxiredoxin 6	Western blotting	1:2000	Santa Cruz Biotechnology
Anti-peroxiredoxin 6-SO_4	Western blotting	1:1000	Santa Cruz Biotechnology
Cofilin1	Western blotting	1:10,000	Abcam (Cambridge Biosciences)
Cofilin-phospho(ser3)	Western blotting	1:50	Abcam (Cambridge Biosciences)

Table 2.3: Primary antibodies used in Western blotting

2.11 VALIDATION OF DIFFERENTIALLY EXPRESSED PROTEINS

2.11.1 GENERATION OF PARAFFIN-EMBEDDED FORMALIN-FIXED COLORECTAL CANCER TISSUE

Six purpose-built colorectal cancer tissue microarrays (TMAs) were constructed, containing 2-6 cores from 404 independent cases of adenocarcinoma in addition to 36 cores of normal colon and 16 cores each of normal kidney, liver and testes, which served as control tissues. TMAs were constructed in the Liverpool Tissue Bank, University of Liverpool between 2005 and 2008. All

patients on the arrays had a signed consent form for the use of their tissue for research and ethical approval from the Knowsley and St Helens ethics committee had been given. Clinicopathological data including age, gender, site of tumour, stage (according to TNM and the American Joint Committee on Cancer, AJCC guidelines(243)) was obtained from hospital computer records. All data was recoded on the secure Liverpool Tissue Bank database and was provided courtesy of Mrs Ann Anderson.

2.11.2 IMMUNOHISTOCHEMISTRY AND CO-IMMUNOFLUORESCENCE

Staining of the colorectal microarray was undertaken as follows; briefly 5µ thick sections of the array were deparaffinised in xylene and rehydrated through alcohol to distilled water. Peroxidase block was performed by incubating with 15% methanol for 12 minutes. Antigen retrieval was perormed by pressure-cooking the slides in 10mmol EDTA solution, (pH 7.0), for 3 min. For immunohistochemical staining, slides were incubated for 60 min with primary antibodies in dilutent. These slides were then rinsed in TBS and visualisation achieved by incubating with a horseradish peroxidase conjugated secondary antibody for 30 min followed by diaminobenzidine for 10 min. Negative controls were incubated with conjugated secondary antibody only. Slides were counterstained with haematoxylin for 30s and coverslips mounted with DPX mountant, (BDH). Detailed protocol outlined in box (page 89) provided courtesy of Mr Andrew Dodson, Histopathology Department, Royal Liverpool University Hospital.

Co-immunofluorescence was performed on duplicate whole formalin-fixed colorectal tumour sections. After antigen retrieval with heat treatment as described, the slides were incubated with primary antibodies overnight at 4°C. Visualisation was achieved using FITC-labelled donkey anti-

mouse (30mg/mL; Santa Cruz Biotechnology) and TRITC labelled swine anti-rabbit (25mg/mL; DakoCytomation) secondary antibodies for 60 min at room temperature. DAPI was used as the counterstain.(244)

Immunohistochemistry protocol

Slide preparation

De-paraffinisation was carried out in xylene for 15-30 minutes. Rehydration with100% ethanol, 70% ethanol then distilled water was carried out for 5 minutes each. Peroxidase block was performed by incubating with 15% methanol for 12 minutes.

Antigen retrieval

Antigen retrieval was performed by pressure-cooking the slides in 10mM EDTA (pH 7.4) for 3 minutes, followed by cooling in distilled water.

Primary antibody

The primary antibody was diluted in ChemoMate (DAKO) at volume of 500µl per slide. Slides were incubated overnight at 4^0 C then washed with Tris buffered saline x 3.

Secondary Antibodies and Substrate

Pre-diluted anti-mouse horse radish peroxidase conjugated secondary antibody from DakoCytomation kit K4010 was applied for 1 hour followed by 3 washes with Tris buffered saline. 20µl of liquid DAB + Chromogen were mixed gently with 1ml of buffer provided in the DakoCytomation kit K4010 to produce the substrate diaminobenzidine in which the slides were incubated for 10 minutes.

Haematoxylin staining

Counterstaining was performed using Erlich's haematoxylin for 30 seconds, followed by bluing agent (2% ammonium hydroxide solution) for 5s. Slides were dehydrated with 100% ethanol for 3 x 1 minute and xylene for 2 x 1 minute. Coverslips were mounted with D.P.X. mountant (BDH).

Antibody	Indication	Dilution	Manufacturer
Polyclonal goat anti-S100A8	Immunohistochemistry Immunofluorescence	1:2000	Santa Cruz Biotechnology
Polyclonal rabbit anti-S100A9	Immunohistochemistry Immunofluorescence	1:4000	Santa Cruz Biotechnology
Monoclonal anti-SMAD4 antibody	Immunohistochemistry	1:50	Santa Cruz Biotechnology
Monoclonal mouse anti-CD68	Immunofluorescence	1:200	Dako
Monoclonal mouse anti-CD14	Immunofluorescence	1:100	Novocastra
Monoclonal mouse anti-HSP27	Immunohistochemistry	1:50	Novocastra
Cofilin1	Immunohistochemistry	1:40,000	Abcam
Cofilin-phospho(ser3)	Immunohistochemistry	1:75	Abcam

Table 2.5: Primary antibodies in immunohistochemistry and co-immunofluorescence

2.11.3 MICROARRAY SCORING AND ANALYSIS

All microarrays were scored by at least two independent scorers, one of whom was a consultant histopathologist (Dr B Azadeh or Dr M Terlizzo). A scoring system was agreed by both pathologists after scrutinizing images from the first colorectal cancer tissue microarray. Scores were then determined independently in separate locations. I compiled and compared the scores from myself and both pathologists; cores with discrepancies in HSP27 intensity and distribution (approximately 10% of cores) were listed for review of the images and discussion. Dr Terlizzo and I viewed the slides on a dual-headed microscope linked directly to her computer; this allowed Dr Azadeh to view emailed images of the cores simultaneously on his office computer. A consensus score for the discrepent cores was allocated following discussion between Dr Terlizzo and Dr Azadeh by telephone.

Smad4 expression was graded using a 0-3 score for both cytoplasmic and nuclear intensity of staining. The extent of both S100A8 and S100A9-expressing cellular infiltrate in the stroma was graded using a similar 0-3 score. In addition, the number of positive S100A8 and S100A9 cells per field at 40x magnification was also determined in each tumour core. Mean S100A8 and S100A9 cell counts were obtained by averaging the number of positive stromal cells across all the tumour cores scored for each patient. HSP27 was scored using intensity of staining (using a 0-3 scale) and the extent of HSP27 staining (0-3 according to the percentage of positive tumour; 0: <5% positive cells, 1: 5-30%, 2: 30-70% and 3: >70%). A combined score or 0-9 HSP27 index was calculated by multiplying the intensity by the extent of staining for every patient.

2.11.4 STATISTICAL ANALYSIS

Comparisons were made using the non-parametric Fisher's exact test (2 groups), or chi-squared test (>2 groups). Life tables and Kaplan-Meier curves were used to evaluate cancer-specific survival; p-values were calculated using the log rank test. Survival was measured from date of diagnosis to date of death, counting death from colorectal cancer as the end point; deaths from other causes were censored in the analysis. If no event occurred, the date of last follow up was used as the end point. Multivariate Cox regression analysis was conducted using stepwise forward selection starting with the most significant variable on univariate analysis and including variables with a *P*-value of <0.10. All analyses were performed using Statview Version 5.01 (SAS Institute Inc). A *P* -value of less than 0.05 was considered significant.

3. RESULTS

3.1 SILVER-STAINING OF GELS

3.1.1 COMPARISON OF SILVER-STAINING PROTOCOLS

Small format Tris-glycine SDS-PAGE gels were prepared as described in section 2.5.2. Reference protein solution was loaded onto the gels in serial dilutions including undiluted, 1:2, 1:4, 1:8 and 1:16, then repeated on the same gel to give a total of 10 lanes, and gels run as previously described. After fixing overnight in 50% ethanol and 12% acetic acid solution, the gels were transected to yield two identical sections each comprising 5 lanes. One gel half was silver-stained using protocol 1, the other side silver-stained with protocol 2 at the same time, Section 2.7.2. The experiment was repeated with the two other gels. The six gel-halves were scanned as described in section 2.8 and the quality and reproducibility of the images compared. Further comparison was made using the 2D-SDS polyacrylamide gels. Serial dilutions of the reference protein sample from 1:2 to 1:16 were run in duplicate, (as described in section 2.6) giving a total of eight gels. Protocol 1 was used to stain one set of four gels and protocol 2 used to stain the duplicate set. The stained gels were scanned as described in section 2.8 and the images compared for sensitivity and reproducibility. Protocol 2 (Blum *et al.*) was found to give more consistent results with less background staining with 1D SDS-PAGE than protocol 1 (Yan *et al.*); figure 3.1. Following 2D SDS-PAGE, a greater number of protein spots were seen at each dilution using protocol 2 than protocol 1, figure 3.2. Blum *et al.* was judged to be more sensitive for the detection of protein and was the staining protocol adopted

for all further silver staining. The Gallyas technique (section 2.7.3) was applied to the previously silver-stained, small format 1D SDS gels described in the same section, see Figure 3.3. Further darkening of the protein bands was seen in all cases; however, a disproportionate darkening of the background rendered the gels difficult to interpret. Furthermore, successive incubations of the gels in the Gallyas solution caused them to become brittle and easily damaged. No further Gallyas staining was performed.

3.1.2 DETERMINATION OF REFERENCE PROTEIN STANDARD

The methodology for visualisation of protein spots was optimised by use of protocol 2, (Blum et al) for silver-staining without Gallyas enhancement. Reference protein gels were produced in serial dilutions from 1:2 to 1:16 concentration, as described. The reference standard was determined by the lowest concentration of reference protein solution which gave the optimal number of protein spots, (>800), without excessive darkening of the background. The 1:4 dilution was adopted as the reference standard (Figure 3.4).

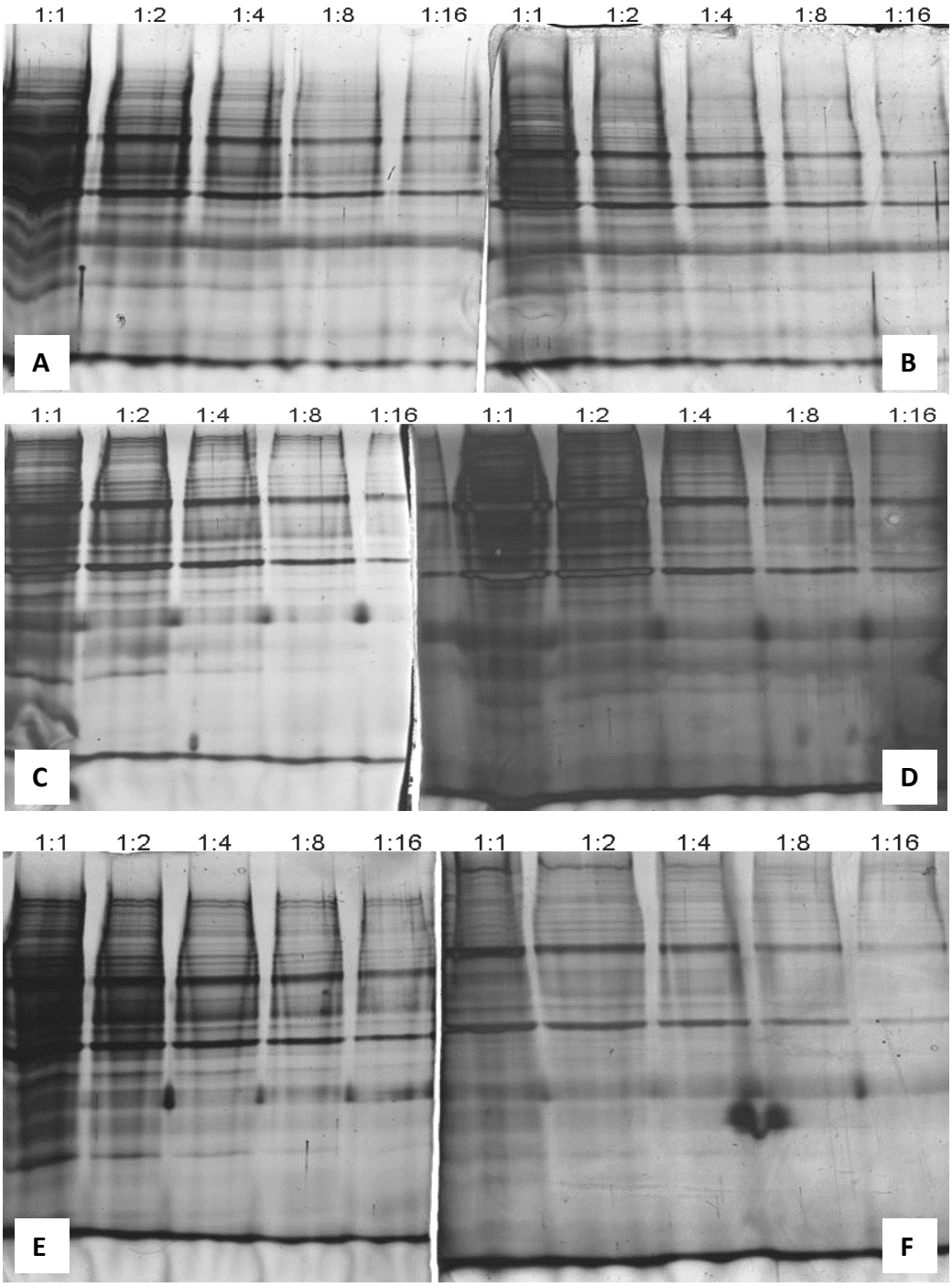


Figure 3.1: Reference protein solution produced from whole tumour lysate (section 2.5.1) in serial dilutions on small format SDS gels. A, C, E stained with protocol 2, (Blum *at el*). B, D, F stained with protocol 1, (Yan *et al*).

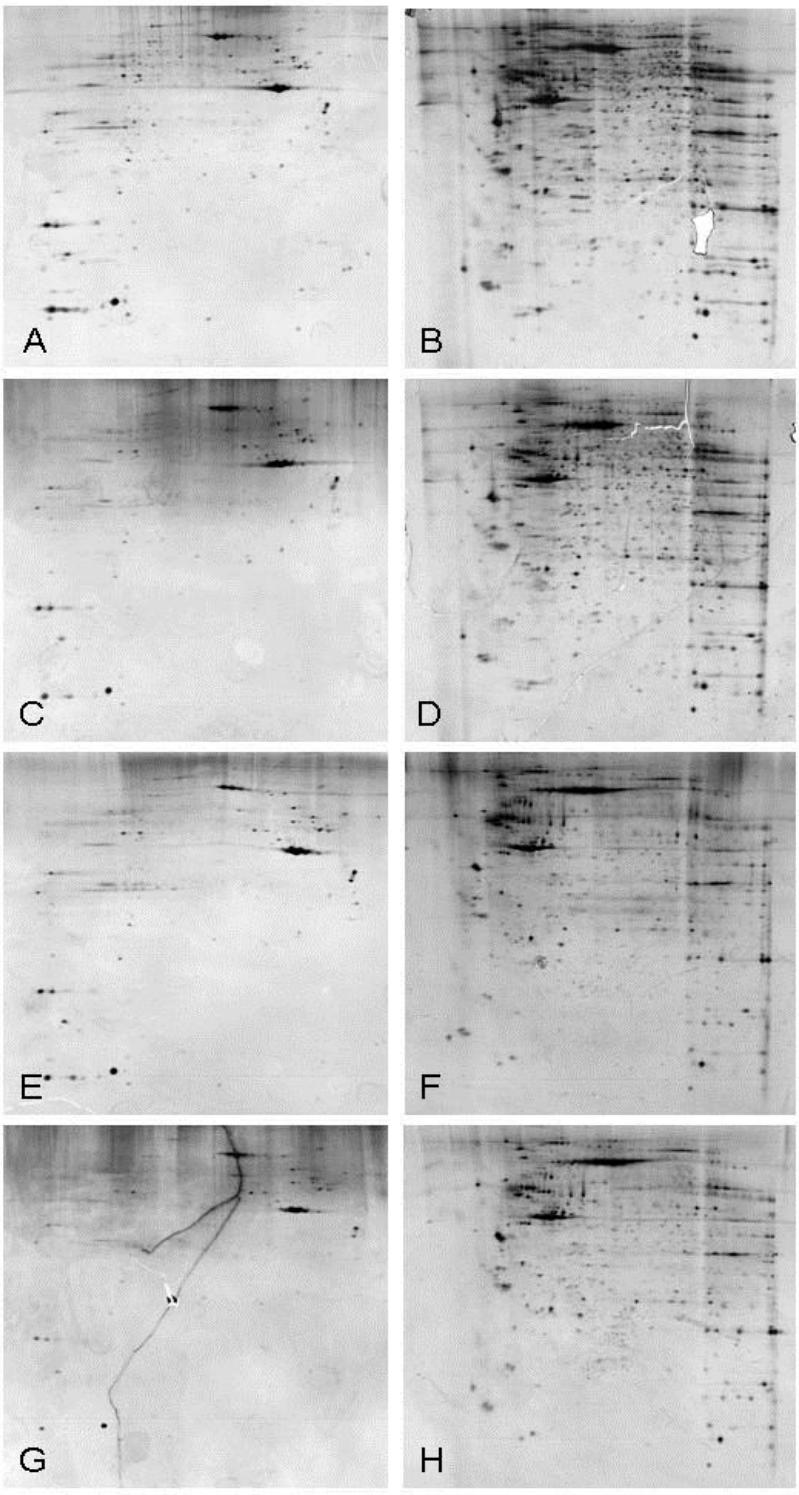


Figure 3.2: Reference protein solution produced from whole tumour lysate (section 2.5.1) in serial dilutions on 2D SDS gels. 1:2 dilution; gels A&B, (A stained protocol 1, B stained protocol 2). 1:4 dilution; gels C&D, (C stained protocol 1, D stained protocol 2). 1:8 dilution; gels E&F, (E stained protocol 1, F stained protocol 2). 1:16 dilution; gels G&H, (G stained protocol 1, H stained protocol 2).

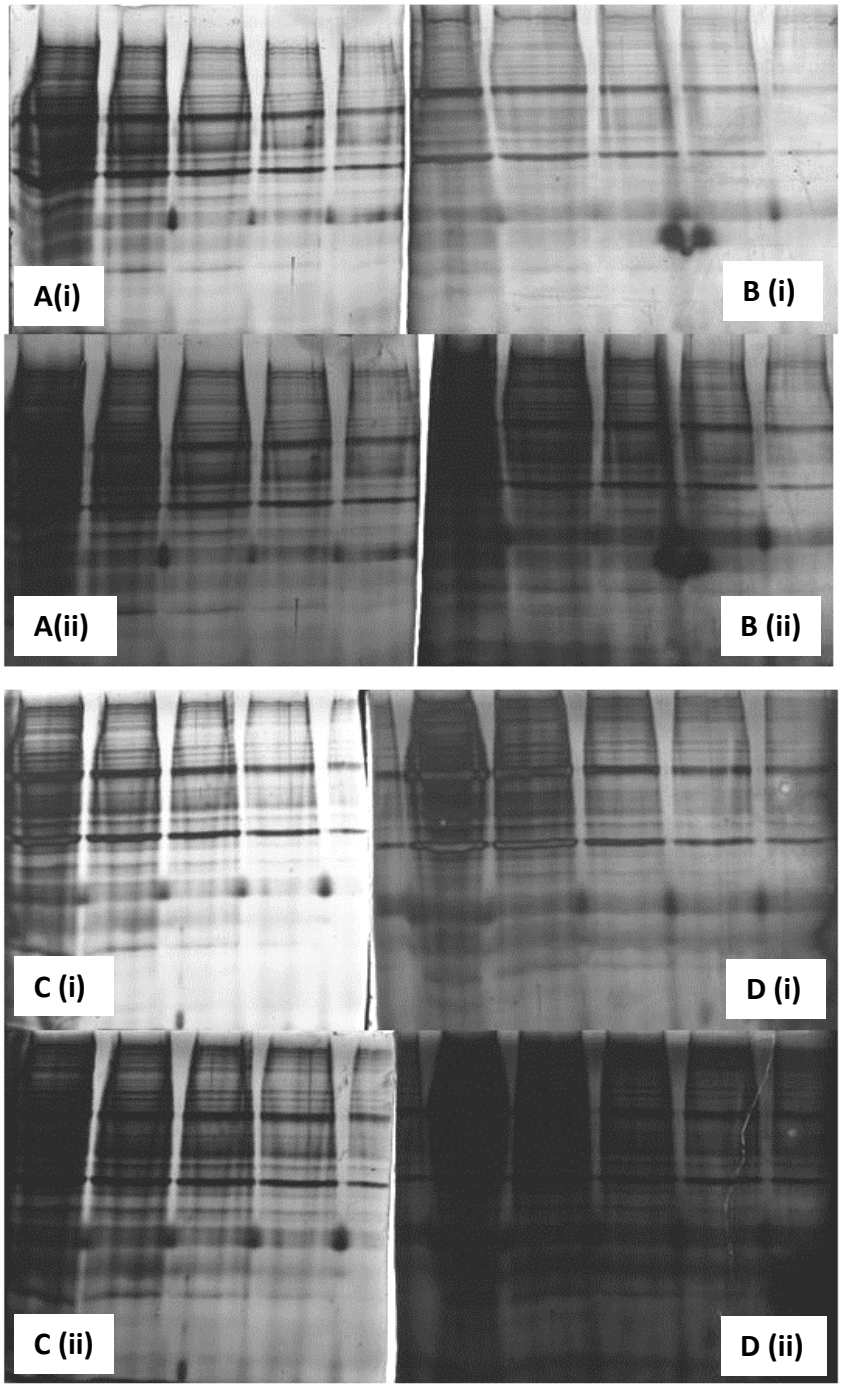


Figure 3.3: Silver-stained 1D gels of reference protein solution in serial dilutions subject to further staining using Gallyas protocol. Gel A corresponds to gel E (Figure 3.1). (i) prior to Gallyas staining; (ii) following Gallyas staining. Gel B corresponds to gel F (Figure 3.1) (i) prior to Gallyas staining; (ii) following Gallyas staining. Gel C corresponds to gel C (Figure 3.1). (i) prior to Gallyas staining; (ii) following Gallyas staining. Gel D corresponds to gel D (Figure 3.1) (i) prior to Gallyas staining; (ii) following Gallyas staining.

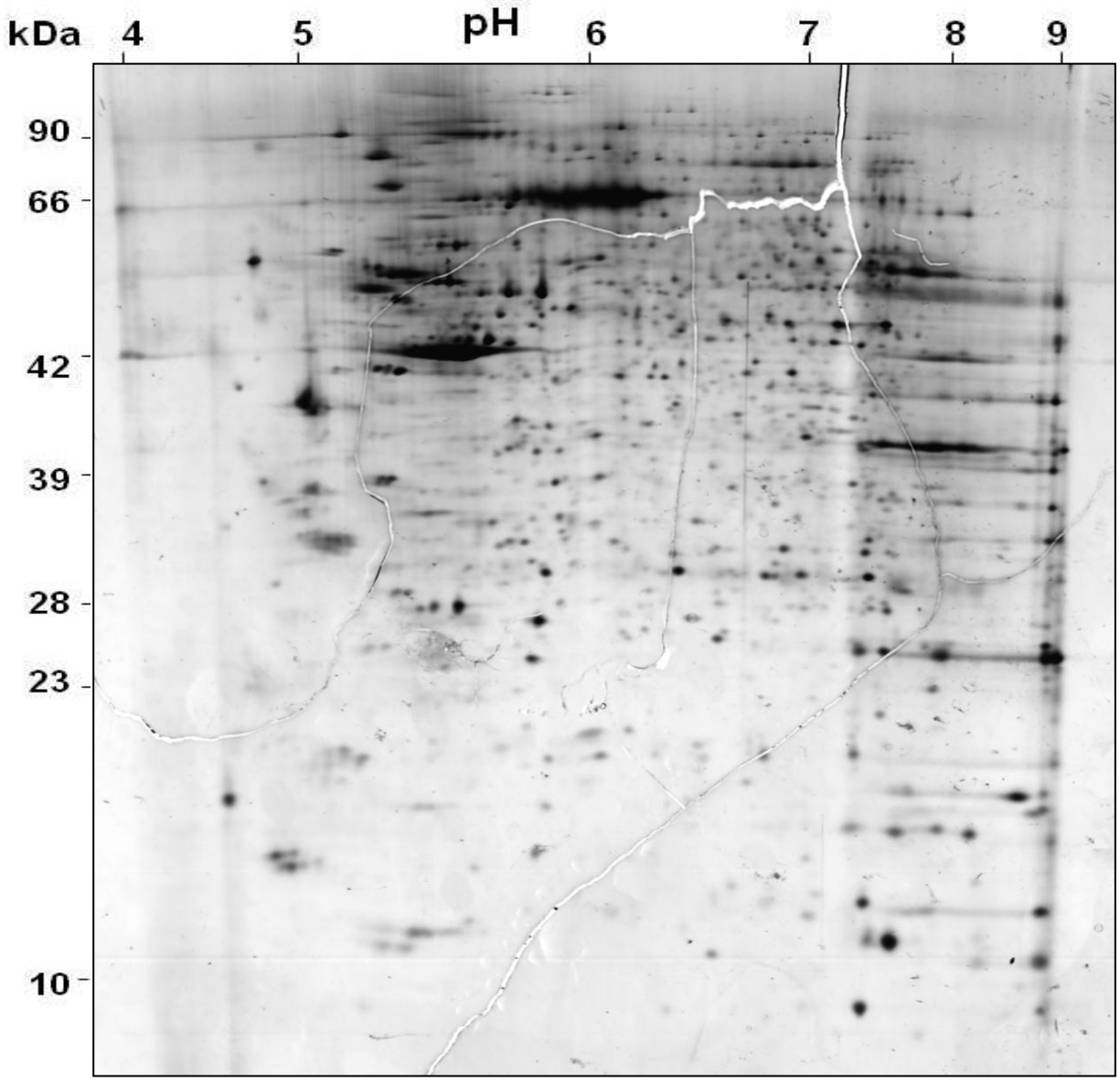


Figure 3.4: 2D SDS-PAGE of reference standard produced from whole tumour lysate (section 2.5.1) at 1:4 dilution. Optimized staining protocol applied (Blum *et al*). Greater than 800 protein spots are visible without over-staining of the background gel indicating that this concentration of lysate is sufficient to produce a good quality 2D gel.

3.2 GEL ANALYSIS

Twenty-one gels were produced from laser-captured frozen tissue, eleven samples of colorectal tumour and ten matched samples of normal colonic epithelium. One gel, sample 010-01T, (a morphologically flat, T1/N0 tumour), had insufficient protein loading to produce a good quality gel. This was in some part due to the small fragment of frozen material available for laser capture; not enough residual tissue remained for further microdissection. A total of 10 usable small tumours were compared with matched normal colonic epithelium in 9 cases. Gel images were analysed using proteomic software Progenesis SameSpots (Nonlinear Dynamics, Newcastle, UK). Samespots allows the manual mapping on gel images onto other gels thus facilitating comparison of spot patterns. A merged gel image constructed from the mapped images of each gel in the group was created; the software then identified differentially expressed spots between the groups of gels. Five merged images were created from this analysis; tumour (n=11 gels), normal (n=10 gels), depressed tumour (n=4 gels), flat tumour (n=3 gels), polypoid tumour (n=4 gels), (Figure 3.5). The software compared morphologically different tumours to produce a list of 30-40 variably-expressed spots (ringed in blue). Visual comparison of identified spots was performed by two independent observers (Dr Eithne Costello and myself). Approximately two thirds of spots were short-listed were excluded due to inaccurate mapping of spots between gels and artifact or background confounding the analysis. A final list of spots with genuine differential expression was drawn up with priority given to those proteins which shown variable expression in different morphology tumours and in tumour compared to normal tissue. Spots of interest after gel comparison are marked on the gel in

figure 3.6. Figures 3.7-3.12 illustrates each spot as expressed in individual gels, including comparison of spot volume determined by Samespots Progenesis software.

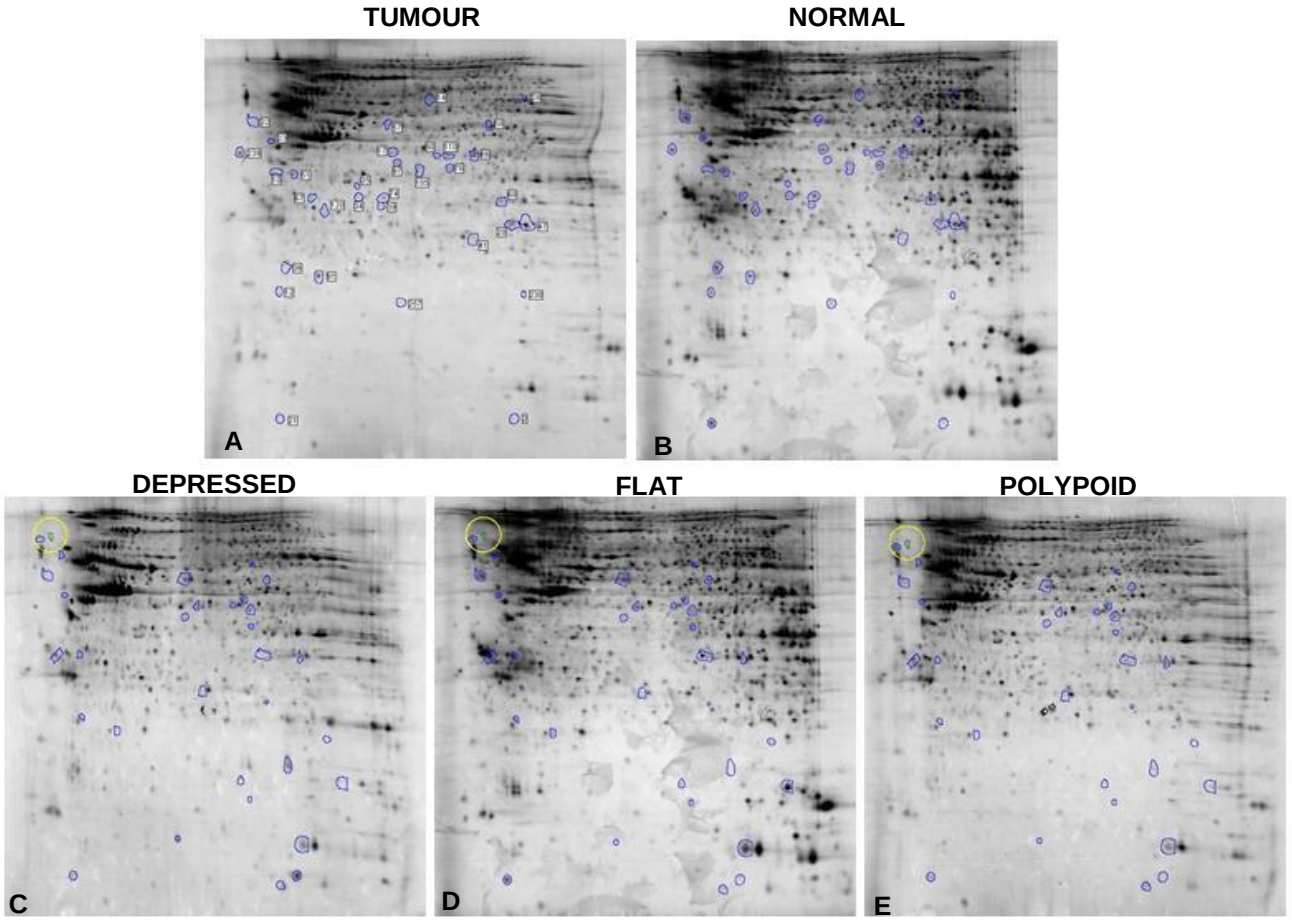


Figure 3.5: Merged images of silver-stained gels created for spot analysis by Samespot software. A: Tumour (n=10) **B:** Normal colon (n=9) **C:** Depressed tumours (n=4) **D:** Flat tumours (n=3) **E:** Polypoid tumours n=3

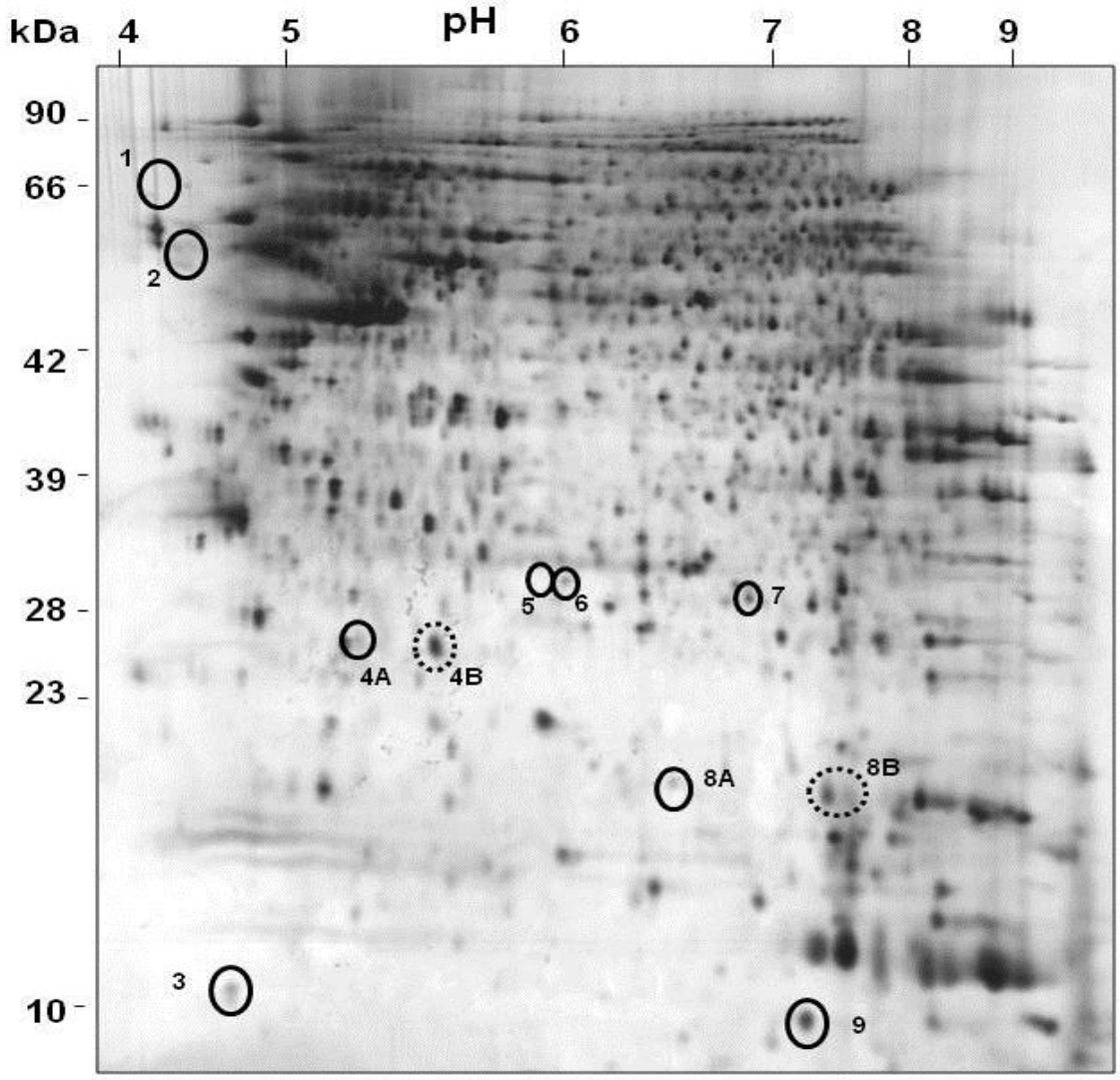


Figure 3.6: 2D gel of laser-captured tumour lysate (sample 233-00T) depicting nine spots found to be differentially expressed and selected for identification using tandem mass spectrometry. Spots were selected after analysis of all tumour gels using Samespots software and two observer visual comparison. Tumours with different morphologies were compared and the findings referenced against matched normal gels. Spots circled in dashed lines were also selected for identification as post-translational modifications of proteins already ringed on the gel.

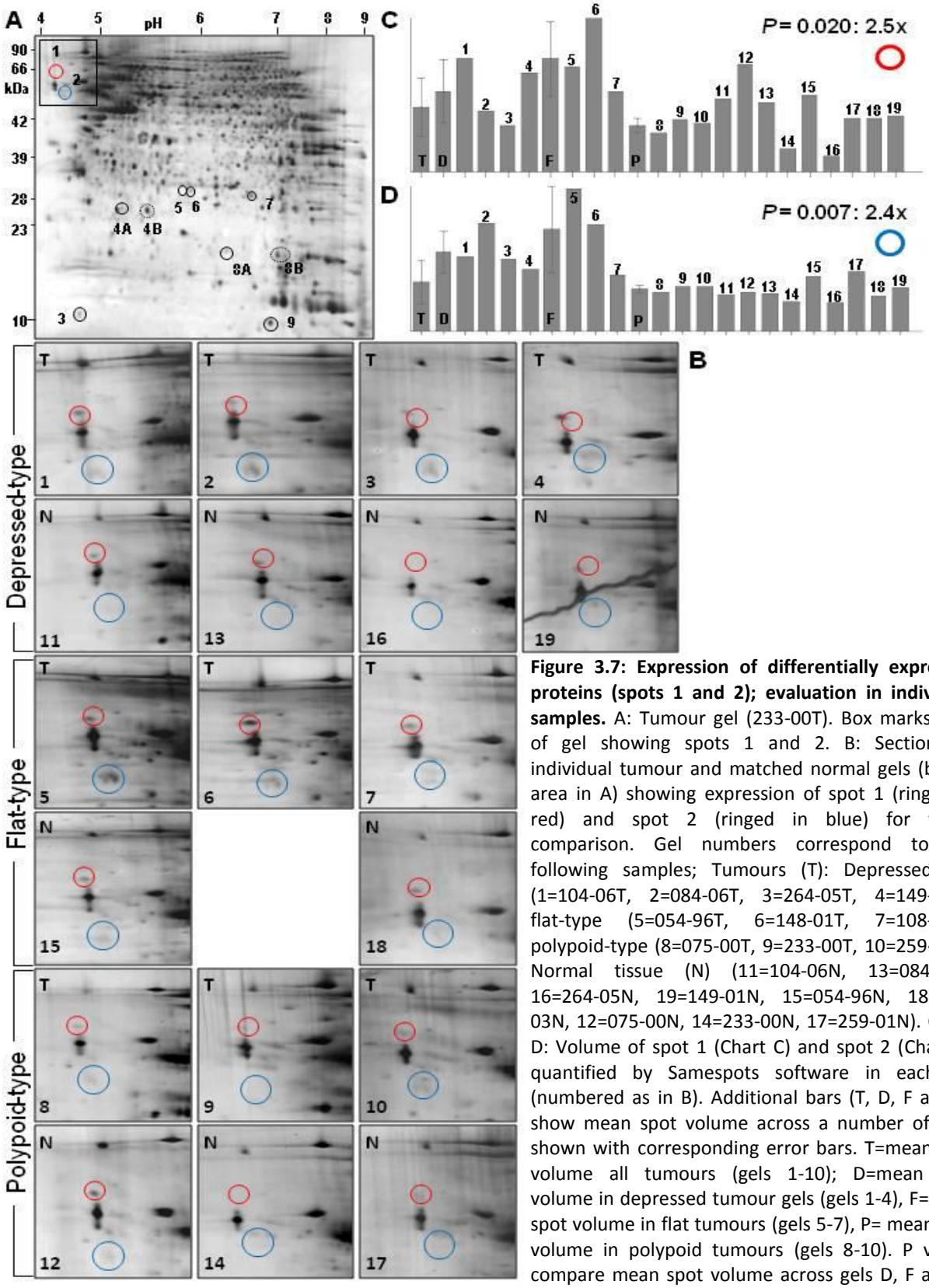


Figure 3.7: Expression of differentially expressed proteins (spots 1 and 2); evaluation in individual samples. A: Tumour gel (233-00T). Box marks area of gel showing spots 1 and 2. B: Sections of individual tumour and matched normal gels (boxed area in A) showing expression of spot 1 (ringed in red) and spot 2 (ringed in blue) for visual comparison. Gel numbers correspond to the following samples; Tumours (T): Depressed-type (1=104-06T, 2=084-06T, 3=264-05T, 4=149-01T); flat-type (5=054-96T, 6=148-01T, 7=108-03T); polypoid-type (8=075-00T, 9=233-00T, 10=259-01T). Normal tissue (N) (11=104-06N, 13=084-06N, 16=264-05N, 19=149-01N, 15=054-96N, 18=108-03N, 12=075-00N, 14=233-00N, 17=259-01N). C and D: Volume of spot 1 (Chart C) and spot 2 (Chart D) quantified by Samespots software in each gel (numbered as in B). Additional bars (T, D, F and P) show mean spot volume across a number of gels, shown with corresponding error bars. T=mean spot volume all tumours (gels 1-10); D=mean spot volume in depressed tumour gels (gels 1-4), F=mean spot volume in flat tumours (gels 5-7), P= mean spot volume in polypoid tumours (gels 8-10). P values compare mean spot volume across gels D, F and P. The fold increase from lowest to highest mean spot volume is shown on each chart.

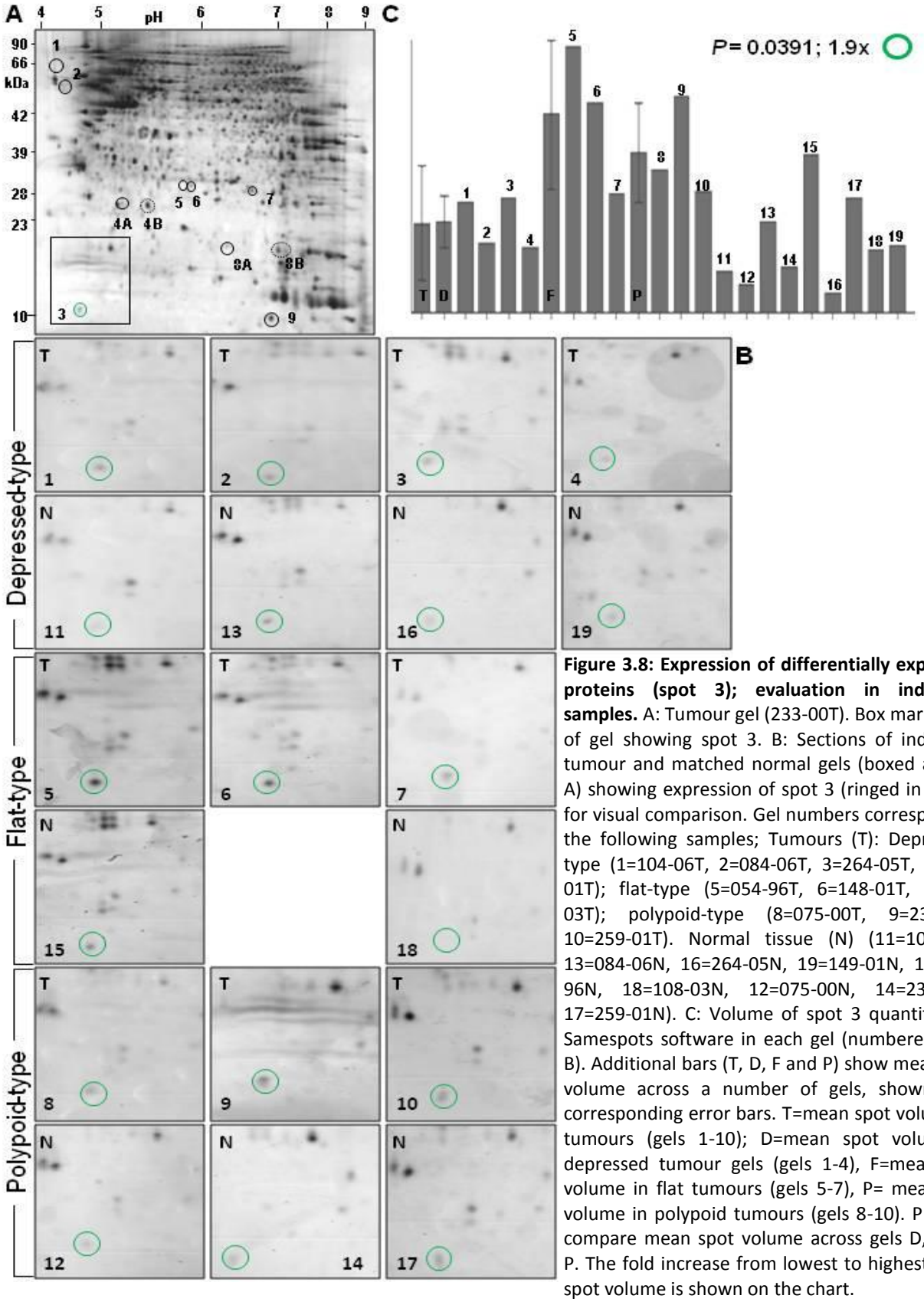


Figure 3.8: Expression of differentially expressed proteins (spot 3); evaluation in individual samples. A: Tumour gel (233-00T). Box marks area of gel showing spot 3. B: Sections of individual tumour and matched normal gels (boxed area in A) showing expression of spot 3 (ringed in green) for visual comparison. Gel numbers correspond to the following samples; Tumours (T): Depressed-type (1=104-06T, 2=084-06T, 3=264-05T, 4=149-01T); flat-type (5=054-96T, 6=148-01T, 7=108-03T); polypoid-type (8=075-00T, 9=233-00T, 10=259-01T). Normal tissue (N) (11=104-06N, 13=084-06N, 16=264-05N, 19=149-01N, 15=054-96N, 18=108-03N, 12=075-00N, 14=233-00N, 17=259-01N). C: Volume of spot 3 quantified by Samespots software in each gel (numbered as in B). Additional bars (T, D, F and P) show mean spot volume across a number of gels, shown with corresponding error bars. T=mean spot volume all tumours (gels 1-10); D=mean spot volume in depressed tumour gels (gels 1-4), F=mean spot volume in flat tumours (gels 5-7), P= mean spot volume in polypoid tumours (gels 8-10). P values compare mean spot volume across gels D, F and P. The fold increase from lowest to highest mean spot volume is shown on the chart.

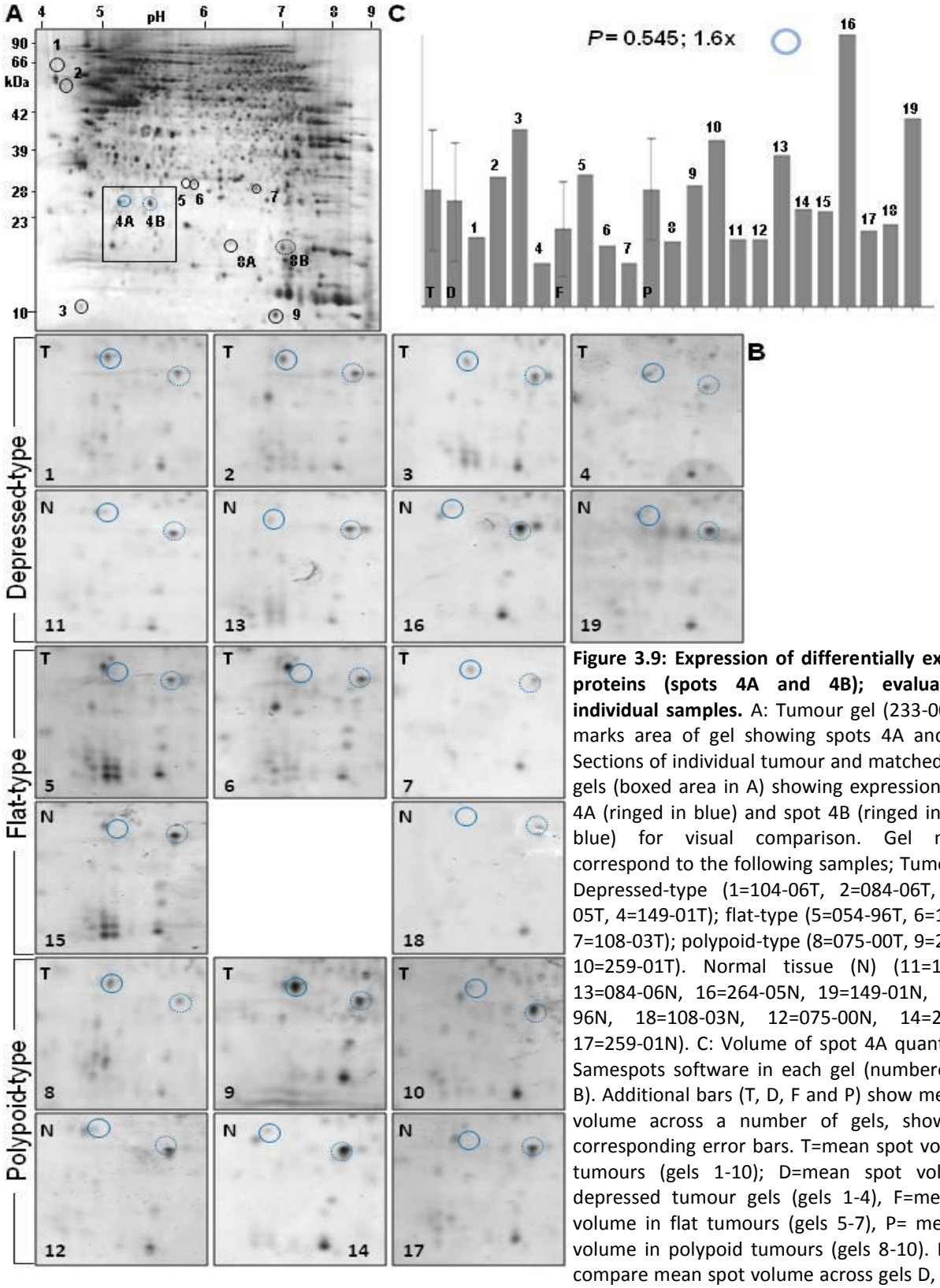


Figure 3.9: Expression of differentially expressed proteins (spots 4A and 4B); evaluation in individual samples. A: Tumour gel (233-00T). Box marks area of gel showing spots 4A and 4B. B: Sections of individual tumour and matched normal gels (boxed area in A) showing expression of spot 4A (ringed in blue) and spot 4B (ringed in dashed blue) for visual comparison. Gel numbers correspond to the following samples; Tumours (T): Depressed-type (1=104-06T, 2=084-06T, 3=264-05T, 4=149-01T); flat-type (5=054-96T, 6=148-01T, 7=108-03T); polypoid-type (8=075-00T, 9=233-00T, 10=259-01T). Normal tissue (N) (11=104-06N, 13=084-06N, 16=264-05N, 19=149-01N, 15=054-96N, 18=108-03N, 12=075-00N, 14=233-00N, 17=259-01N). C: Volume of spot 4A quantified by Samespots software in each gel (numbered as in B). Additional bars (T, D, F and P) show mean spot volume across a number of gels, shown with corresponding error bars. T=mean spot volume all tumours (gels 1-10); D=mean spot volume in depressed tumour gels (gels 1-4), F=mean spot volume in flat tumours (gels 5-7), P= mean spot volume in polypoid tumours (gels 8-10). P values compare mean spot volume across gels D, F and P. The fold increase from lowest to highest mean spot volume is shown on the chart.

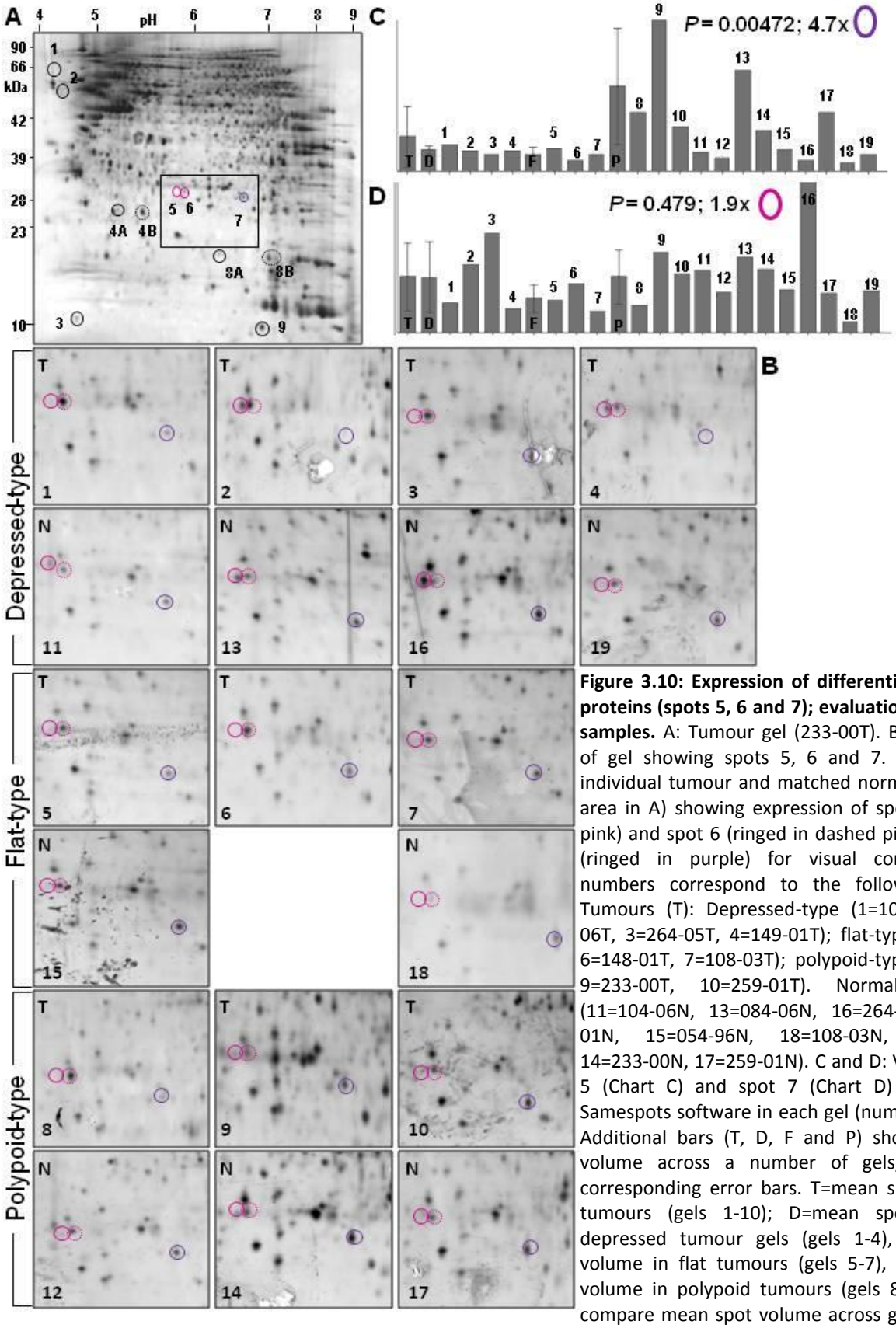


Figure 3.10: Expression of differentially expressed proteins (spots 5, 6 and 7); evaluation in individual samples. A: Tumour gel (233-00T). Box marks area of gel showing spots 5, 6 and 7. B: Sections of individual tumour and matched normal gels (boxed area in A) showing expression of spot 5 (ringed in pink) and spot 6 (ringed in dashed pink) and spot 7 (ringed in purple) for visual comparison. Gel numbers correspond to the following samples; Tumours (T): Depressed-type (1=104-06T, 2=084-06T, 3=264-05T, 4=149-01T); flat-type (5=054-96T, 6=148-01T, 7=108-03T); polypoid-type (8=075-00T, 9=233-00T, 10=259-01T). Normal tissue (N) (11=104-06N, 13=084-06N, 16=264-05N, 19=149-01N, 15=054-96N, 18=108-03N, 12=075-00N, 14=233-00N, 17=259-01N). C and D: Volume of spot 5 (Chart C) and spot 7 (Chart D) quantified by Samespots software in each gel (numbered as in B). Additional bars (T, D, F and P) show mean spot volume across a number of gels, shown with corresponding error bars. T=mean spot volume all tumours (gels 1-10); D=mean spot volume in depressed tumour gels (gels 1-4), F=mean spot volume in flat tumours (gels 5-7), P= mean spot volume in polypoid tumours (gels 8-10). P values compare mean spot volume across gels D, F and P. The fold increase from lowest to highest mean spot volume is shown on each chart.

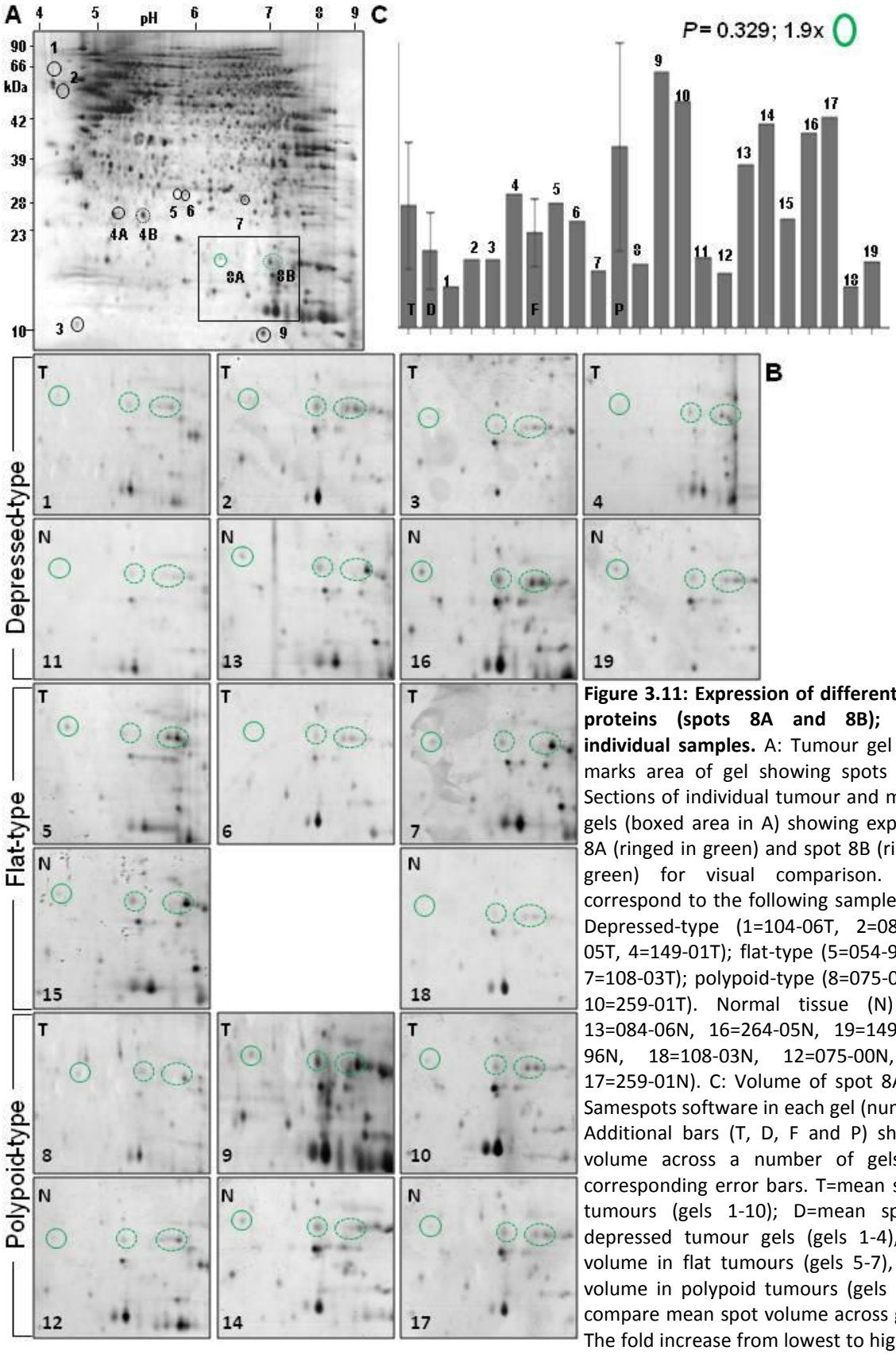


Figure 3.11: Expression of differentially expressed proteins (spots 8A and 8B); evaluation in individual samples. A: Tumour gel (233-00T). Box marks area of gel showing spots 8A and 8B. B: Sections of individual tumour and matched normal gels (boxed area in A) showing expression of spot 8A (ringed in green) and spot 8B (ringed in dashed green) for visual comparison. Gel numbers correspond to the following samples; Tumours (T): Depressed-type (1=104-06T, 2=084-06T, 3=264-05T, 4=149-01T); flat-type (5=054-96T, 6=148-01T, 7=108-03T); polypoid-type (8=075-00T, 9=233-00T, 10=259-01T). Normal tissue (N) (11=104-06N, 13=084-06N, 16=264-05N, 19=149-01N, 15=054-96N, 18=108-03N, 12=075-00N, 14=233-00N, 17=259-01N). C: Volume of spot 8A quantified by Samespots software in each gel (numbered as in B). Additional bars (T, D, F and P) show mean spot volume across a number of gels, shown with corresponding error bars. T=mean spot volume all tumours (gels 1-10); D=mean spot volume in depressed tumour gels (gels 1-4), F=mean spot volume in flat tumours (gels 5-7), P= mean spot volume in polypoid tumours (gels 8-10). P values compare mean spot volume across gels D, F and P. The fold increase from lowest to highest mean spot volume is shown on the chart.

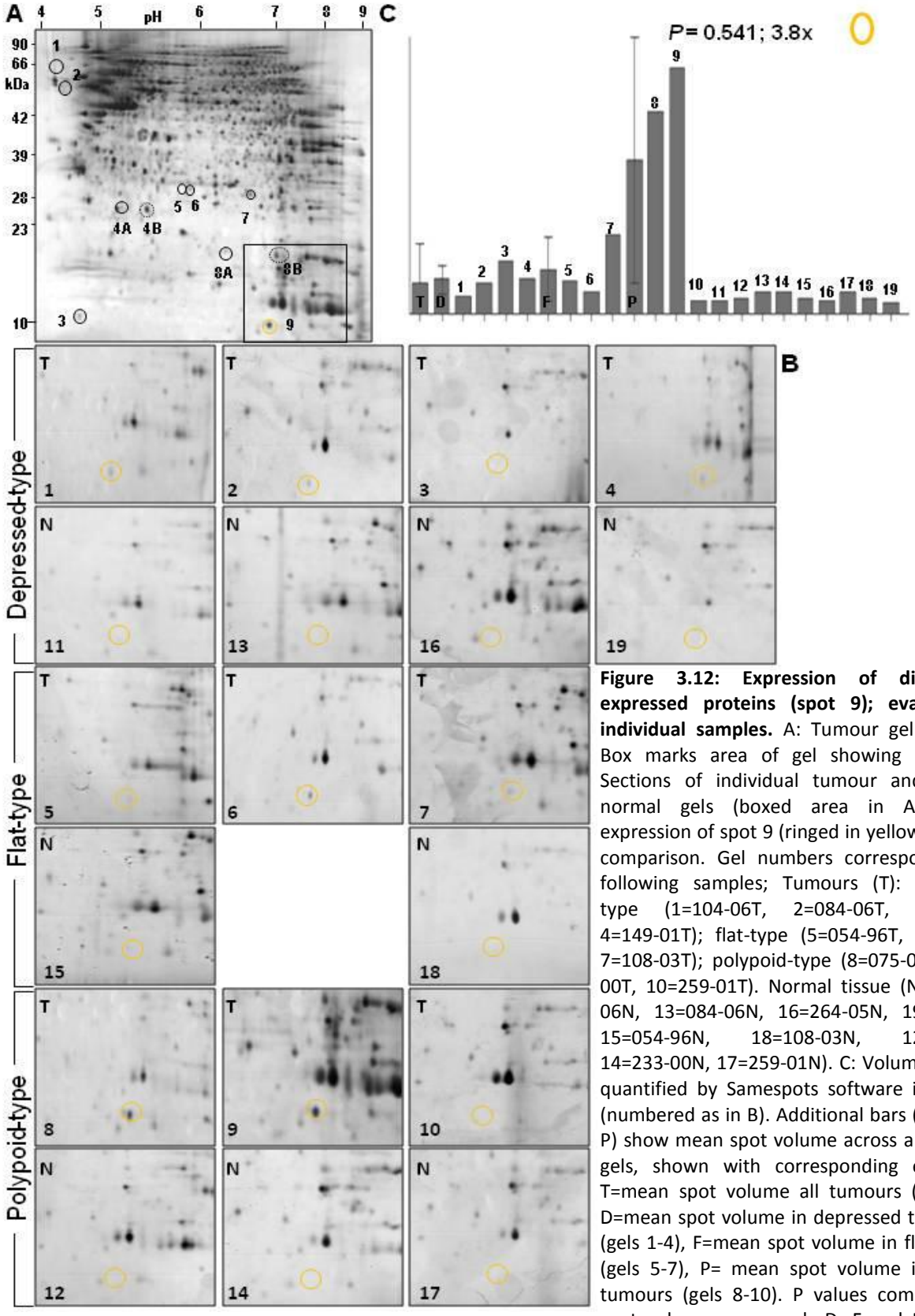


Figure 3.12: Expression of differentially expressed proteins (spot 9); evaluation in individual samples. A: Tumour gel (233-00T). Box marks area of gel showing spot 9. B: Sections of individual tumour and matched normal gels (boxed area in A) showing expression of spot 9 (ringed in yellow) for visual comparison. Gel numbers correspond to the following samples; Tumours (T): Depressed-type (1=104-06T, 2=084-06T, 3=264-05T, 4=149-01T); flat-type (5=054-96T, 6=148-01T, 7=108-03T); polypoid-type (8=075-00T, 9=233-00T, 10=259-01T). Normal tissue (N) (11=104-06N, 13=084-06N, 16=264-05N, 19=149-01N, 15=054-96N, 18=108-03N, 12=075-00N, 14=233-00N, 17=259-01N). C: Volume of spot 9 quantified by Samespots software in each gel (numbered as in B). Additional bars (T, D, F and P) show mean spot volume across a number of gels, shown with corresponding error bars. T=mean spot volume all tumours (gels 1-10); D=mean spot volume in depressed tumour gels (gels 1-4), F=mean spot volume in flat tumours (gels 5-7), P= mean spot volume in polypoid tumours (gels 8-10). P values compare mean spot volume across gels D, F and P. The fold increase from lowest to highest mean spot volume is shown on the chart.

3.2 IDENTIFICATION OF PROTEIN SPOTS

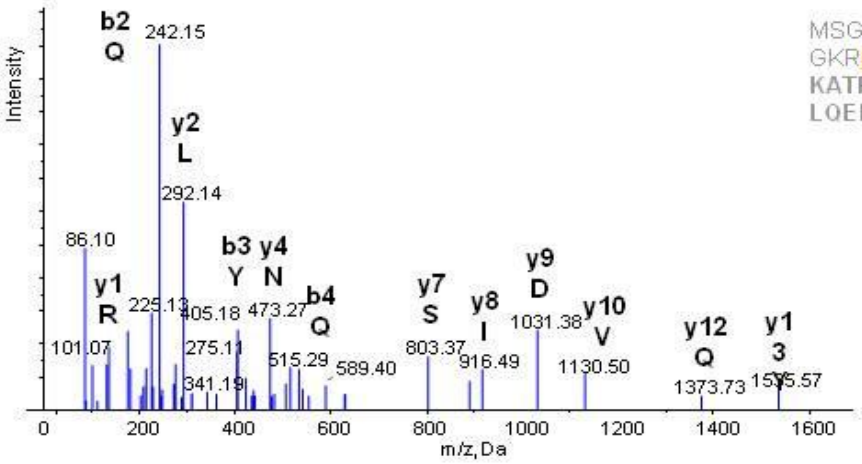
Silver stained 2D gels were matched with well-loaded undissected Coomassie-stained gels of tumour and normal tissue lysate. Spots were cut out from gels and subject to tandem mass spectrometry analysis as described in Section 2.10. Three spots were not identified using this analysis. Spectra from the remaining spots are shown in Figure 3.13. Identities of each protein spot are shown in table 3.1 with corresponding average spot volumes calculated by SameSpots analysis according to tumour morphology. *P*-values were generated by comparing average spot volume.

Table 3.1: Spots identified on 2D gel analysis

Spot no	Spot volume			Fold increase	*P*-value	Identity
	Depressed	Flat	Polypoid			
1	34,327	48,372	19,968	2.5x	0.02	No identity
2	60,842	78,045	32,730	2.4x	0.007	No identity
3	25,581	56,291	45,546	1.9x	0.039	SH3 domain-binding glutamic acid-rich-like protein 3
4A	26,596	19,842	29,709	1.6x	0.545	Peroxiredoxin 2 (Oxidised)
4B	Analysed due to proximity to spot 4A					Peroxiredoxin 2 (Reduced)
5	6,402	10,690	11,901	1.9x	0.479	Peroxiredoxin 6
6	Analysed due to proximity to spot 5					Heat Shock Protein 27
7	1,929	1,548	7,710	4.7x	0.005	No identity
8A	9,269	19,286	19,419	1.7x	0.744	Cofilin (phosphorylated serine 3)
8B	Identified on western blotting					Cofilin
9	22,768	29,968	102,292	3.8x	0.541	S100A8

A. SH3-binding glutamic acid rich protein-like 3

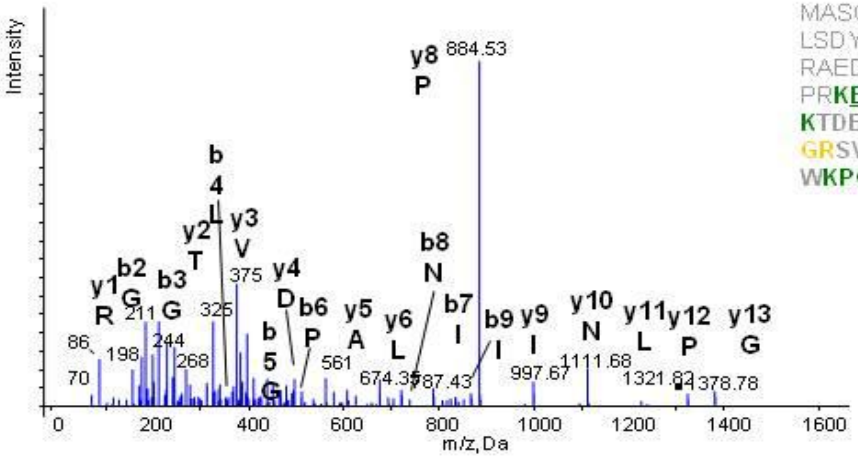
Sequence coverage at 50% confidence = 20.4



MSGLRVYSTSVTGSREIKSQQSEVTRILD
GKRIQYQLVDISQDNALRDEMRALAGNP
KATPPQIVNGDQYCGDYELFVEAVEQNT
LQEFLKLA

B. Peroxiredoxin 2

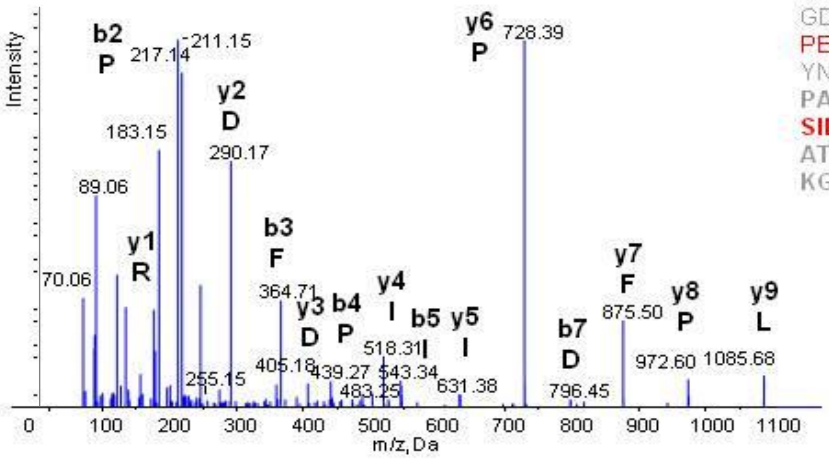
Sequence coverage at 95% confidence = 24.2



MASGNARIGKPAPDFKATAVVDGAFKEVK
LSDYKGKYVVLFFYPLDFTFVCPTEIIAFSN
RAEDFRKLGCEVLGVSVDSQFTHLAWINT
PRKEGGLGPLNIPLLADVTRRLSEDYGVL
KTDEGIAYRGLFIIDGKGVLRQITVNDLPV
GRSVDEALRLVQAFQYTDEHGEVCPAG
WKPGSDTIKPNVDDSKEYFSKHN

C. Peroxiredoxin 6

Sequence coverage at 95% confidence = 4.0



MPGGLLLGDVAPNFEANTTVGRIRFHDFL
GDSWGILFSHPRDFTPVCTTELGRAAKLA
PEFAKRNVKLIALSIDSVEDHLAWSKDINA
YNCEEPTEKLPFPIIDDRNRELAILLGMLD
PAEKDEKGMPGTARVVFVFGPDKKLKL
SILYPATTGRNFDEILRVVISLQLTAEKRV
ATPVDWKDGDSVMVLPTIPEEEAKKLFP
KGVFTKELPSGRKYLRYTPQP

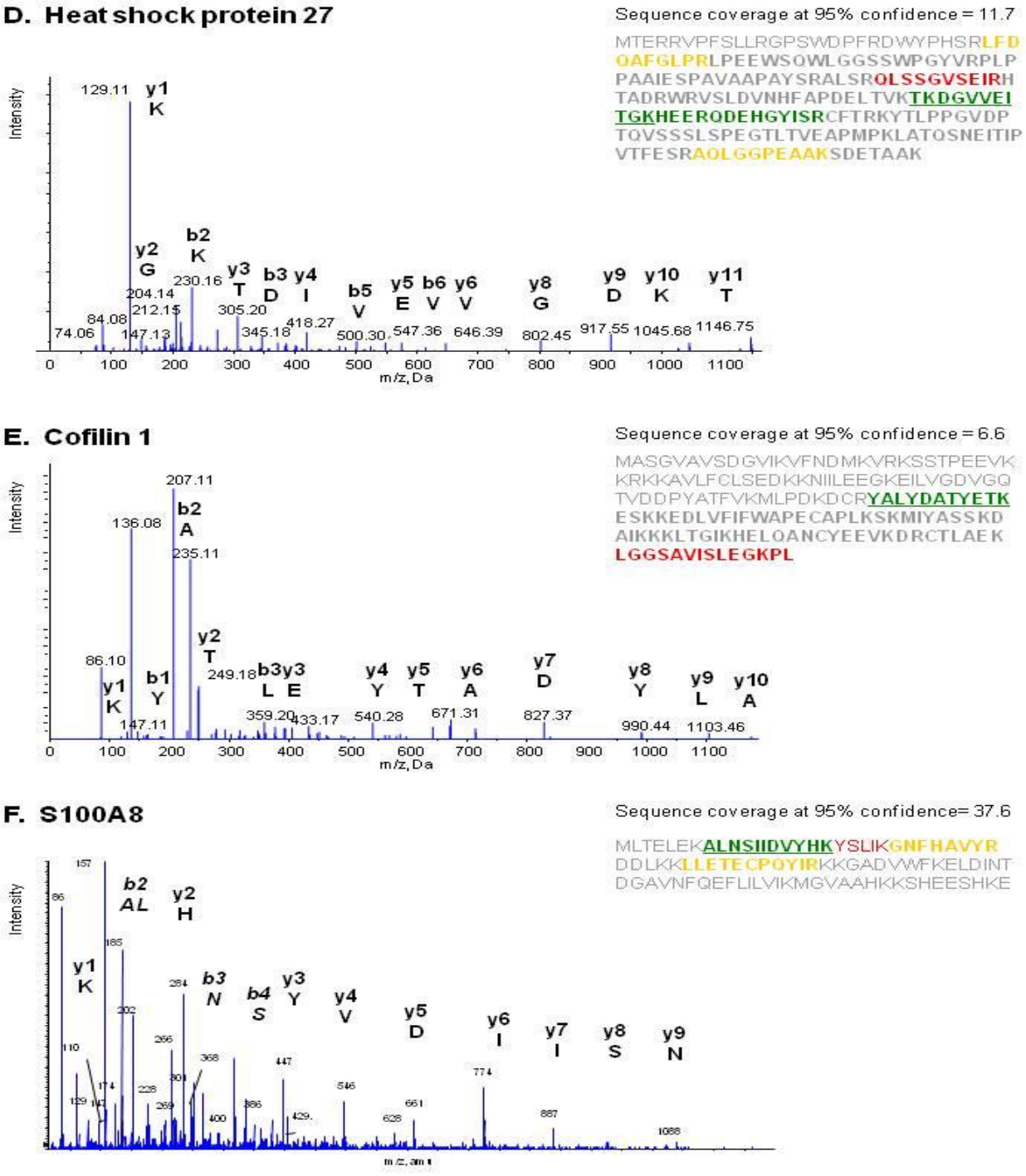


Figure 3.13A-F: MS/MS spectra for identified protein spots. Complete sequences are shown on the right with peptide masses identified in the first chamber shown in colour. Spectra shown correspond to underlined peptide sequenced in second chamber. A: SH3BGRPL3 B: Peroxiredoxin 2 C: Peroxiredoxin 6 D: HSP27 E: Cofilin1 F:S100A8

3.3 VALIDATION OF DIFFERENTIALLY-EXPRESSED PROTEINS

Five identified differentially-expressed proteins were taken forward for validation. Commercial antibodies for heat shock protein 27 and S100A8 immunohistochemistry were in use in the Diagnostic Molecular Pathology Department in the Royal Liverpool University Hospital. Staining protocols for these two antibodies were kindly obtained from the Lead Biomedical Scientist in Pathology, Mr Andy Dodson. Validation of the expression of these proteins in colorectal adenocarcinoma is described in sections 3.6 and 3.8 respectively. Commercial antibodies for Peroxiredoxin 6 and Cofilin1 were purchased and protocols for use were developed as described in sections 3.5 and 3.7 respectively. No commercial antibody was available for SH3 domain-binding glutamic acid-rich like protein 3 (SH3BGRLP3). Dr Zhang (University of Sceince and Technology of China) was contacted and kindly agreed to donate a sample of mouse ascites for investigation, described in section 3.4. Peroxiredoxin 2 was not selected for investigation as published results in other tumour types showed no correlation between total protein levels and prognosis or stage. No antibody was available for the for sulphide (hyperoxidised) form of the protein (section 3.5).

3.4 SH3 DOMAIN-BINDING GLUTAMIC ACID-RICH-LIKE PROTEIN 3

3.4.1 INTRODUCTION

The 10.5kDa protein SH3 binding glutamic acid-rich protein-like 3 has an isoelectric point of 5.0 corresponding well with the position of the protein spot identified on 2D gel analysis (figure 3.8). SH3 binding glutamic acid-rich (SH3BGR) gene is located to human chromosome 21. Two homologous genes, SH3BGRL and SH3BGRL3 are located to chromosome Xq13.3 and 1p34.3-35, respectively and code for small proteins similar to the N-terminal region of the SH3BGR protein.(245) SH3BGRL3 protein shows a significant similarity to Glutaredoxin 1 of Escherichia coli, and all the three proteins are predicted to belong to Thioredoxin-like protein family. Glutaredoxins (GRXs) are ubiquitous oxidoreductases, which catalyze the reduction of many intra-cellular protein disulfides and play an important role in many redox pathways. However, the SH3BGRL3 protein lacks the enzymatic function of glutaredoxins and may have a role as a regulator of redox activity.(246) Proteins such as glutaredoxin and thioredoxin are reported as up-regulated in many cancers such as lung and pancreatic; they have been implicated in increased resistance of cancer cells to free-radicals. There is little current evidence which directly links SH3GRPL3 with survival in cancer cells, however the protein has recently been identified as up-regulated in glioblastoma multiform compared to normal cerebral tissue on proteomic analysis.(247) Studies of acute promyelocytic leukemia cell line NB4 have also reported up-regulation of the protein.(248) Conversely, the related protein SH3BGRL is reported to be downregulated in fibroblasts, lymphoid cells, and splenic tumor cells transformed by the viral oncogene v-Rel. Co-expression of SH3BGRL

with v-Rel in primary splenic lymphocytes reduced the number of colonies formed by 76%.(249) Xu *et al.* reported SH3BGRPL3 protein as a post-translational modification of the 27kDa tumor necrosis factor alpha (TNF-α) inhibitory protein, TIP-B1. This protein is potentially involved in resistance of cells to the apoptosis-inducing affect of TNF-α.(248)

3.4.2 VALIDATION OF SH3BGRPL3

In order to validate both the identity of the protein spot thought to be SH3BGRPL3, and to determine the relationship of the protein to survival in colorectal cancer we sought an antibody which could be utilised for immunohistochemistry and western blotting. No antibody was available commercially; however a kind gift from Dr Q Zhang, University of Science and Technology of China of mouse ascites was investigated for antibody activity. Unfortunately, no antibody activity was detected on western blotting in colorectal cancer cell line or tissue lysate; or on direct spotting of the antibody onto nitrocellulose membrane (Figure 3.14).

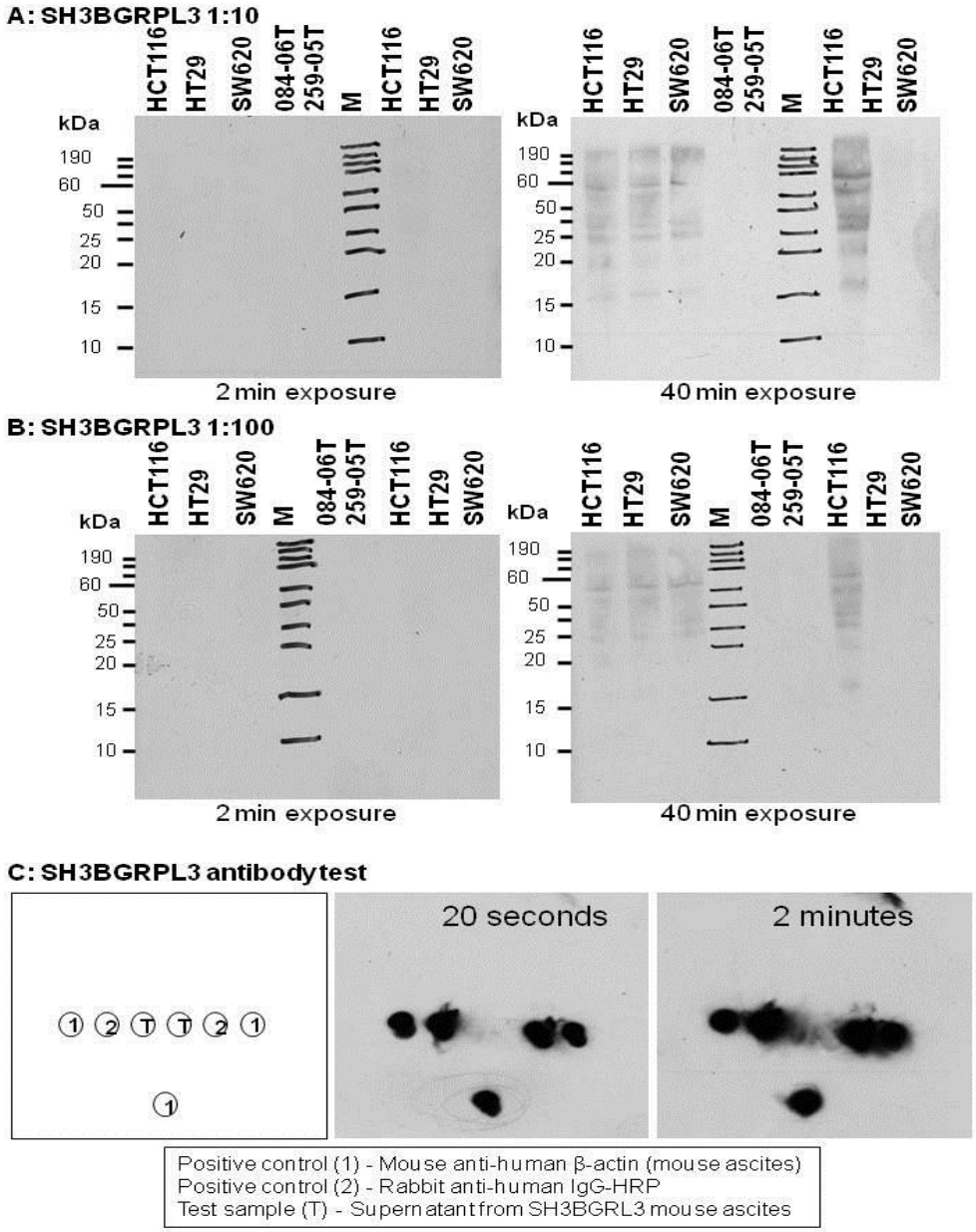


Figure 3.14: Western blotting for SH3BGRPL3. A&B: Proteins from colorectal cancer cell lines HCT116, HT29, SW620 and whole tissue lysate from colorectal cancers 084-06 and 259-05 were separated by 1D SDS-PAGE, transferred to membrane and western analysis undertaken with anti-SH3BGRPL3 mouse ascites. Primary antibody concentration was 1:10 (A) and 1:100 (B) with exposure times of 2 and 40min. No specific protein band was detected. **C:** In order to ascertain whether the anti-SH3BGRPL3 mouse ascites contained active antibody the ascites was spotted directly onto nitrocellulose membrane with two commercial antibody solutions, (mouse anti-human β-actin and rabbit anti-human 1gG-HRP) in the positions marked. The membrane was incubated with anti-mouse secondary. No signal was seen from the donated anti-SH3BGRPL3 mouse ascites at 20s or 2min exposure which confirmed that no antibody was present in the ascites.

3.5 PEROXIREDOXINS II AND VI

3.5.1 INTRODUCTION

Peroxiredoxins, Prx are a highly conserved group of proteins in eukaryotes with six isoforms, I-VI. Peroxiredoxin II has a molecular weight of 21.8kDa and a theoretical PI of 5.7, whereas peroxiredoxin VI has a molecular weight of 24kDa and PI of 6.2; both correspond well to the spots identified following gel analysis (Figure 3.9 and 3.10 respectively). There are 3 forms of the peroxiredoxin protein, typical 2-Cys (I-IV), atypical 2-Cys (V) and 1-Cys (VI). Specific isoforms occupy discrete intra-cellular compartments; I and V are located in all compartments, II in cytoplasm and nucleus, III in mitochondria, IV in lysosomes and VI in cytoplasm.(250)

Peroxiredoxins are responsible for reducing hydrogen peroxide within the cell (Figure 3.15). Cysteinyl residues in the protein are oxidised to form the short-lived and unstable form Prx-SOH. The protein can interact with another peroxiredoxin molecule to form a Prx-S-S-Prx disulfide bond. The disulphide bonded Prx dimers are reduced and recycled in a rapid reaction catalysed by thioredoxin. Alternatively, an over-oxidised form of peroxiredoxin can be generated at times of high peroxide concentrations by the formation of sulfenic acid residues, Prx-SO_2H. This stable protein has a more acidic PI then the reduced form of the protein on 2D gel analysis and was thought to be irreversible. However, evidence suggests that it can be slowly recycled to the reduced form by the enzyme sulfiredoxin.(251)

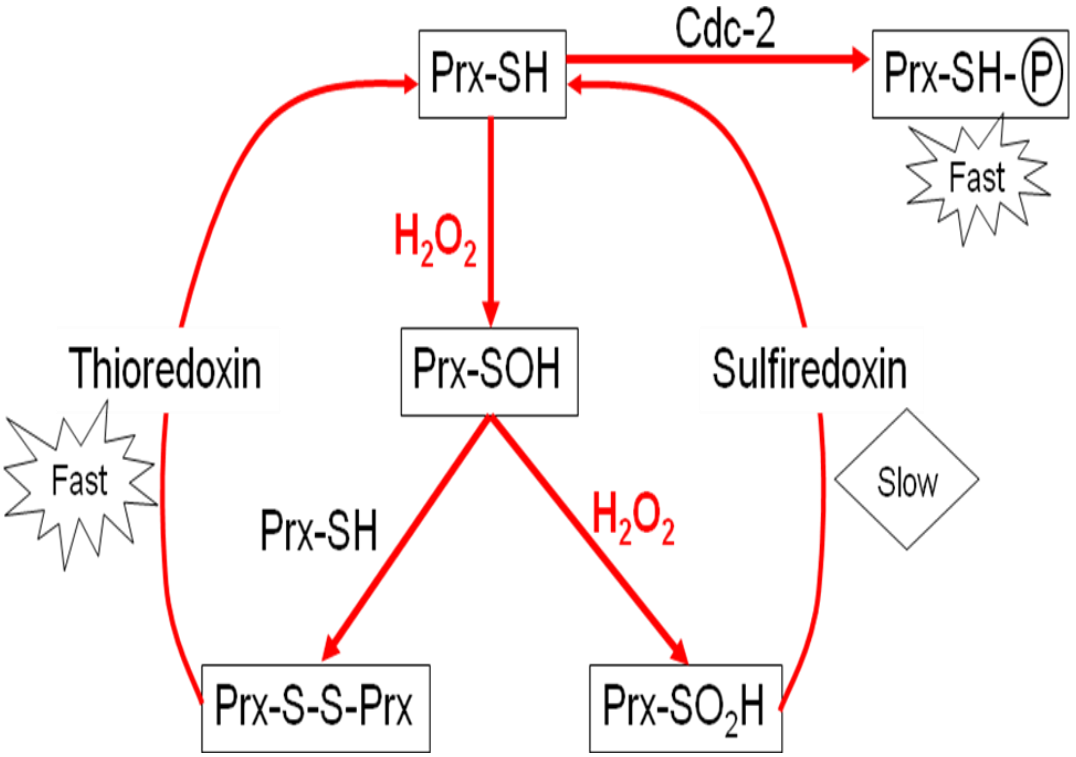


Figure 3.15: Pathways of Peroxiredoxin metabolism. Reduced Peroxiredoxin (Prx-SH) binds hydrogen peroxide (H_2O_2) to form oxidized Peroxiredoxin (Prx-SOH). This unstable protein can interact with another Peroxiredoxin molecule to form a dimer (Prx-S-S-Prx) when it is reduced and recycled by thioredoxin. During periods of high hydrogen peroxide concentrations Prx-SOH can combine with another H_2O_2 to form a stable hyperoxidised peroxiredoxin (Prx-SO2H).

Peroxiredoxins scavenge low level hydrogen peroxide in the cytoplasm generated as by-products of metabolism. However, peroxiredoxins can be inactivated by hyper-oxidation during periods of high H_2O_2 exposure. Evidence is mounting that ROS (reactive oxygen species) such as H_2O_2 act as signalling molecules. Growth factors and cytokines applied to cells *in vitro* generate a pulse of ROS, and addition of oxidants to cells to induce ROS can activate growth factor signalling pathways. Hyperoxidation of peroxiredoxins can facilitate ROS-mediated cell signalling pathways by enabling transient pulses of H_2O_2 to occur.

Two types of signalling pathways have been shown to utilise ROS such as H_2O_2: TNF-β1 induced apoptosis by generation of mitochondrial ROS, and cell growth and proliferation mediated by EGF and PDGF signalling.(252) Therefore, elevated peroxiredoxin expression may protect cells from apoptosis, whereas disproportionate elevation of the inactive Prx-sulfide form of peroxiredoxin could also indicate high levels of cell growth and proliferation.

Total expression of Peroxiredoxin-II has been analysed using immunohistochemistry in many cancers including colon, lung, breast and renal. No association between stage or five-year survival has been demonstrated in lung(253) or breast cancer;(254, 255) correlation with good prognosis was reported in a small series of renal cell carcinoma patients.(256) In a small series of colon cancers (n=32), Prx II was found to be over-expressed in cancer tissue compared to normal tissue (n=23/32, 72%). A correlation was described between elevated tumour Prx II levels and incidence of nodal metastases (*P*=0.023).(257)

Total PrxVI has been found to be up-regulated in the sera of individuals with both squamous cell carcinoma of the oesophagus(258) and lung cancer.(259) It is up-regulated in breast cancer cells

and is associated with increased invasiveness and pulmonary metastases.(260) In colon cancer tissue (n=32 cases) Prx VI was over-expressed with a frequency of 18/32, (56%). No association was seen in this study between expression of Prx VI and stage at presentation or prognosis in colon cancer.(257)

Inability to distinguish between reduced and oxidised forms of peroxiredoxin using immunohistochemistry is a disadvantage when assessing their impact on cancer behavior as the ratio of reduced: oxidised isoforms may be more informative that total levels of the protein.

We identified two isoforms of Peroxiredoxin II on 2D gel analysis. The form that was differentially expressed was more acidic, suggesting the oxidised Prx-II-sulfide protein.(261) No commercially available antibody has been generated which is specific for the sulphide form of Prx-II and therefore this supposition could not be confirmed. Given the lack of association between overall Prx-II levels in cancer tissue and prognosis, no further analysis of Prx-II was carried out in colorectal cancer tissue. One isoform of Peroxiredoxin-VI was differentially expressed in our cohort of colorectal cancer specimens, identified following 2D gel analysis and tandem mass spectrometry. It was not possible to determine which Prx-VI isoform (reduced or oxidised) was differentially expressed in silver-stained gels. Antibodies to both proteins were commercially available and were utilised in 1D and 2D western analysis of colorectal cancer cell lysate with a view to validating our findings on SDS-PAGE. Immunohistochemical analysis in colorectal cancer tissue could not be performed as the antibodies proved not to be sensitive or specific (section 3.5.2).

3.5.2 RESULTS OF PRX-VI ANALYSIS

Lysate from cell lines HT-29, SW480, HCT116 and SW620 was quantified for protein and equal quantities loaded onto a series of 1D gels as previously described in section 2.10.2. Protein on electrophoresed gels was transferred onto nitrocellulose membrane and blots probed for both Peroxiredoxin-VI and Peroxiredoxin-VI-sulfide using primary antibodies at concentrations recommended by the manufacturer (see table 2.3). Adjustments were made to the protocol including alteration of blocking solutions, incubation time of the primary antibody and concentration of primary antibody utilised. Prx-VI antibody was found to detect a protein band at the appropriate molecular weight (24 kDa) which was equally expressed in all colorectal cancer cell lysate (Figure 3.16). However, cross reactivity was seen between the antibody and other proteins producing two bands at around 19kDa and 35kDa, figure 3.16A. Two-dimensional western blot analysis for Prx-VI was performed on colorectal cancer cell lysate to better determine the nature of the cross-reactivity seen on 1D western blot (results not shown). No convincing spot was highlighted at the expected located of Prx-VI on the 2D gel and cross-reactivity was seen with a multitude of different spots confirming that the reactivity seen on 1D western blotting was non-specific in nature. Similarly, 1D analysis of Prx-VI-sulfide showed a faint band at the appropriate molecular weight (24kDa), but multiple additional bands and a high background reactivity (Figure 3.16C). Alterations to the protocol used were made as outlined above, but cross-reactivity could not be eliminated and the decision was made that the antibodies were not suitable for further analysis in tissue.

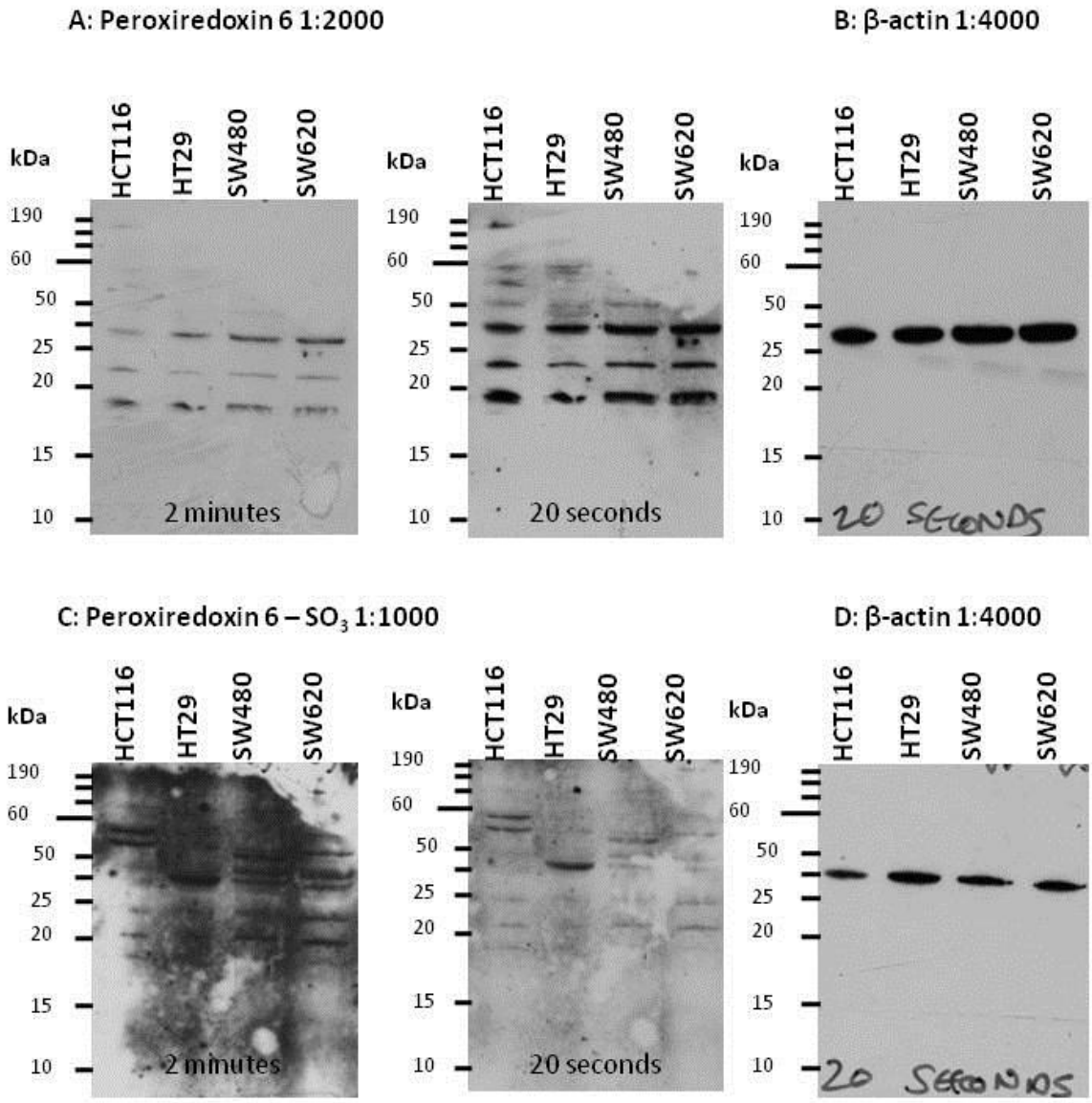


Figure 3.16: Western blot of peroxiredoxin 6 and peroxiredoxin 6-SO_4 expression. Protein lysate from colorectal cancer cell lines HCT116, HT29, SW480 and SW620 were separated by 1D SDS-PAGE, transferred to membrane and western analysis undertaken with anti-PrxVI and anti-PrxVI-SO_3, β-actin was used as control. Primary antibody concentrations were Prx-VI 1:2000 (A), Prx-VI-SO_3 1:1000 (C) and β-actin 1:4000 (B&D). **A:** Blot probed for Prx-VI shows a band at the appropriate molecular weight (28kDa) but additional bands at 40kDa and 19kDa. **B**: B-actin control. Underloading HCT116. **C:** Blot probed for Prx-VI-SO_3 shows multiple bands and heavy background despite blocking with both milk protein and goat serum. **D**: B-actin control. Underloading HCT116, overloading HT29.

3.6 HEAT SHOCK PROTEIN 27

3.6.1 INTRODUCTION

The HSP27 gene encodes a 27kDa mass, 199-amino acid polypeptide which, in unstressed cells exists predominantly as a single charged isoform (HSP27 A) with a PI of approximately 6.8.(262) After exposure to heat or chemical shock HSP27 synthesis increases, and the protein accumulates in the cytoplasm over 12-hours to levels that are approximately 10-fold higher than the basal level found in unstressed cells. Physiological transcription is mediated by Heat Shock Transcription Factor 1 (HSF1) which translocates to the nucleus after heat shock and interacts with the heat shock element (HSE) in the promoter region of the HSP27 gene.(263) HSP27 is also subject to more rapid post-transcriptional modifications after cellular stress. Within minutes of exposure, two acidic isoforms of HSP27 are generated, (HSP27 B and C), which represent phosphorylation at the serine 78 and 82 residues respectively by S6 kinase.(264) Additional agents which induce phosphorylation, but not transcription of HSP27 have been identified. These are predominantly mitogenic agents, such as serum, thrombin, fibroblast growth factor, TNF-α and TGF-β which exert their affect through p38 MAP kinase.(265)

Up-regulation of HSP27 (and other HSP proteins) has been correlated with the acquisition of thermotolerance.(266) This state is induced by a spectrum of activity broadly categorised into 1. Direct inhibition of death pathways and 2. Repair of protein damage and resolution of protein aggregates. HSP27 inhibits at multiple levels both the intrinsic (mitochondrial death) pathway and the extrinsic (receptor-mediated) cell death pathways; both of which activate effector caspases to

execute cell death.(267) HSP27 prevents loss of mitochondrial membrane potential and release of mitochondrial proteins. It also prevents the interaction between cytochrome c and pro-caspase-9 thus preventing apoptosome formation. HSP27 targets the extrinsic pathway through Daxx by inhibiting its binding to both the Fas cell death receptor and apoptosis signal regulating kinase, Ask1.(268) In conjunction with other members of the HSP family, HSP27 functions as a molecular chaperon. It forms complexes with damaged proteins preventing their non-specific aggregation, and then enables refolding in an ATP-dependant process mediated by HSP70.(269) F-actin is one such crucial protein stabilised by HSP27 during periods of cellular stress. HSP27 binds to denatured actin monomers dissociated from F-actin and protects them forming large insoluble aggregates under conditions of cellular stress. This has a potent anti-apoptotic affect by maintaining compartment integrity and supporting protein trafficking within the cell.(270) An overview of HSP27 regulation is shown in Figure 3.17.

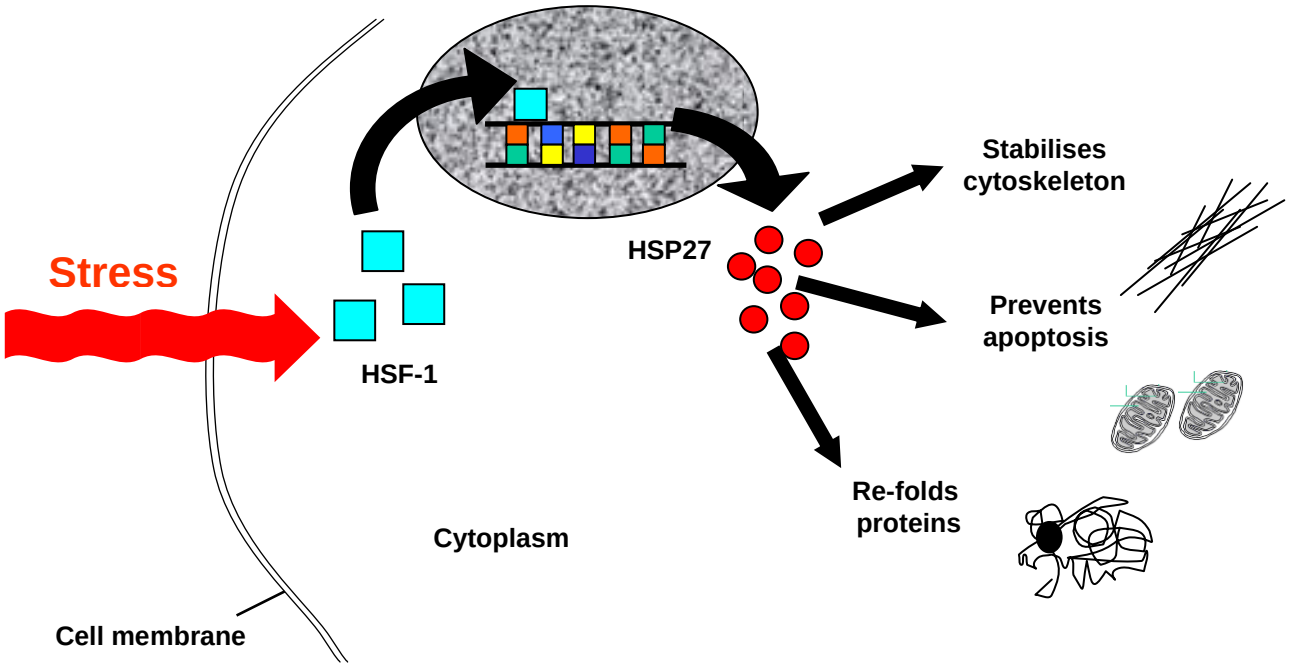


Figure 3.17: The regulation and actions of heat shock protein 27. HSF1 migrates into the nucleus following a stressor event. HSF1 interacts with the HSE on the promoter region of the HSP27 gene causing transcription of the HSP27 protein

Given its inhibitory influence on programmed cell death, it is not surprising that HSP27 has been reported as elevated in a number of human cancers, principally prostate, breast and ovarian.(271) One putative mechanism for these observations has centred on micro-environmental stress imposed by tumour hypoxia; however evidence for this hypothesis is lacking. One model of prostate tumour spheroids cultured with embryoid bodies observed little HSP27 expression during tumour hypoxia, but an increase after neo-vascularisation and restoration of normal oxygen tension.(272) Indeed, tumours cells growing as xenografts exhibited inhibition rather than enhancement of HSP27 expression.(273) There is emerging evidence to suggest that genomic stress contributes to the up-regulation of HSP27 in cancer through direct activation of the HSE, removal of transcriptional repression and stabilisation of HSF1.(274) p53 reportedly attaches to the binding site for the transcription factor NF-Y in the promoter region of HSP genes causing repression of transcription. Proteomic analysis of p53-null cell lines documented increased expression of both HSP70 and HSP27 compared to cells with wild-type p53.(275) This is a potentially important mechanism of regulation in colorectal cancer which has a 50% rate of p53 mutation.(276) Stabilisation of HSF1, prolonging transcription of HSP27 has been reported following heregulinβ1 activation of the c-erbB receptor,(277) a factor associated with poor survival in colorectal cancer.(278) In addition, direct transcription of HSP through the HSE can occur via the proto-oncogene c-Myc, which is also induced by heregulin and HER2.(279)

3.6.2 HSP27 IS A PROMISING TARGET FOR VALIDATION IN COLORECTAL CANCER

Spot 6 was picked for analysis due its proximity to spot 5, felt to be a potential isoform of the previously identified Peroxiredoxin-VI. The identification of the unrelated protein HSP27 was unexpected, yet interesting. Re-analysis of the gel images suggested that HSP27 was in fact differentially-expressed between different tumour samples, although not in relation to morphology (Figure 3.10). A protocol for use of a commercially available anti-HSP27 antibody was already employed for immunohistochemistry of prostate adenocarcinoma by the Lead Biomedical Scientist in Pathology, Mr Andy Dodson in the Diagnostic Molecular Pathology Department in the Royal Liverpool University Hospital. The antibody had previously been validated in our institute in a series of paraffin-embedded, formalin-fixed prostate cancers and was found to be specific for HSP27, easily scored and correlated with stage at presentation and prognosis of prostate cancer. Our group had also found HSP27 to be differentially expressed in a series of 2D gels of undissected Duke's C colorectal cancers (work by Mr Ilyas Khattak). Initial staining of 98 cases of formalin-fixed colorectal cancers had identified an association between HSP27 expression and survival in node-positive colorectal cancer. Further validation of HSP27 expression in colorectal cancer was we felt merited by these early results and kind permission to utilise data from the first 98 cases was given by Mr Khattak and is included in the results discussed in this chapter.

3.6.3 PATIENT DEMOGRAPHICS

Formalin-fixed, paraffin-embedded, primary colorectal adenocarcinomas, n=404 (199 colon, 205 rectal) from fully-consented patients, who underwent surgery at the Royal Liverpool University Hospital, UK between 1993 and 2003, were obtained from the Liverpool Tissue Bank, University of Liverpool. The tissue was microarrayed in eight separate blocks utilizing between two and six cores per tumour in addition to normal control tissue from kidney, liver, testes, tonsil and colon. Patient demographic details are provided in Table 3.2. Thirty percent of patients (121/404) underwent chemotherapy. In the 103/121 (85%) patients in whom the chemotherapy agents were known, all received 5-fluorouracil; 4/103 (4%) received an additional agent, (irinotecan or oxaliplatin). Second-line and third-line chemotherapy was given in 37/103 (36%) and 19/103 (18%) of patients respectively. Additional agents (irinotecan, oxaliplatin, mitomycin C or levamisole) were administered in 9/37 (24%) of second-round and 10/19 (53%) of third-round patients. Thirteen percent of rectal cancer patients underwent short-course radiotherapy as part of the CR07 trial(280) (25 Gray in 5 fractions over 1 week; surgery within 1 week of treatment), while 15% underwent long-course radiotherapy (offered to patients with bulky tumours and consisting of 45 Gray, administered in 25 daily fractions over 5 weeks followed by surgery after 6–10 weeks). The median follow-up was 49.5 months (IQR 19.6-74.6 months, range 0.23-146.5 months) and 284 deaths from any cause were reported, including 176 deaths due to colorectal cancer. Seven patients died within 30 days of surgery and were censored in the survival analysis. Four additional patients were censored at the point of last follow up as no date of death was recorded.

Clinical parameter	All cases, n=404 (%)	Colon cancer n=199 (%)	Rectal cancer n=205 (%)
Gender			
Female	153 (38)	95 (48)	58 (28)
Male	251 (62)	104 (52)	147 (72)
Age			
<70 years	209 (52)	90 (45)	119 (58)
≥70 years	195 (48)	109 (55)	86 (42)
Tumour size			
<40mm	98 (24)	42 (21)	56 (27)
40-59mm	178 (44)	83 (42)	95 (46)
≥60mm	128 (32)	74 (37)	54 (27)
Resection margins			
Clear	356 (88)	181 (91)	175 (85)
Involved	47 (12)	17 (9)	30 (15)
Unrecorded	1	1	0
Differentiation			
Well	10 (2)	4 (2)	6 (3)
Moderate	359 (89)	181 (91)	181 (88)
Poor	31 (8)	15 (7)	16 (8)
Unrecorded	4 (1)	2	2 (1)
Depth of invasion			
T1	20 (5)	12 (6)	8 (4)
T2	60 (15)	19 (9)	41(20)
T3	273 (67)	131 (66)	142 (69)
T4	51 (13)	37 (19)	14 (7)
Nodal status			
N0	225 (55)	117 (59)	108 (53)
N1	99 (25)	43 (22)	56 (27)
N2	80 (20)	39 (19)	41 (20)
Adjuvant therapy			
None	279 (69)	152 (76)	127 (62)
Chemotherapy	121 (30)	46 (23)	75 (37)
Unrecorded	4 (1)	1 (1)	3 (1)
Neoadjuvant therapy			
None	-	-	147 (72)
Short course	-	-	27 (13)
Long course	-	-	31 (15)

Table 3.2: Clinicopathological data for n=404 patients on the microarrays

3.6.4 HSP27 INTENSITY MORE PREDICTIVE OF SURVIVAL THAN PERCENTAGE OF STAINED CELLS

Scoring of HSP27 expression was performed using a protocol which included intensity of staining from 0-3 (based on staining of internal benign control tissue) and the percentage of cells staining grouped into 0: <5%, 1: 5-30%, 2: 30-70%, 3:>70%. The scoring system is illustrated in Figure 3.18 using a selection of variably-staining colorectal adenocarcinomas. A composite value or 0-9 index was calculated by multiplying the intensity and percentage staining. The median score in each field was calculated and used to dichotomise the data for analysis; intensity (low≤1.5 versus high>1.5), percentage (low≤2 versus high>2) and index (low≤3 versus high>3). These factors were subject to univariate Cox regression to assess which aspects of HSP27 staining were most strongly correlated with overall survival, table 3.3. Intensity of staining, but not percentage staining was related to overall survival, *P*=0.0171 versus *P*=0.9261 respectively; however, index was most predictive of survival, *P*=0.0078 and was utilised in the further construction of Kaplan-Meier curves and life tables.

Variable		**Cases**	**Univariate analysis**		
		n=404 (%)	**HR(95%CI)**	**χ^2**	**p-value**
HSP27 intensity (median score 1.5)	≤1.5	232 (57)	-		
	>1.5	172 (43)	1.286 (0.957-1.730)	2.780	0.0955
HSP27 percentage (median score 2.0)	≤2.0	234 (58)	-		
	>2.0	170 (42)	0.944 (0.699-1.274)	0.143	0.7056
HSP27 index (median score 3.0)	≤3.0	228 (56)			
	>3.0	176 (44)	1.382 (1.028-1.858)	4.600	**0.0320**

Table 3.3: Univariate Cox regression of HSP27 intensity and percentage

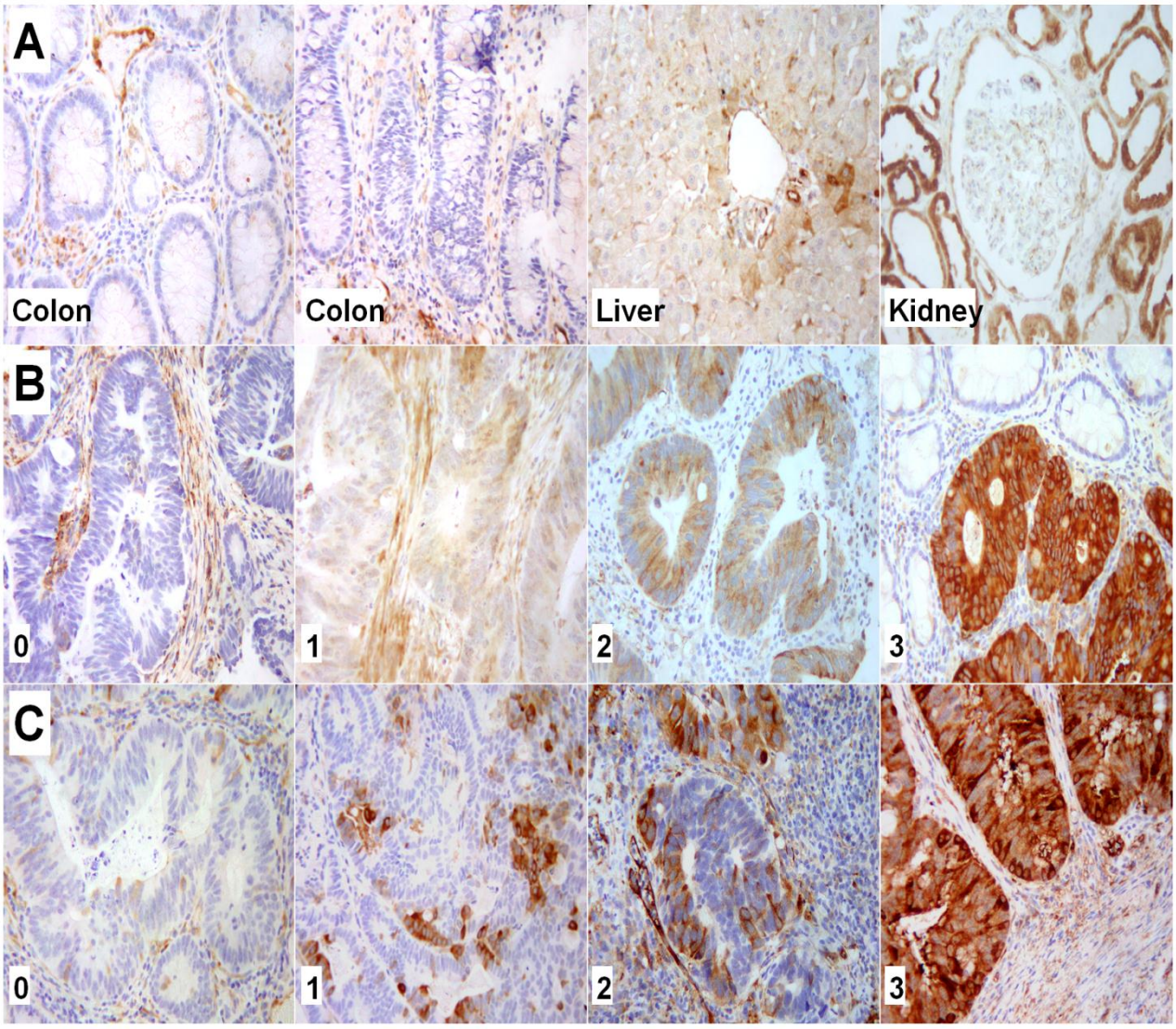


Figure 3.18: Immunohistochemistry for HSP27. (A) Benign control tissue showing absence of detectable HSP27 in normal colonic epithelium, weak staining in normal liver and intense staining of normal renal tubules. (B) Colorectal cancer tissue illustrating the range of intensities of HSP27 immunostaining, from 0 to 3. (C) Colorectal cancer tissue depicting varying percentages of HSP27 staining in different tumours.

3.6.5 HSP27 DOES NOT CORRELATE WITH STAGE AT PRESENTATION OR METASTASES

We proceeded to assess the relationship between HSP27 index score and clinicopathological variables related to the 404 tumours on the tissue microarray. TNM and the American Joint Committee on Cancer, AJCC guidelines were used to stage tumours.(243) Associations were sought between HSP27 expression (combined index) and gender, age, anatomical location, size of tumour, resection margin status, differentiation grade, depth of invasion (T-stage), presence of nodal metastases (N-stage) and treatment (adjuvant chemotherapy and neoadjuvant radiotherapy). The entire cohort of 404 patients was examined, in addition to separate analyses of the subgroups of patients with colon cancer (n=199) and rectal cancer (n=205). No significant correlations were demonstrated between HSP27 index and these parameters in any of groups analysed, with one exception (Table 3.4). High HSP27 expression was associated with incomplete resection margins in rectal cancer patients (P=0.009; Table 3.4).

	All cases				Colon cancers				Rectal cancers			
Clinical parameter	Cases n=404 (%)	HSP27 –ve n=228(%)	HSP27 +ve n=176(%)	P value*	Cases n=199 (%)	HSP27 –ve n=115(%)	HSP27 +ve n=84(%)	P value*	Cases n=205 (%)	HSP27 –ve n=113(%)	HSP27 +ve n=92(%)	P value*
Gender												
Female	153 (38)	83 (36)	70 (40)	0.489	95 (48)	51 (44)	44 (52)	0.262	58 (28)	32 (28)	26 (28)	0.993
Male	251 (62)	145 (64)	106 (60)		104 (52)	64 (56)	40 (48)		147 (72)	81 (72)	66 (72)	
Age												
<70 years	209 (52)	113 (50)	96 (55)	0.320	90 (45)	51 (44)	39 (46)	0.771	119 (58)	62 (55)	57 (62)	0.306
≥70 years	195 (48)	115 (50)	80 (45)		109 (55)	64 (56)	45 (54)		86 (42)	51 (45)	35 (38)	
Tumour size												
<40mm	98 (24)	52 (23)	46 (26)	0.439	42 (21)	25 (22)	17 (20)	0.683	56 (27)	27 (24)	29 (31)	0.391
40-59mm	178 (44)	98(43)	80 (46)		83 (42)	45 (39)	38 (45)		95 (46)	53 (47)	42 (46)	
≥60mm	128 (32)	78 (34)	50 (28)		74 (37)	45 (39)	29 (35)		54 (27)	33 (29)	21 (23)	
Resection margins												
Clear	356 (88)	207 (91)	149 (84)	0.080	181 (91)	104 (90)	77 (91)	0.563	175 (85)	103 (91)	72 (78)	**0.009**
Involved	47 (12)	21 (9)	26 (15)		17 (9)	11 (10)	6 (8)		30 (15)	10 (9)	20 (22)	
Unrecorded	1	0	1 (1)		1	0	1 (1)		0	0	0	
Differentiation												
Well	10 (2)	4 (2)	6 (4)	0.562	4 (2)	1 (1)	3 (4)	0.087	6 (3)	3 (3)	3 (3)	0.316
Moderate	359 (89)	204 (89)	155 (88)		181 (91)	101 (88)	77 (91)		181 (88)	103 (91)	78 (85)	
Poor	31 (8)	18 (8)	13 (7)		15 (7)	12 (10)	3 (4)		16 (8)	6 (5)	10 (11)	
Unrecorded	4 (1)	2 (1)	2 (1)		2	1 (1)	1 (1)		2 (1)	1 (1)	1 (1)	
Depth of invasion												
T1	20 (5)	11 (5)	9 (5)	0.763	12 (6)	7 (6)	5 (6)	0.964	8 (4)	4 (3)	4 (4)	
T2	60 (15)	34 (15)	26 (15)		19 (9)	10 (9)	9 (11)		41(20)	24 (21)	17 (19)	
T3	273 (67)	157 (69)	116 (66)		131 (66)	77 (67)	54 (64)		142 (69)	80 (71)	62 (67)	
T4	51 (13)	26 (11)	25 (14)		37 (19)	21 (18)	16 (19)		14 (7)	5 (4)	9 (10)	
Nodal status												
N0	225 (55)	131 (57)	94 (53)	0.558	117 (59)	65 (56)	52 (62)	0.744	108 (53)	66 (58)	42 (46)	0.095
N1	99 (25)	56 (25)	43 (25)		43 (22)	26 (23)	17 (20)		56 (27)	30 (27)	26 (28)	
N2	80 (20)	41 (18)	39 (22)		39 (19)	24 (21)	15 (18)		41 (20)	17 (15)	24 (26)	
Adjuvant therapy												
None	279 (69)	161 (71)	118 (67)	0.297	152 (76)	89 (77)	63 (75)	0.613	127 (62)	72 (64)	55 (60)	0.406
Chemotherapy	121 (30)	63 (27)	58 (33)		46 (23)	25 (22)	21 (25)		75 (37)	38 (33)	37 (40)	
Unrecorded	4 (1)	4 (2)	0		1 (1)	1 (1)	0		3 (1)	3 (3)	0	
Neoadjuvant therapy												
None	-	-	-		-	-	-		147 (72)	84 (74)	63 (68)	0.629
Short course	-	-	-		-	-	-		27 (13)	14 (12)	13 (14)	
Long course	-	-	-		-	-	-		31 (15)	15 (14)	16 (18)	

Table 3.4: Clinicopathological features associated with HSP27 expression

3.6.6 HSP27 STATUS INDEPENDENTLY PREDICTS SURVIVAL IN RECTAL CANCER

Elevated HSP27 was associated with poor survival (*P*=0.0312) when the entire cohort of 404 patients was examined (Fig. 3.19A). However, when the colon and rectal cancer patients were analysed separately, HSP27 expression was not associated with survival in the colon cancer group (*P*=0.7385; Figure 3.19B), but was significantly associated with poor survival in the rectal cancer group (*P*=0.0063; Figure 3.19C). At five-years, rectal cancer-specific survival of high HSP27-expressers was 63% versus 73% for low HSP27-expressers. Survival was assessed across all stages at presentation in rectal and colon cancer. Elevated HSP27 predicted poor cancer-specific survival in rectal cancer patients with early stage I/II tumours (Figure 3.19D). HSP27 however, was not predictive of poor survival for rectal cancer patients with stage III (N+/M0) disease (*P*=0.2132, Figure 3.19E). Kaplan-Meier curves were constructed for stage I/II colon cancer (Figure 3.20A) and stage III colon cancer (Figure 3.20B). No difference in survival according to HSP27 expression was noted either group; *P*=0.8569 and *P*=0.4365 respectively (Figure 3.20A&B). The benefits of adjuvant therapy for patients with stage III disease are well established, whilst management of patients with stage II rectal tumours (T3-4/N0/M0) remains controversial.(281) We next determined whether high HSP27 expression might be useful in identifying a 'high-risk' group of patients with stage II rectal tumours (T3-4/N0/M0) who might benefit from adjuvant therapy. We found that the overall survival of patients with stage II (T3-4/N0/M0) disease who had elevated HSP27, n=29 was similar to that of patients with N1 disease, n=56 (*P*=0.6523, Fig. 3.19F). This was not observed in colon cancer, where the overall survival of patients with stage II (T3-4/N0/M0) disease who had elevated HSP27, n=41 was significantly better than that of patients with N1 disease, n=43 (*P*=0.0268, Figure 3.20C).

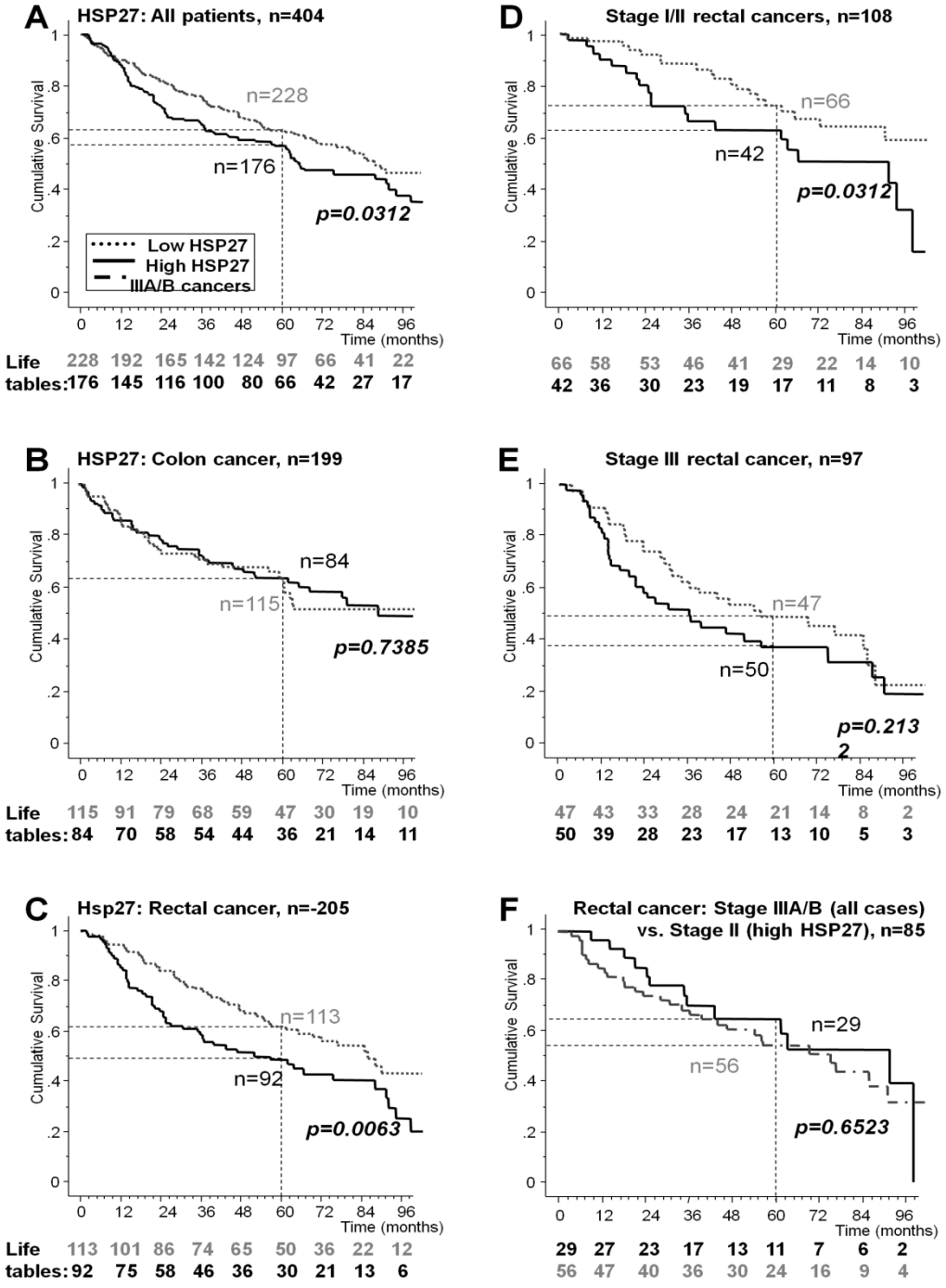


Figure 3.19: Kaplan-Meier graphical analysis of 8-year survival in (A) all patients, (B) colon cancer patients, (C) rectal cancer patients, (D) Stage I/II rectal cancer patients, (E) Stage III rectal cancer patients. (F) Stage IIIA/B rectal cancer patients versus Stage II high HSP27 expressors.

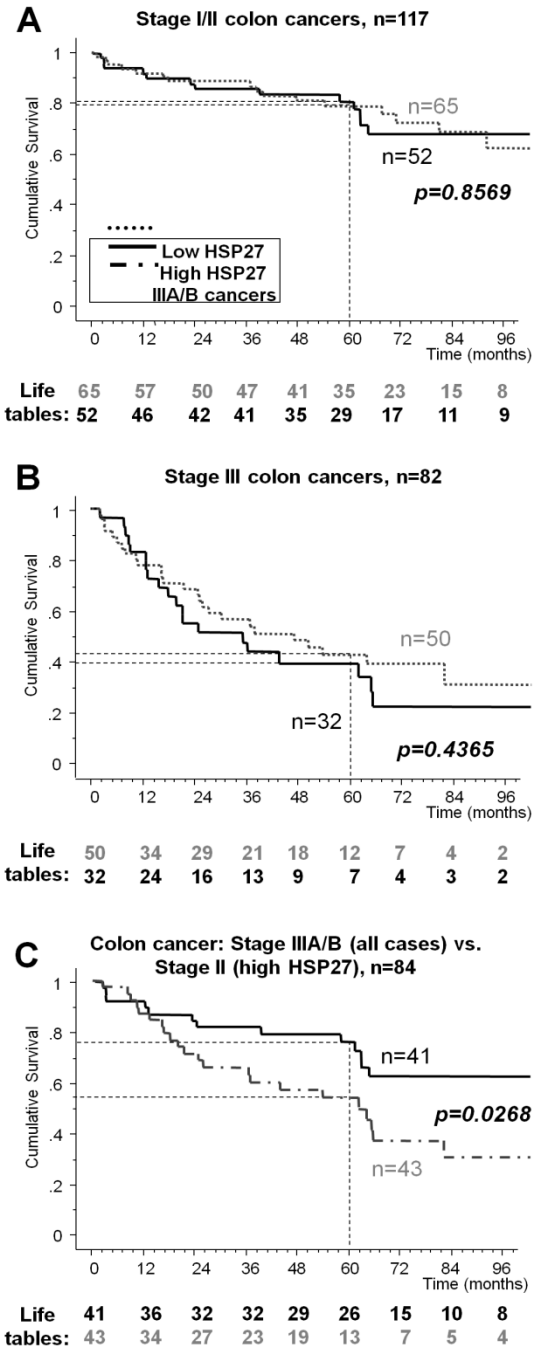


Figure 3.20: Kaplan-Meier curves of HSP27 index in colon cancer (A) Stge I/II colon cancer; high HSP27-expressors (n=52) showed similar survival to low expressors (n=65; log-rank, *P*=0.8569). **(B)** Stage III colon cancer; high HSP27-expressors (n=32) showed similar survival to low expressors (n=50; log-rank, *P*=0.4365). **(C)** Poor survival for stage IIIA/B colon cancer (all cases; n=43) versus Stage II high HSP27 expressors (n=41, log-rank, *P*=0.0268.

3.6.7 MULTIVARIATE ANALYSIS OF SURVIVAL

Multivariate Cox proportional hazards regression analysis was performed to derive risk estimates related to survival for all clinicopathologic characteristics and HSP27 expression. Elements associated with cancer-specific survival on univariate analysis with a *P*-value<0.10 were included in the multivariate model. Univariate analysis of 205 rectal cancer patients revealed that resection margin status (*P*=0.0894), N-stage (*P*=0.0001) and HSP27 expression (*P*=0.0069) were significantly associated with survival (Table 3.5A). Only N-stage and HSP27 expression remained independently predictive of survival in rectal cancer with *P*-values of 0.0001 and 0.0233 respectively. Univariate analysis of 199 colon cancers revealed significant factors as differentiation grade (*P*=0.0278), T-stage (*P*=0.0008), resection margin status (*P*=0.0001) and N-stage (*P*=0.0001), (Table 3.5B). HSP27 was not associated with survival (*P*=0.7382). On multivariate analysis only resection margin status (*P*=0.0001) and N-stage (*P*=0.0001) remained independently significant for colon cancer (Table 3.5B).

Variables	Group	Cases n=205 (%)	Univariate			Multivariate		
			HR(95%CI)	χ^2	p-value	HR(95%CI)	χ^2	p-value
Age (years)	68yrs (60-74)	-	1.000 (0.981-1.018)	0.001	0.973	-	-	-
Size (mm)	45mm (35–60)	-	1.001 (0.998-1.004)	0.115	0.693	-	-	-
Diff grade	Well-Mod	187 (91)						
	Poor	16 (9)	1.355 (0.682-2.693)	0.753	0.386	-	-	-
T-stage	T1	8 (4)	-	2.686	0.261	-	-	-
	T2	41 (20)	1.154 (0.336-3.967)	0.052	0.820	-	-	-
	T3	156 (76)	1.708 (0.539-5.416)	0.827	0.363	-	-	-
Resection margins	Clear	175 (85)						
	Involved	30 (15)	1.563 (0.933-2.618)	2.884	**0.089**	0.808 (0.450-1.453)	0.506	0.477
N-stage	N0	108 (53)	-	23.892	**0.001**	-	20.227	**0.001**
	N1	56 (27)	1.557 (0.962-2.521)	3.250	0.071	1.539 (0.947-2.500)	3.026	0.082
	N2	41 (20)	3.259 (2.027-5.240)	23.766	0.001	3.289 (1.956-5.532)	20.150	0.001
HSP27 expression	Negative	113 (55)						
	Positive	92 (45)	1.720 (1.160-2.550)	7.293	**0.007**	1.607 (1.066-2.422)	5.143	**0.023**

Table 3.5 A: Univariate and multivariate analysis of survival in rectal cancer

Variable	Group	Cases n=199 (%)	Univariate			Multivariate		
			HR(95%CI)	χ^2	p-value	HR(95%CI)	χ^2	p-value
Age (years)	71 yrs (64 – 77)	-	1.002 (0.982-1.022)	0.023	0.879	-	-	-
Size (mm)	50mm (40 - 60)	-	1.001 (0.998-1.003)	0.246	0.620	-	-	-
Diff grade	Well-Mod	182 (91)						
	Poor	15 (8)	2.192 (1.089-4.412)	4.838	**0.028**	1.315 (0.605-2.857)	0.478	0.489
T-stage	T1	12 (6)	-	16.738	**0.001**	-	7.181	0.066
	T2	19 (10)	1.476 (0.134-16.29)	0.101	0.751	1.905 (0.172-21.15)	0.276	0.600
	T3	131 (66)	4.818 (0.665-34.89)	2.423	0.120	3.257 (0.445-23.85)	1.352	0.245
	T4	37 (18)	10.56 (1.425-78.29)	5.322	0.021	5.825 (0.771-43.99)	2.918	0.088
Resection margins	Clear	181 (91)						
	Involved	17 (9)	4.776 (2.532-9.008)	23.326	**0.001**	4.002 (2.055-7.792)	16.644	**0.001**
N-stage	N0	117 (59)	-	31.717	**0.001**	-	19.423	**0.001**
	N1	43 (22)	2.622 (1.510-4.555)	11.712	0.001	2.021 (1.136-3.597)	5.726	0.017
	N2	39 (19)	4.671 (2.702-8.075)	30.466	0.001	3.705 (2.062-6.658)	19.191	0.001
HSP27 expression	Negative	115 (58)						
	Positive	84 (42)	0.926 (0.588-1.456)	0.111	0.739	1.354 (0.497-1.290)	0.833	0.361

Table 3.5 B: Univariate and multivariate analysis of survival in colon cancer

3.6.8 RELATIONSHIP BETWEEN HSP27 EXPRESSION AND NEO-ADJUVANT TREATMENT

The difference in survival observed in the rectal, but not the colon cancer patients, prompted us to question whether HSP27 expression was related to administration of neo-adjuvant radiotherapy. Our rectal cancer patients underwent two forms of treatment; long-course and short-course radiotherapy. Short-course pre-operative radiotherapy was offered as part of the CR07 trial which recruited patients with operable tumours and administered 25 Gray in five daily fractions over one week, with surgery within one week of treatment. Long-course radiotherapy was offered to patients with bulky tumours which, on pre-operative imaging, was felt to compromise the proposed mesorectal excision margin. The aim was to downsize the tumour rendering it operable, and 45 Gray was administered in 25 daily fractions over five weeks followed by surgery after 6–10 weeks. We analysed a cohort of rectal cancer patients in order to determine whether pre-operative radiotherapy was associated with a change in HSP27 expression. Data were obtained for 80 patients; 49 (61%) had no neo-adjuvant therapy, 17 (21%) had short-course and 14 (18%) had long-course radiotherapy. Matched diagnostic biopsy material was obtained for all 80 patients and analysed for HSP27 expression. In 65/80 (81%) of cases the HSP27 index was the same in the biopsy and the tumour. Four tumours (6%) showed up-regulation of HSP27 compared to the biopsy and 9/67 (13%) down-regulated HSP27 in the tumour compared to the biopsy, (Figures 3.21A and B, Table 3.6). Expression of HSP27 in the primary tumour was not correlated with neo-adjuvant therapy received, P=0.802, neither was there an associated change in HSP27 expression between the biopsy and tumour specimens following pre-operative therapy, P=0.602, (Table 3.7).

3.6.9 NEO-ADJUVANT THERAPY IS NOT ASSOCIATED WITH OVERALL SURVIVAL

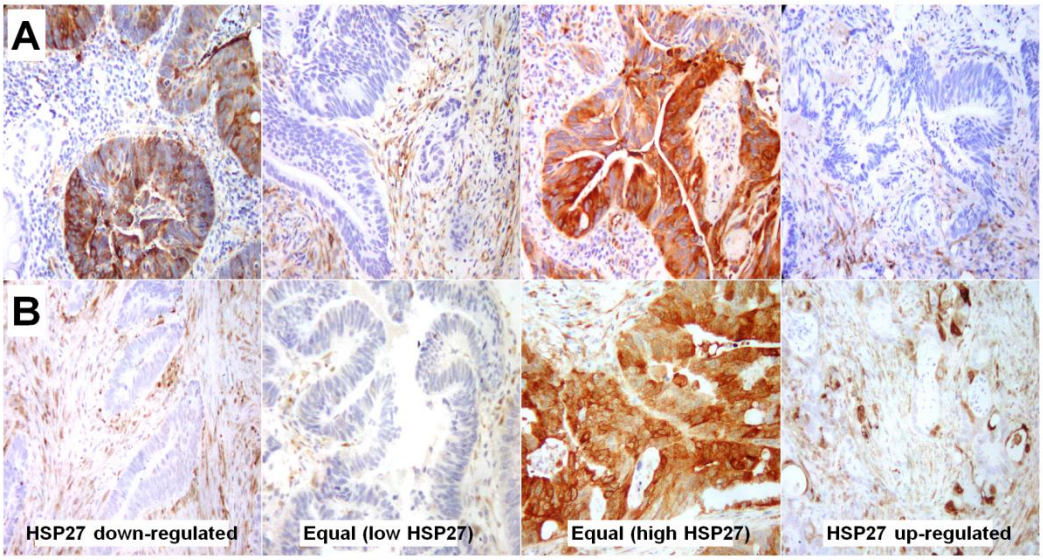
Having demonstrated that neo-adjuvant radiotherapy did not influence HSP27 expression, we sought to determine whether the type of neo-adjuvant radiotherapy received contributed to the survival difference observed in the rectal cancer group. Survival analysis performed for the 147 rectal cancer patients who did not receive radiotherapy treatment confirmed that high HSP27 expression in this group was associated with poor survival (*P*=0.0078, Figure 3.21C). No difference in cancer-specific survival was seen in the rectal cancer group according to type of radiotherapy received (*P*=0.4317, Figure 3.21D).

HSP27 expression in diagnostic biopsies	No of cases, n=80 (%)	Low HSP27 index, n=42 (%)	High HSP27 index, n=38 (%)	P-value
Low HSP27 in biopsy	37 (46)	32 (76)	5 (14)	<0.0001
High HSP27 in biopsy	43 (54)	10 (24)	33 (86)	

Table 3.6: Comparative HSP27 expression between diagnostic biopsy and tumour

Levels of HSP27 in tumour compared to biopsy	No of cases, n=80(%)	Neo-adjuvant radiotherapy			P-value
		None, n=49	Short course, n=17	Long-course, n=14	
Up-regulated in tumour	5 (6)	3 (6)	2 (12)	0	0.6423
Equal expression	65 (81)	39 (80)	14 (82)	12 (86)	
Down-regulated in tumour	10 (13)	7 (14)	1 (6)	2 (14)	

Table 3.7: Change in HSP27 expression according to pre-operative radiotherapy



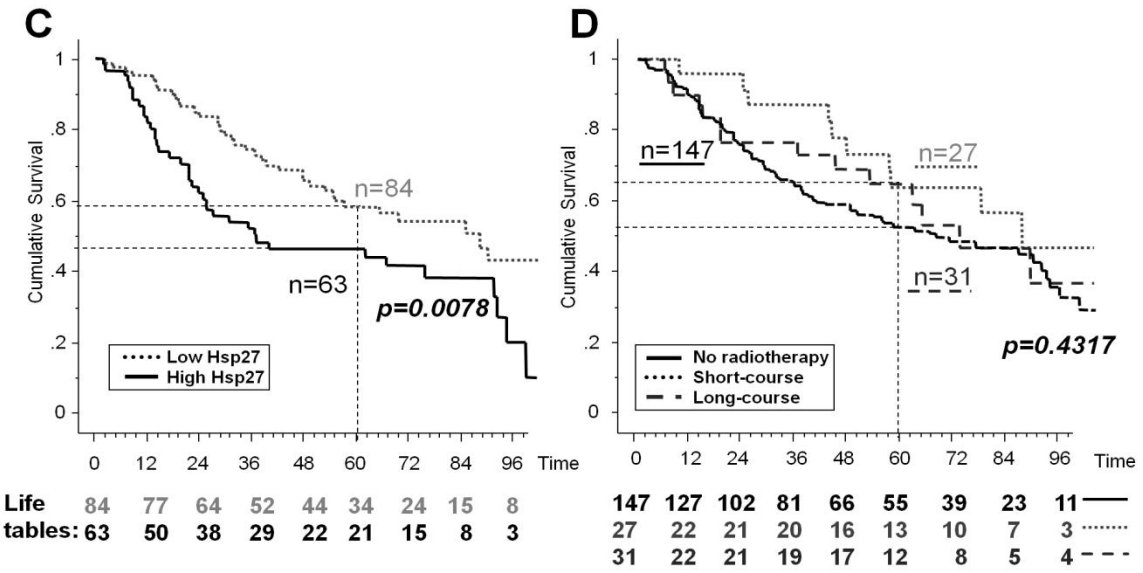


Figure 3.21: (A and B): HSP27 staining in diagnostic biopsy material (upper panel) compared to matched tumour cores (lower panel)**. (C)** Kaplan-Meier survival curves demonstrating that the level of HSP27 remains predictive of cancer-specific survival in rectal cancer patients who have not received neoadjuvant radiotherapy and **(D)** that mode of neoadjuvant radiotherapy received did not correlate with cancer-specific survival.

3.7 COFILIN1 AND COFILIN-PHOSPHO(SER3)

3.7.1 INTRODUCTION

Cofilin is one of a group of universally-expressed actin-binding proteins which co-operate to control the structure of the cytoskeleton. The actin-binding region takes the form of a long α-helix which is highly conserved between isoforms. Cofilin-1 (non-muscle cofilin or n-cofilin) is an 18kDa protein consisting of 166 amino acids. It is the most abundant and ubiquitously expressed member of the cofilin family which also incorporates cofilin-2 (muscle cofilin or m-cofilin) and ADF (actin-depolymerising factor or destrin). Expression of the different forms of the cofilin family varies according to tissue type; cofilin-1 and ADF are the predominant isoforms in most adult tissues whereas cofilin-2 is the chief isoform present in skeletal and cardiac muscle.(282) The protein is subject to a number of post-translational modifications including phosphorylation at residues 3, 25, 41, 68, 140 and 156 and acetylation at 2 and 132. Phosphorylation at the serine 3 residue is the only known functional modification which prevents cofilin from binding to actin.(283)

Actin filaments (F-actin) are formed by polymerization from actin monomers (G-actin); these helices intertwine generating actin microfilaments.(284) Filaments are polarised, with polymerisation taking place at the barbed-end and dissociation at the pointed-end of actin molecules. Polymerization in vivo is dependent on the formation of free barbed-ends that act as nuclei for elongation. Rapid nucleation is created by two complementary processes; uncapping or severing of existing filaments by cofilin and generation of novel nuclei on the body of filaments by the complex Arp 2/3 producing new branches.(285) Polymerisation proceeds once ATP-G-actin binds to the

nucleation point; the ATP is hydrolyzed to ADP releasing the inorganic phosphate. At the pointed-end, ADP-F-actin dissociates slowly in a process which is catalysed by cofilin. Cofilin releases old ADP-F-actin into the cytoplasm to enable ADP-to-ATP exchange to take place, re-generating the cellular pool of ATP-G-actin. In the presence of plentiful G-actin, cofilin facilitates actin polymerization via its actin-severing activity; providing free barbed ends for further polymerization and nucleation. When the concentration of G-actin is limited, the net result of severing by cofilin is depolymerisation, not nucleation.(286)

Cell motility is dependent on synchronised activity of the actin cytoskeleton. The cell initiates a protrusion at the front, which subsequently attaches to the substratum. This is followed by contraction of the cell body and tail detachment, resulting in movement in the direction of the protrusion. The initiating event in this cycle is appears to be activation of cell surface receptors by chemotactic factors, which establish a signalling cascade culminating in the polymerization of new actin at the leading edge.(287) The site of signal activation on the cell surface determines the direction of cell migration. Within 60 seconds of stimulation with a chemoattractant, Arp2/3 complex and cofilin are recruited to the membrane. As described, both factors act synergistically to generate multiple dendritic actin filaments with free barbed ends. Function-blocking antibodies directed against either protein significantly decrease barbed-end generation and cell protrusion.(288) Polymerisation of the actin filaments is dependent on a high concentration of G-actin to act as substrate. Cofilin depolymerises filaments at the base of membrane protrusions to replenish G-actin and facilitate polymerisation at the leading edge in a process called treadmilling.(289)

Cofilin activity can be regulated within a cell in a number of ways. Firstly, cofilin can be inactivated by phosphorylation of the serine 3 residue by the LIM family of kinases. LIM kinase 1 and 2 (LIMK1 and LIMK 2) are regulated by Rho GTPases through their downstream effectors Rho-associated protein kinase (ROCK) and p21-activated kinases 1 and 4 (PAK1 and PAK4). These activate LIMK1 and LIMK2 by phosphorylation at residues Thr508 and Thr505, respectively. Active cofilin can be sequestered by phosphatidylinositol (4,5)-bisphosphate, PIP_2 forming a pool of the protein close to the cell membrane unavailable for actin binding. Cofilin is also regulated by pH; the actin binding efficiency is markedly reduced when the pH drops below 7.0.(290, 291) Activation of cofilin can take place through increasing pH, dephosphorylation by various phosphatases including Slingshot (SSH) and chronophin or hydrolysis of PIP_2 due to increasing phospholipase C (PLC) activity which releases cofilin from sequestration.(292) An overview of cofilin regulation is shown in Figure 3.22.

The central position of cofilin in co-ordinating membrane protrusion and directional motility has led many to investigate its role in cancer cell invasion. Expression of cofilin has been reported as up-regulated in tumour tissue including oral squamous cell,(293) ovarian(294) and renal cell carcinoma;(295) and in human cancer cell lines for example breast (MDA-MB435S),(296) pancreatic (EPP85-181RDB)(297) and lung adenocarcinoma (A549).(298) Phospho(ser3)-cofilin has been quantified and found at reduced levels in various human cancer cell lines including T-cell lymphoma (Jurkat), cervical (HeLa), colon (KM12), liver (HepG2) and kidney (COS1). A study of 13 different colorectal cancer cell lines confirmed universally high levels of cofilin-1 and ADF on Western blot. SiRNA induced knock-down of both cofilin and ADF was associated with enhanced adhesion to laminin I and collagen I/IV. Migration of cells through Matrigel was reduced in colorectal cancer cell

lines HCT116 and LS174T, but not the Isrecol cell line following reduction of cofilin-1 expression; whereas Isrecol cell migration was reduced by ADF depletion.(299) However, several studies have found cofilin to be down-regulated in cancer, specifically hepatocellular carcinoma cell line (MCHCC97-H),(300) and increasing cofilin expression in H1299 human lung cancer cells reduces invasion by disrupting the actin cytoskeleton at the leading edge.(301) In breast cancer cells, EGFR activation results in PLC-mediated hydrolysis of PIP_2, releasing cofilin; simultaneously LIM kinase and phosphorylated cofilin levels are seen to increase.(302) This points to formation of an equilibrium between active cofilin1 and inactive cofilin-phospho(ser3) within a cell mediated by a number of different factors including LIM kinase and PLC. Following stimulation of the cell by chemotactic factors a new equilibrium may be reached.(303)

Cofilin has been linked to cell invasion and an increase in malignant phenotype of breast cancer cells.(303) Little is known about the expression of cofilin in actual tumours. Only one study evaluated immunohistochemical levels of cofilin1 in paraffin-embedded cancer tissue. The paper reports an increase in depth of invasion in neuroendocrine gastrointestinal tumours with increasing expression of cofilin1, although the number of cases involved was small (n=34).(304) We have made preliminary investigations of commercially available antibodies to cofilin and cofilin-phospho(ser3) with a view to conducting the first large scale study of cofilin expression in colorectal cancer tissue.

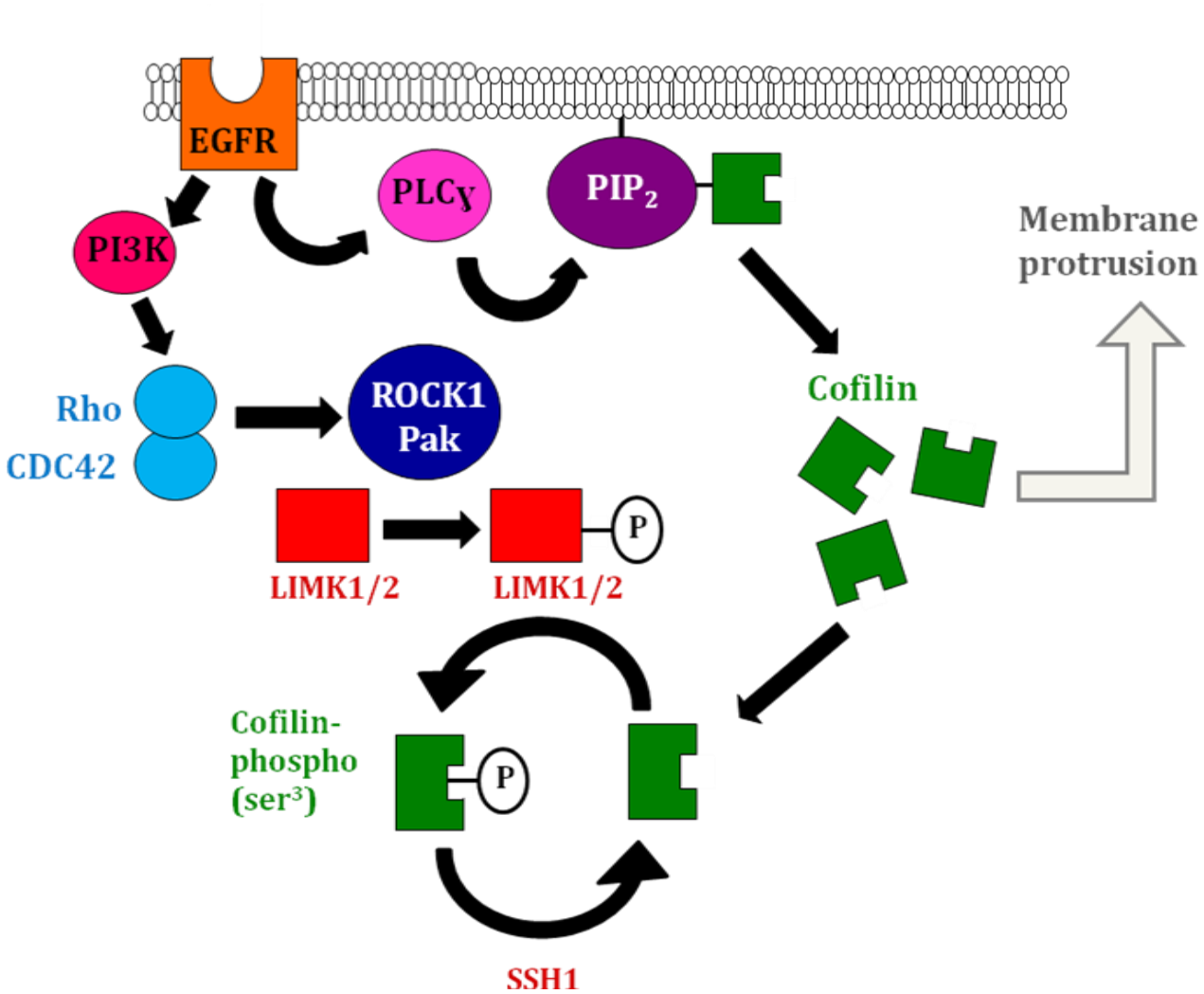


Figure 3.22: Regulation of cofilin

3.7.2 COFILIN AND COFILIN-PHOSPHO(SER3) EXPRESSED IN COLORECTAL CANCER CELL LYSATE

Colorectal cancer cell lines HCT116, HT29, SW480 and SW620 cell lines were cultivated in L15 in 5% CO2 to confluence. Cell lysate was prepared as described in Section 2.5.1 from each cell line and protein quantified using the BCA method. An equal quantity of protein (30µg) was loaded onto each lane of a small format 1D gel and run as described in section 2.5.1. Anti-cofilin antibody and anti-cofilin-phospho(ser3) antibodies were used as primaries in concentration 1:10,000 and 1:50 respectively. The secondary antibody was horseradish-conjugated rabbit anti-mouse at 1:4000 concentration. Development of blots revealed a band at the appropriate molecular weight (17kDa) in all cell lines probed for cofilin. Loading was variable in actin control films with underloading in HCT116 lane and overloading in SW480 lane. When probed for cofilin-phospho(ser3) only HT-29 (non-metastastic) colorectal cancer cell line showed a band at the appropriate molecular weight at 2 minutes with weaker bands visible at 15 minutes in SW480 and SW620 samples. Anti-cofilin and cofilin-phospho(ser3) antibodies are capable of detecting protein bands of the relevant size in tumour cell lysate and show little cross-reactivity with other proteins. Inconsistent gel loading, evidenced by variable expression of β–actin control, indicated that relative expression of cofilin between cell lines could not be commented upon (Figure 3.23A&B). Despite inconsistent gel loading in evidence on β–actin control blot, the expression of cofilin-phospho(ser3) was felt to be more variable, with highest expression in cell line HT29, followed by SW620 and SW480; lowest expression was seen in the cell line HCT116 (Figure 3.23C&D).

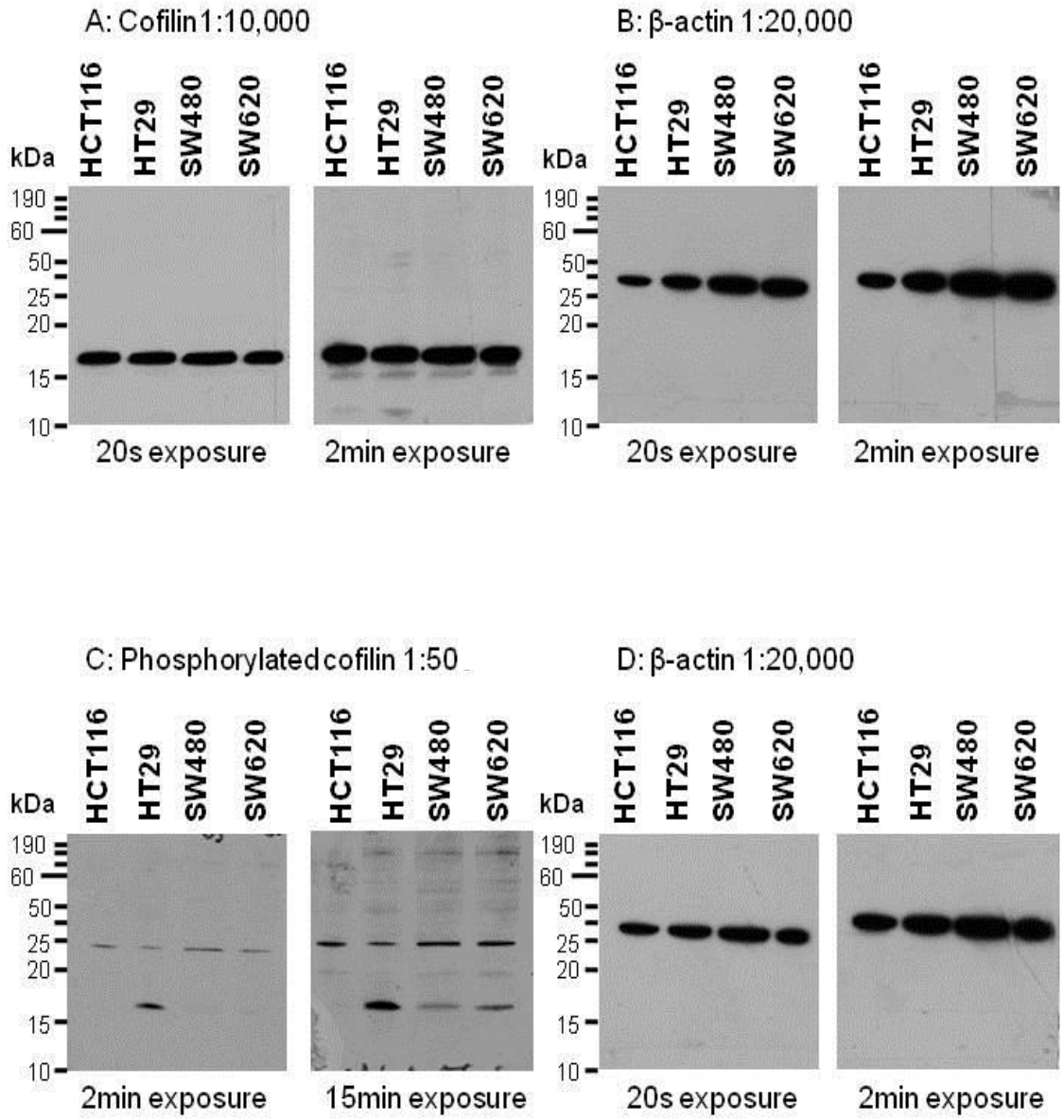


Figure 3.23: 1D Western blots of colorectal cancer cell lysate probed for cofilin and cofilin-phospho(ser3). Protein lysate from colorectal cancer cell lines HCT116, HT29, SW480 and SW620 were separated by 1D SDS-PAGE, transferred to membrane and western analysis undertaken with anti-cofilin1 and anti-phosphylated cofilin. β-actin was used as control. Primary antibody concentrations were cofilin1 1:10000 (A), cofilin-P 1:50 (C) and β-actin 1:20000 (B&D). A/B: Blot probed for cofilin1 and β-actin showing greater expression of the protein in HCT116 cells. C/D: Blot probed for cofilin-phospho(ser3) and β-actin showing greatest expression in HT29 and lowest expression in HCT116 cells

3.7.3 CONFIRMATION OF SPOT IDENTITY USING 2D WESTERN BLOTTING IN CELL LINES AND TISSUE

In order to confirm the identity of cofilin1 on tandem mass spectrometry I sought to validate the findings on 2D Western blot. Western blot analysis could give a more accurate impression of post-translational modifications in the protein of interest. Two samples from each of four lysates were subject to 2D electrophoresis as described in section 2.3; cell lines SW480 and HT29 in addition to undissected material from patient 264-05 (tumour and normal colon). One gel from each pair was fixed and stained with coomassie blue as control: proteins on the second gel were electro-phorectically transferred onto nitrocellulose membrane in preparation for Western blotting as described in section 2.5. Membranes were stained with Ponceau stain to check adequate transfer prior to being probed with anti-cofilin-phospho(ser3) at 1:50 overnight at 4°C. Following development of the blots, membranes were stripped with stripping buffer (20ml SDS 10%, 12.5 ml 0.5 M Tris HCl (pH 6.8), 0.8 ml ß-mercaptoethanol, 67.5 ml ultra pure water) and re-probed with anti-cofilin1 at 1:10,000 for 1hr at room temperature.

Colorectal cancer cell lysate, undissected tumour and undissected normal colon showed similar pattern of cofilin1 staining. A string of proteins of similar molecular weight (17kDa) was seen extending from PI 6.5-8.0. Little non-specific cross-reactivity was seen with other proteins on the blot. Cofilin-phospho(ser3) showed activity in a single spot at 17kDa, PI 6.5 which corresponded well with the spot picked as variably-expressed on the silver-stained gels. Staining was more variable across samples with the metastatic cancer cell line SW480 showing heaviest staining, frozen tumour tissue and non-metastatic cell line HT29 showing moderate staining and normal colon showing minimal staining; (Figures 3.24 and 3.25).

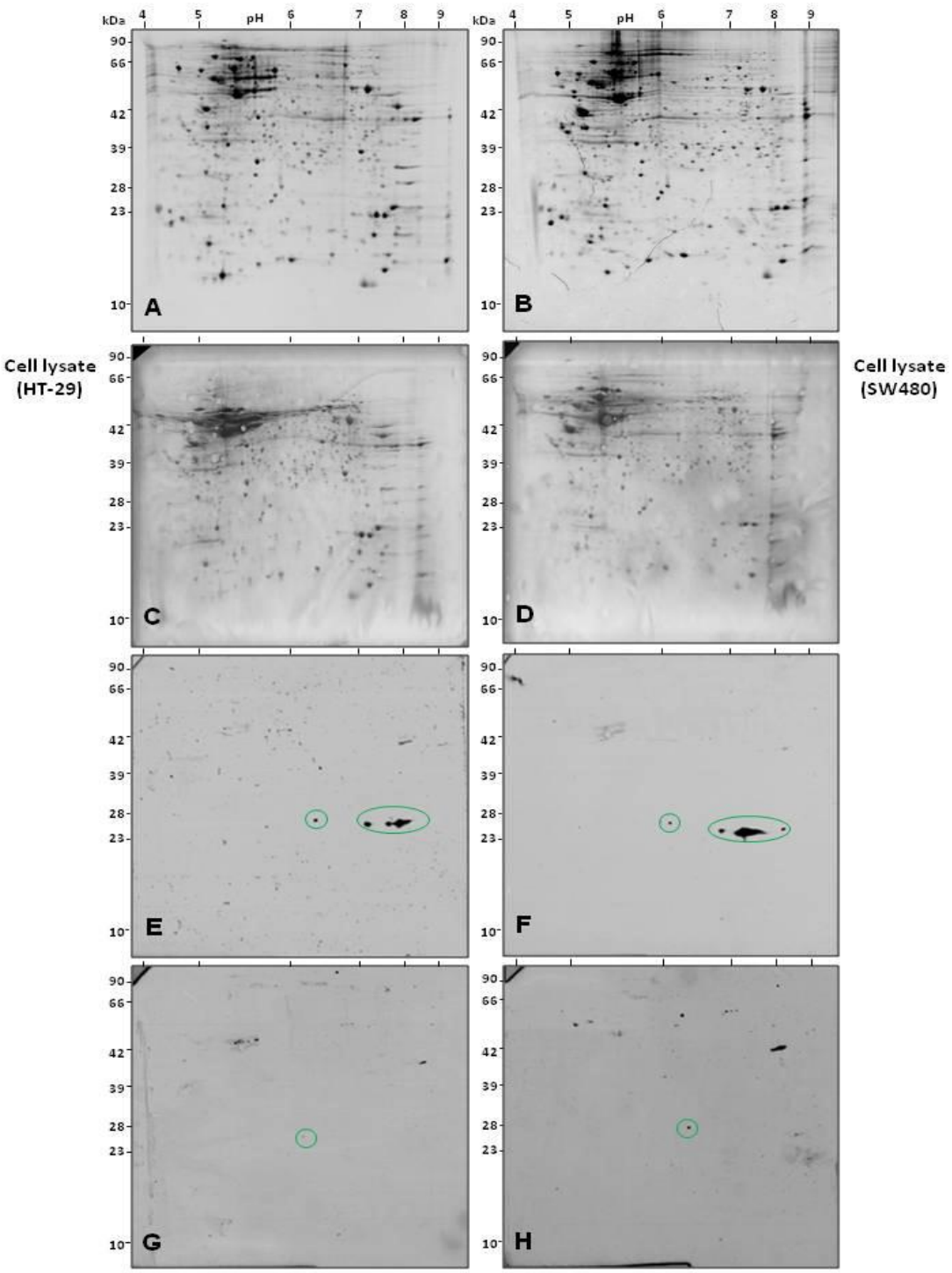


Figure 3.24: 2D Western blot of cofilin and cofilin-phospho(ser3) in colorectal cancer cell lines. A&B. Coomassie-stained gels C&D: Ponceau-stained membrane E&F: Cofilin1 blot showing string of proteins at 17kDa, PI 6.5-8.0. Ringed in green. G&H: cofilin-phospho(ser3) blot showing one spot corresponding top the most acidic form of cofilin1 with variable expression. Ringed in green.

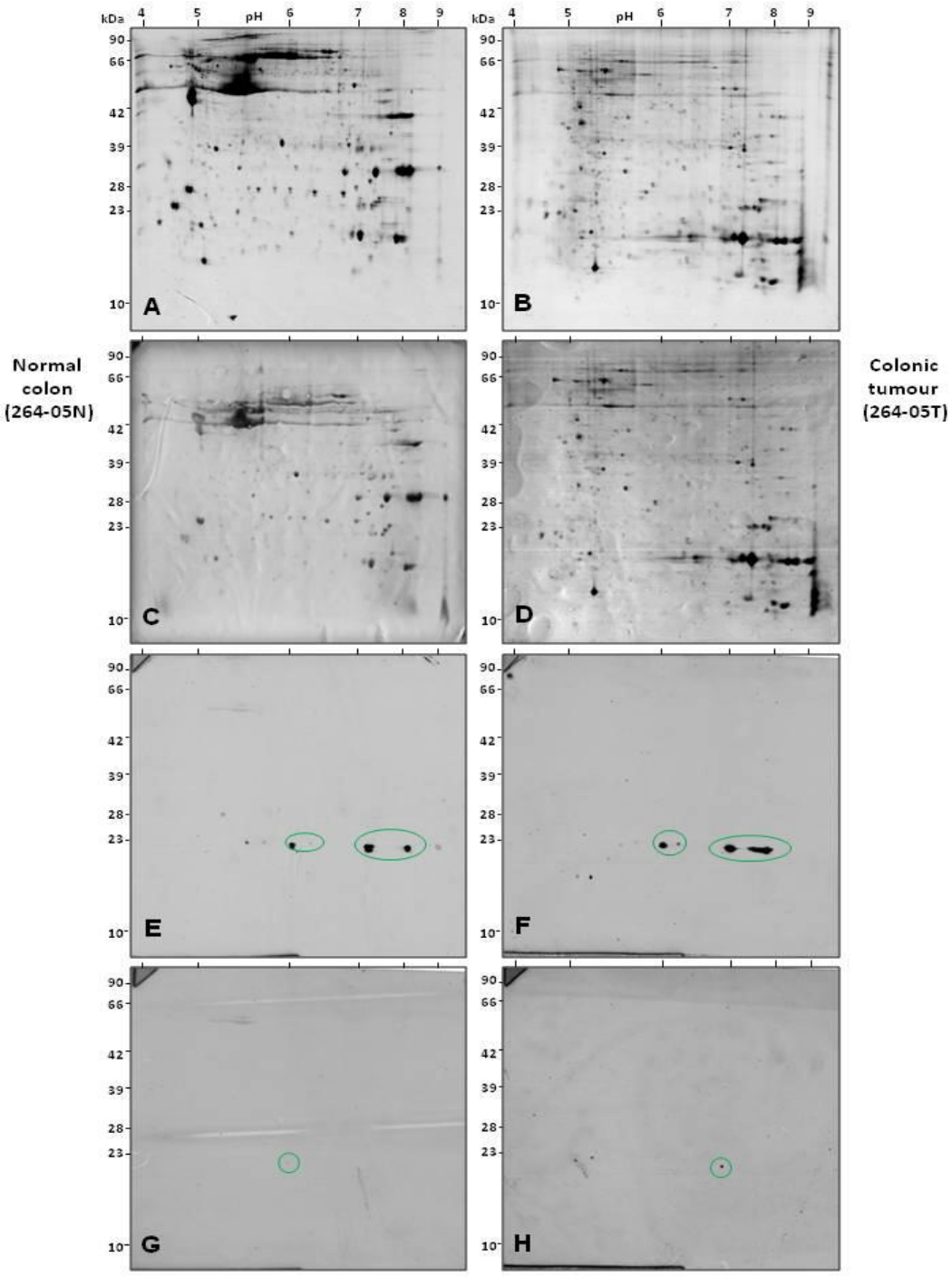


Figure 3.25: 2D Western blot of cofilin and cofilin-phospho(ser3) in frozen tumour and normal colon. A&B. Coomassie-stained gels C&D: Ponceau-stained membrane E&F: Cofilin1 blot showing string of proteins at 17kDa, PI 6.5-8.0. Ringed in green. G&H: cofilin-phospho(ser3) blot showing one spot corresponding top the most acidic form of cofilin1 with variable expression. Ringed in green.

3.7.4 DETECTION OF COFILIN1 AND IN PARAFFIN-EMBEDDED COLORECTAL TISSUE

Sections of paraffin-embedded colorectal cancer tissue were stained using the protocol outlined in methods (Section 2.1). Primary antibody was monoclonal rabbit anti-cofilin1 at a concentration 1:40,000. Staining of whole tumour and benign tissue sections was undertaken in order to compare immunohistochemistry levels with expression in laser-captured 2D gels. Initial results on whole tumour staining were promising with variable expression seen in tumour cytoplasm; no staining in tumour nucleus or membranes was identified. In benign tissue strong staining was seen in muscle; no or light staining at the base and stronger staining at the neck of normal colonic epithelial crypts, (Figure 3.26).

The second stage of immunohistochemical evaluation was staining of the colorectal cancer tissue microarray using the same antibody conditions. Unfortunately, staining of a larger number (n=313) of specimens demonstrated that the antibody utilised was not sensitive enough to detect the small differences in expression levels between the majority of tumours, images not shown. Approximately 80-90% of the arrayed specimens had scores of 2 or 3 in the cytoplasm. The array was assessed by a consultant histopathologist, Dr Bahram Azadeh who felt that the pattern of staining given by this antibody was not sensitive or specific enough to define different groups of colorectal adenocarcinomas. Further evaluation of the cofilin pathway is planned using anti-cofilin-phospho(ser3) antibody in paraffin-embedded tissue.

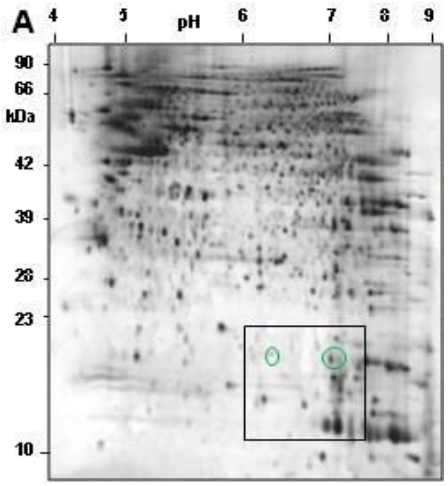
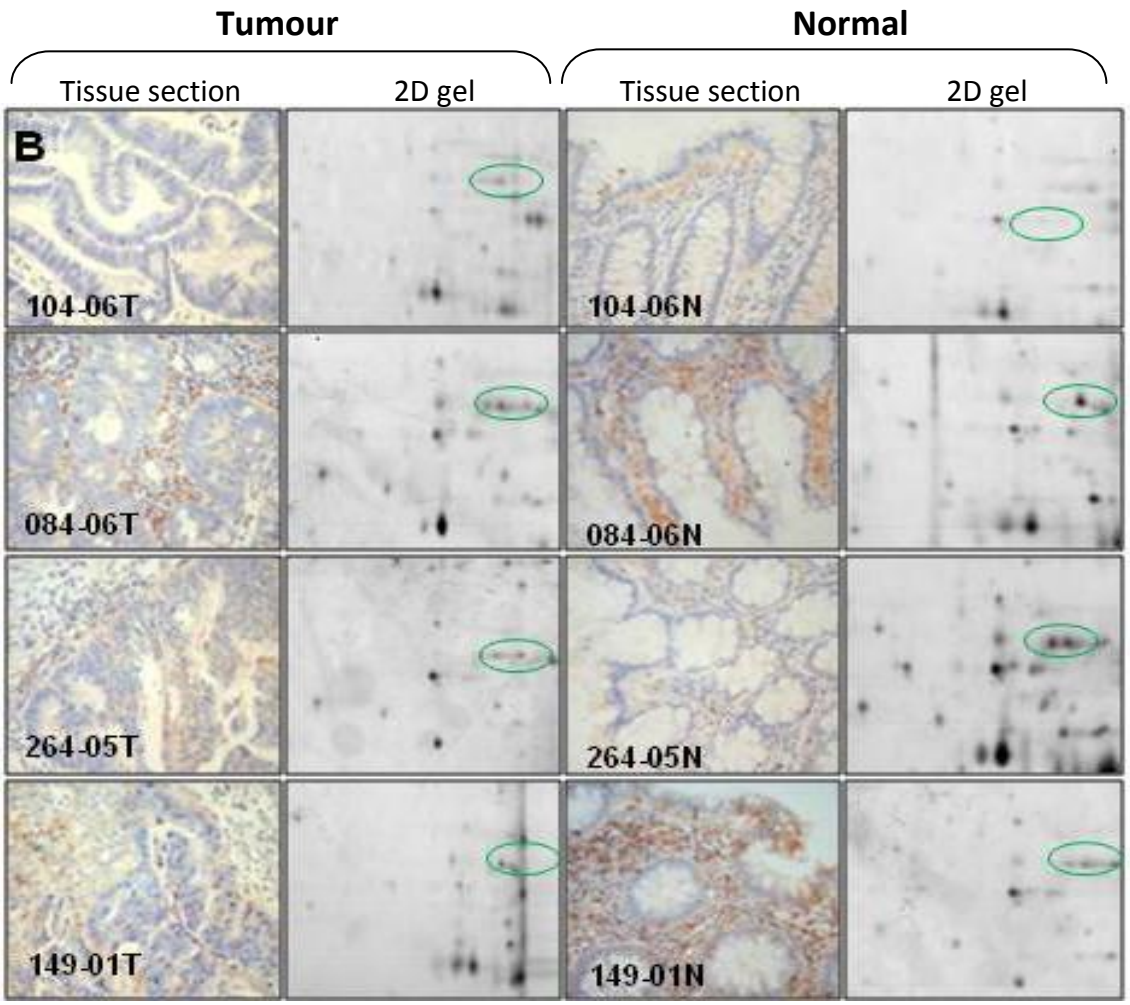


Figure 3.26A: Immunohisto-chemistry (IHC) of cofilin1 in normal colon and depressed-type tumour tissue with corresponding 2D gel images.

A: Whole tumour gel with area of gel shown in B marked with box. **B:** Immunohistochemistry for cofilin1 in normal colon and colorectal cancer tissue matched with 2D gel analysis of the same tissue (samples 104-06, 084-06, 264-05, 149-01). Good concordance demonstrated between expression of cofilin1 in tumour or epithelial cells on IHC (brown staining) and silver-staining of spots on 2D gel analysis.



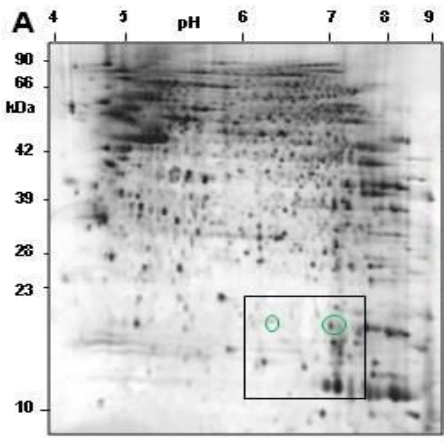
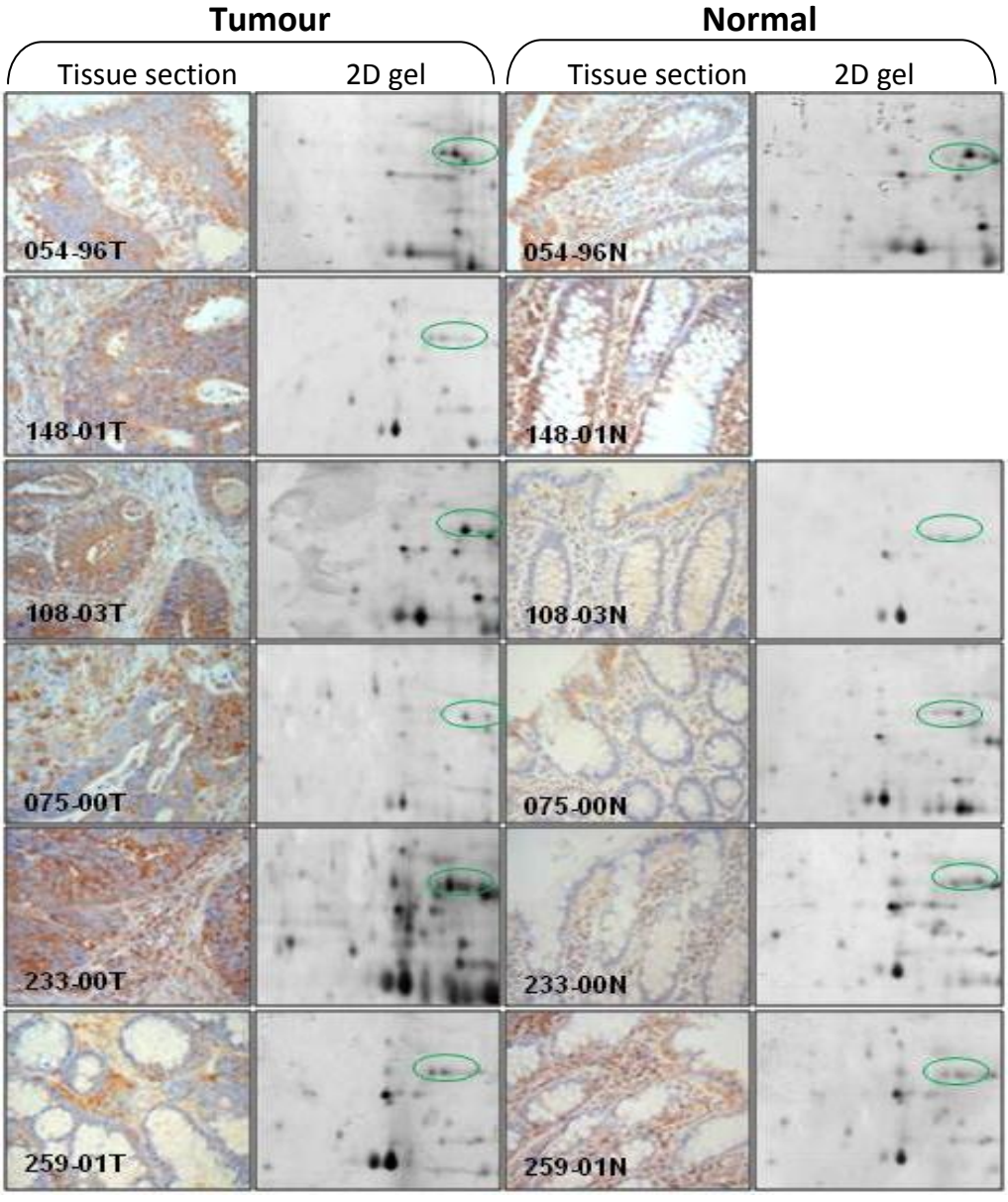


Figure 3.26B: Immunohistochemistry (IHC) of cofilin1 in normal colon and polypoid and flat-type tumour tissue with corresponding 2D gels images.

A: Whole tumour gel with area of gel shown in B marked with box. **B:** Immunohistochemistry for cofilin1 in normal colon and colorectal cancer tissue matched with 2D gel analysis of the same tissue (samples 054-96, 148-01, 108-03, 075-00, 233-00, 259-01). Good concordance demonstrated between expression of cofilin1 in tumour or epithelial cells on IHC (brown staining) and silver-staining of spots on 2D gel analysis.



3.8 S100A8

3.8.1 INTRODUCTION

The calcium-binding proteins S100A8 and S100A9 are expressed on the surface of myeloid cells and are secreted at the site of inflammation as stable heterodimeric complexes.(305, 306) Monocytes are found predominantly in the circulation, but can be recruited to areas of inflammation or tumour whereon they differentiate into macrophages.(307) The factors that promote monocyte recruitment and differentiation, and the affect this may have on the composition of the tumour-associated stroma are not yet clear. There is some evidence to suggest that certain subsets of circulating monocytes can be recruited to the tumour by hypoxia and ligand expression on the surface of cancer cells; and that they are capable of promoting angiogenesis in the stroma.(308) S100A8 and S100A9, which are secreted by infiltrating monocytes have demonstrable cytokine-like properties, acting as a chemotactic factor for other inflammatory cells.(309) More recently, both proteins have been shown to activate signalling cascades through cell-surface receptors and promote motility of various cancer cell types in vitro. This affect has been reported to occur through activation of the multi-ligand receptor RAGE, (receptor for advanced glycation end-products).(310)

Colorectal cancers show varying degrees of desmoplastic stroma response. The cellular content of the surrounding stroma principally comprises myofibroblasts, myeloid cells, lymphocytes and macrophages. The composition of the tumour-associated stroma can affect tumour growth, metastasis and patient survival.(311, 312) One type of stromal response is characterised by a strong

'cytotoxic' (CD8+) lymphocytic infiltrate around the tumour margin which is closely associated with dendritic cells and macrophages. This type of response has been linked to tumour regression, inhibition of metastases and good prognosis, and is frequently associated with MSI tumours.(313) Other forms of stromal response have been reported in which myofibroblasts and large numbers of infiltrating macrophages are associated with nodal metastases and poor prognosis.(314, 315) These observations have been partially explained by the macrophage balance hypothesis, which proposes two different macrophage populations; M1 type characterised by potent cytotoxic activity, and M2 type characterised by angiogenesis and matrix remodeling.(316) The role of the cancer cell in recruitment, differentiation and maintenance of the stroma is not yet fully understood.

In 1990, Fearon and Vogelstein proposed multistage genetic alterations in colorectal carcinogenesis which underpin the transformation of a benign adenoma to a malignant lesion.(33) The genes associated with various stages of transformation were identified as the APC (5q), the *K-ras* gene, p53 gene (17p) and a gene located on 18q21. In the 18q21 region Smad4 was one of the proposed candidate genes. Smad4 forms a cytoplasmic complex with other Smad proteins following TGF-β signalling, causing them to migrate into the nucleus initiating gene transcription. Loss of expression of the Smad4 protein in colorectal cancers occurs in 10-15% of cases overall,(317) but is reported to increase in frequency in to 30-35% in those tumours with distant or nodal metastases.(318) The largest study of prognosis for those with 18q deletions in colorectal cancer noted a reduction in long-term survival predominantly in node-negative (stage II) tumours.(46) Chromosomal instability, CIN is the most common abnormality in colorectal cancer, however approximately 15% of sporadic colorectal cancers show microsatellite instability, MSI as a result of a functional mis-match repair

defect (commonly as a result of promoter methylation of the hMSH2, hMLH1, hPMS1 or hPMS2 genes).(71) These tumours generally have a characteristic pattern of increased stromal inflammatory response and better prognosis than similar tumours displaying chromosomal instability.(233, 234)

My results in laser-captured colorectal cancer tissue suggest there is low level over-expression of S100A8 in colorectal cancer tissue compared to normal colonic epithelium in all tumours analysed, however, over-expression was more pronounced in the polypoid tumour group (Figure 3.11). Our group has previously identified the proteins S100A8 and S100A9 as abundant in tumour-associated stroma of pancreatic ductal adenocarcinoma on 2D gel analysis. Immunohistochemical validation of the findings revealed that the numbers of S100A8 and S100A9-expressing cells in the tumour stroma were related to the Smad4 status of the tumour. Comparison was made between my n=11 2D gels of laser-captured colorectal tumours and n=12 gels of undissected Duke's C colorectal cancer produced in the Division of Surgery and Oncology (work by Mr I Khattack) in which S100A8 was also identified as differentially expressed. S100A8 was more strongly and variably expressed in the n=12 gels containing tumour plus associated stroma; we postulated therefore that the majority of S100A8 was expressed in the stroma. We undertook immunohistochemical staining for S100A8, S100A9 and Smad4 using our pre-existing protocol in n=313 paraffin-embedded colorectal cancer cases in order to assess expression of these proteins in both tumour and stromal tissue.

3.8.2 DISTRIBUTION OF S100A8 AND S100A9 AT IMMUNOHISTOCHEMISTRY

As expected, normal colonic epithelium did not stain positive for either S100A8 or S100A9. There was universal expression of S100A8 in the cytoplasm of colorectal cancer cells which show low to moderate staining in all cases (intensity score 1-2). Differences in the S100A8 levels detected in laser-captured tumour tissue may have been due to the variable presence of inflammatory infiltrate within tumour glands (rather than stroma) in these cases. S100A9 was not expressed at a detectable level in cancer cells which corresponds with the pattern of protein expression seen on 2D gel analysis. Similar stroma staining was seen in both colorectal and pancreatic ductal adenocarcinomas with cells strongly positive for S100A8 and S100A9 scattered throughout the tumour stroma (Figure 3.27A-D). Co-immunofluorescence was undertaken in colorectal cancer sections to delineate the nature of these positive stromal cells. Co-immunofluorescence indicated extensive co-localization of S100A8 and S100A9 in stromal cells (Figure 3.28A). Furthermore, co-localization of S100A9 was observed in some cells with the monocyte marker CD14 (Figure 3.28B) but not the macrophage marker CD68 (Figure 3.28C).These images suggest that a population of tumour-associated monocytes co-express S100A8 and S100A9, but these proteins are not expressed in tissue-associated macrophages.

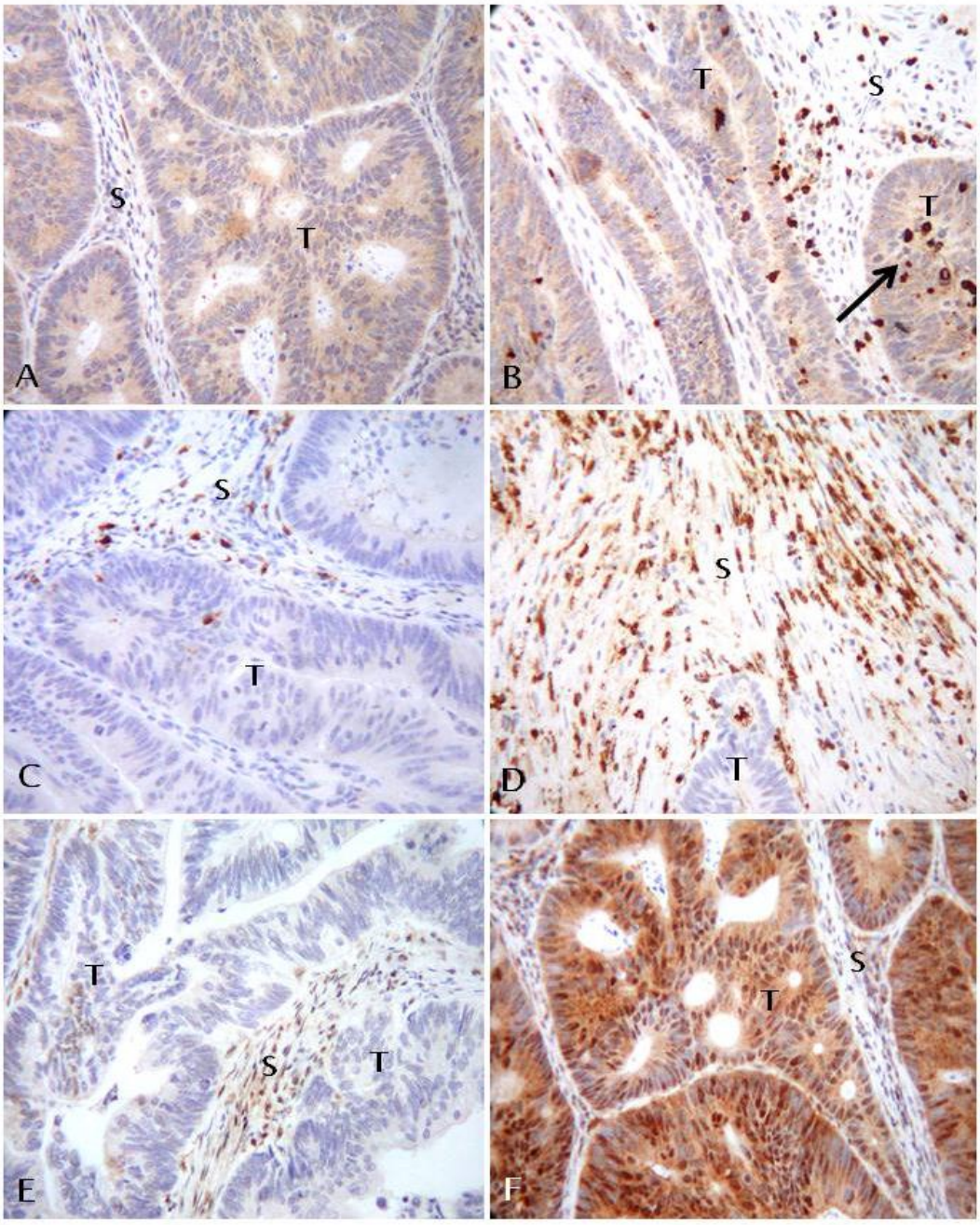


Figure 3.27: Immunohistochemistry in paraffin-embedded colorectal cancer tissue. T=tumour, S=stroma. A: Tumour stained for S100A8 showing low expression in tumour cytoplasm but no S100A8 positive stroma cells. B: Tumour showing higher numbers of S100A8-positive stromal cells in addition to several intra-tumour inflammatory cells (arrowed). C: S100A9 staining confirming no expression in tumour cells and low numbers of positive stromal cells. D: High numbers of S100A9 positive stromal cells. E: Tumour stained for Smad4 showing loss of expression. F: Tumour showing Smad4 expression in cytoplasm.

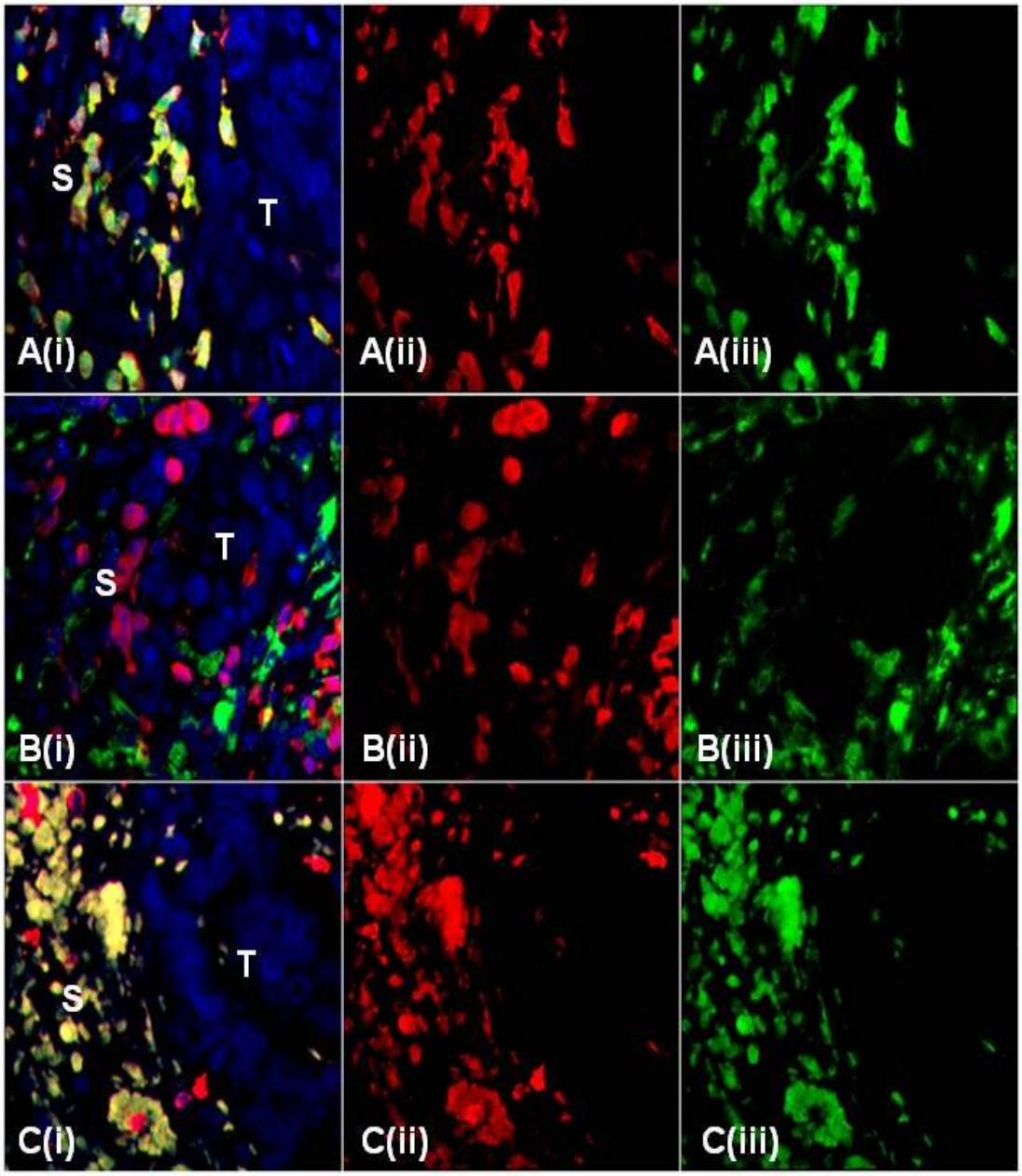


Figure 3.28 Co-immunofluorescence. S100A9 labelled with red fluorescence, DAPI used as nuclear counterstain (blue). (i) Co-localisation between red/green fluorescence plus blue nuclear counterstaining. (ii) Red fluorescence alone in same tissue section. (iii) Green fluorescence alone in same tissue section. **A(i-iii):** S100A8 labelled with green fluorescence and shows co-expression in the same S100A9-positive stromal cells. **B(i-iii):** CD68 labelled with green fluorescence showing no co-localization between CD68+ macrophages and S100A9+ cells. **C(i-iii):** CD14 labelled with green fluorescence showing co-expression of S100A9 in CD14+ monocytes.

3.8.3 QUANTIFICATION OF S100A8, S100A9 AND SMAD4 EXPRESSION

The S100A8 and S100A9 positive cells present in each tumour on the colorectal microarray were quantified by counting the number of stained cells in a field at 40x magnification. Tumour cores on the microarrays were visualized in entirety in one field at 40x magnification making the cell count per field equivalent to the cell count per core. The mean number of positively stained S100A8 and S100A9 cells was calculated by averaging the number of positive stromal cells across all the tumour cores scored for each patient. The number of S100A8-positive or S100A9-positive cells in each of 313 patient tumors (mean of at least 2 TMA cores per tumor case) was determined. Numbers ranged from 0 (4.5% of cases) to 288, and from 0 (0.65% of cases) to 882 for S100A8 and S100A9 respectively. The median number of stromal S100A8-expressing cells was 23 (IQR 6-70), while the median number of stromal S100A9-expressing cells was 65 (IQR 27-126) with 284/308 (92%) tumors showing more S100A9 than S100A8-expressing cells in the stroma. Although tumors generally contained fewer S100A8-positive than S100A9-positive cells (P<0.0001, Wilcoxon Signed Rank Test), there was a strong positive relationship between S100A8 and S100A9 counts (n=302 independent tumor cases; R^2=0.76, P<0.0001). This is entirely consistent with our observation that S100A8 co-localized with S100A9. The median S100A9/S100A8 ratio was 2.2 (IQR 1.3-4.4). To examine whether a relationship existed between the numbers of stromal S100A8-positive or S100A9-positive cells and the Smad4 tumor status, the expression of Smad4 protein was determined by immunohistochemistry (Figure 3.27E-F). Forty-two of 304 patients (14%) were categorized as Smad4-negative based on mean cytoplasmic intensity scores of ≤ 0.5. The remaining 262/304 (86%) were categorized as Smad4 positive.

3.8.4 LOSS OF SMAD4 EXPRESSION IS RELATED TO STROMAL LEVELS OF S100A8

We found that loss of Smad4 expression in the primary tumour was associated with significantly lower median counts of S100A8-positive monocytes in the stroma; 14 (IQR 5-37) in Smad4-negative group compared to 25 (IQR 6-76) in the Smad4-positive group, *P*=0.036, Mann-Whitney U Test, (figure 3.29C). A Receiver Operating Characteristic, ROC curve was constructed to establish whether stromal S100A8 counts were predictive of Smad4 expression in the tumour, and was found to be conclusive with a threshold of <42 S100A8+ cells, (Area Under the Curve, AUC=0.602, *P*=0.024), figure 3.29D. There was no difference in the S100A9-positive count between Smad4 negative and positive tumours, 48 (IQR 23-96) versus 65 (IQR 27-129), *P*=0.266, Mann-Whitney U Test, figure 3.29E. The ROC curve constructed to establish whether stromal S100A9 counts were predictive of Smad4 expression in the tumour showed no significant relationship between the two factors, (AUC=0.554, *P*=0.250), figure 3.29F. The S100A8 and S100A9-positive cell counts were divided into two groups; S100A8 was divided by the predicted threshold for prediction of Smad4 status (<42 cells versus ≥42 cells) and S100A9 was divided by the median count (≤65 cells versus >65 cells) as the ROC curve did not predict a significant threshold point. Low numbers of S100A8-positive cells, but not S100A9-positive cells are linked to loss of Smad4 expression in the primary tumour, *P*=0.015, Fisher's exact test, (Table 3.8).

SMAD4 staining	**Cases with S100A8 scoring, n=300 (%)**	**No of S100A8 +ve cells**		**p-value**	**Cases with S100A9 scoring, n=298 (%)**	**No of S100A9 +ve cells**		**p-value**
		Low <42 (%), n=190	High ≥42 (%), n=110			Low <52 (%), n=127	High ≥52 (%), n=171	
Negative , n=42	41 (14)	33 (17)	8 (7)	**0.015**	42 (14)	23 (18)	19 (11)	0.094
Positive, n=262	259 (86)	157 (83)	102 (93)		256 (86)	104 (82)	152 (89)	

Table 3.8: Correlation between Smad4 expression in the tumour and S100A8 and S100A9+ cells in the stroma

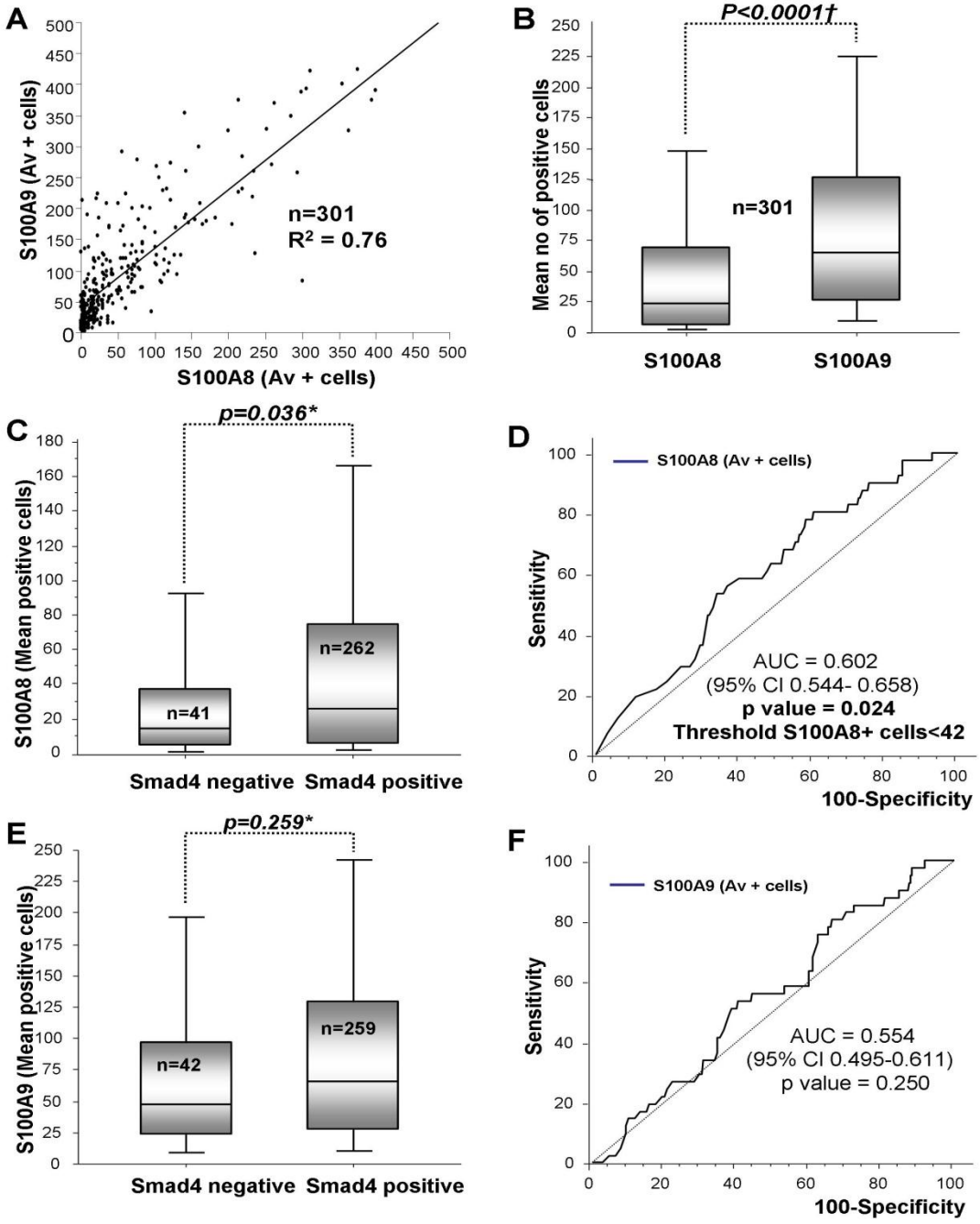


Figure 3.29: correlation between stromal S100A8 and S100A9 and Smad4 in the tumour. A: Correlation between number of stromal S100A8 and S100A9+ cells in the same tumour, B: comparison of S100A8 and S100A9+ stromal cells, C: numbers of S100A8+ cells in relation to Smad4 status of tumour, D: Roc curves to predict Smad4 expression using S100A8+ cell counts, E: numbers of S100A9+ cells in relation to Smad4 status of tumour, D: Roc curves to predict Smad4 expression using S100A9+ cell counts

3.8.5 RELATIONSHIP OF SMAD4 TUMOUR EXPRESSION AND S100A8 AND S100A9 STROMAL EXPRESSION WITH CLINICOPATHOLOGICAL PARAMETERS

We aimed to establish whether a relationship existed between the various tumour and stromal proteins identified on the tissue microarray and the clinical and histological parameters of those tumours, Table 3.9. Patients were categorized as having mean S100A8-positive cell counts that were low (≤median of 23), n=156 or high (>23), n=153. Similarly, for S100A9, patients were categorized as having low (≤median of 65), n=159 or high (>65), n=147 cell counts. Smad4 expression was categorised as negative (≤0.5 mean score, n=42) and positive (>0.5 mean score, n=262). No correlation was demonstrated between degree of stromal S100A8 and S100A9 infiltration with age at surgery, gender, site of tumour, depth of tumour invasion or nodal metastases (Table 3.9). However, high cell S100A8 and S100A9 counts were associated with larger tumour size (*P*=0.01 and 0.0006 respectively; chi-squared test). High S100A9 counts were associated with poor differentiation grade, *P*=0.036, (Table 3.9, chi-squared test). Smad4-negative tumours were significantly associated with greater depth of tumour infiltration than Smad4 positive tumours; *P*=0.022, (Table 3.10, Chi-squared test). Loss of Smad4 expression was more frequent in rectal tumours compared to colonic tumours, 55% versus 37%, *P*=0.027, (Table 3.10, Chi-squared test). No relationship between Smad4 expression and differentiation grade, size of tumour or presence of nodal metastases was established in our patient cohort (Table 3.10).

Clinicopathological parameter	All cases, n=313 (%)	S100A8 +ve cells, n=309		*p* value*	S100A9 +ve cells, n=306		*p* value*
		Low ≤23 cells (%), n=156	High >23 cells (%), n=153		Low ≤65 cells (%), n=159	High >65 cells (%), n=147	
Age 70 (IQR 62-76 years)							
Young (<median)	155 (49)	75 (48)	80 (52)	0.459	75 (47)	78 (53)	0.303
Old (> median)	158 (51)	81 (52)	73 (48)		84 (53)	69 (47)	
Gender							
Male	188 (60)	93 (60)	92 (60)	0.926	97 (61)	85 (58)	0.570
Female	125 (40)	63 (40)	61 (40)		62 (39)	62 (42)	
Site of tumour							
Colon	188 (60)	89 (57)	96 (63)	0.307	94 (59)	90 (61)	0.707
Rectum	125 (40)	67 (43)	57 (47)		65 (41)	57 (39)	
Size 50 (IQR 38-60mm)							
Small-Medium (<60mm)	214 (69)	116 (74)	94 (61)	**0.014**	123 (77)	87 (59)	**0.0006**
Large (≥60mm)	99 (31)	40 (26)	59 (39)		36 (23)	60 (41)	
Differentiation grade							
Well	5 (2)	2 (1)	2 (1)	0.819	2 (1)	3 (2)	**0.036**
Moderate	283 (90)	140 (90)	140 (92)		150 (94)	127 (86)	
Poor	21 (7)	12 (8)	9 (6)		5 (4)	15 (11)	
Uncategorised	4 (1)	2 (1)	2 (1)		2 (1)	2 (1)	
Excision Margin							
Clear	270 (86)	130 (83)	138 (90)	0.055	134 (84)	130 (88)	0.199
Involved	39 (13)	25 (16)	12 (8)		24 (15)	14 (10)	
Uncategorised	4 (1)	1 (1)	3 (2)		1 (1)	3 (2)	
T-stage							
T1	13 (4)	8 (5)	5 (3)	0.766	8 (5)	5 (3)	0.356
T2	47 (15)	22 (14)	25 (16)		21 (13)	24 (16)	
T3	206 (66)	104 (67)	101 (66)		110 (69)	92 (63)	
T4	44 (14)	19 (12)	22 (15)		18 (12)	25 (17)	
Uncategorised	3 (1)	3 (2)	0		2 (1)	1 (1)	
N-stage							
N0	177 (56)	82 (53)	93 (61)	0.446	90 (57)	82 (56)	0.332
N1	71 (23)	38 (24)	32 (21)		40 (25)	30 (20)	
N2	62 (20)	33 (21)	28 (18)		27 (17)	34 (23)	
Uncategorised	3 (1)	3 (2)	0		2 (1)	1 (1)	

Table 3.9: Clinicopathological characteristics and correlation with S100A8+ and S100A9+ cell infiltrates in the stroma. S100A8 and S100A9 cell counts were dichotomized by median counts and analysed according to clinical and pathological variables. Statistical analysis was performed using chi-squared test.

Clinicopathological parameter	All cases, n = 303 (%)	SMAD4 status		*p* value
		Negative (%), n=42	Positive (%), n=261	
Age (median 70, ranged 33-93)				
Young (<median)	148 (49)	23 (55)	125 (48)	0.409
Old (≥ median)	155 (51)	19 (45)	136 (52)	
Gender				
Male	183 (60)	26 (62)	157 (60)	0.829
Female	120 (40)	16 (38)	104 (40)	
Site of tumor				
Colon	184 (61)	19 (45)	165 (63)	**0.027**
Rectum	119 (39)	23 (55)	96 (37)	
Size 50 (IQR 38-60mm)				
Small (<38mm)	72 (24)	9 (21)	63 (24)	0.863
Medium (38-59mm)	133 (44)	20 (48)	113 (43)	
Large (≥60mm)	98 (32)	13 (31)	85 (33)	
Differentiation grade				
Well	5 (2)	1 (3)	4 (1)	0.848
Moderate	273 (90)	38 (90)	235 (90)	
Poor	21 (7)	3 (7)	18 (8)	
Uncategorized	4 (1)	0	4 (1)	
Excision Margin				
Clear	262 (87)	36 (86)	226 (86)	0.848
Involved	37 (12)	5 (12)	32 (13)	
Uncategorized	4 (1)	1 (2)	3 (1)	
pT				
T1	13 (4)	1 (2)	12 (5)	**0.022**
T2	43 (14)	4 (10)	39 (15)	
T3	203 (67)	25 (59)	178 (68)	
T4	41 (14)	12 (29)	29 (11)	
Uncategorized	3 (1)	0	3 (1)	
pN				
N0	171 (56)	22 (52)	149 (57)	0.547
N1	70 (23)	13 (31)	57 (22)	
N2	59 (20)	7 (17)	52 (20)	
Uncategorized	3 (1)	0	3 (1)	
Stage (AJCC)				
I	37 (12)	4 (9.5)	33 (13)	0.697
II	133 (44)	18 (42.9)	115 (44)	
III	128 (42)	20 (47.6)	108 (41)	
Uncategorized	5 (2)	0	5 (2)	

Table 3.10: Association between Smad4 status and clinicopathological parameters. Tumours were dichotomized by Smad4 expression into negative and positive tumours and compared to clinical and pathological tumour features. Chi-squared test used for statistical analysis.

3.8.6 ASSOCIATION BETWEEN NUMBERS OF STROMAL S100A8 AND S100A9-POSITIVE CELLS AND CANCER-SPECIFIC SURVIVAL

Following the confirmation of an association between stromal S100A8 and S100A9 and tumour size, we endeavored to determine whether a relationship existed between these proteins and overall patient survival. Kaplan-Meier curves were constructed of overall 96-month survival for n=302 patients according to stromal S100A8 expression and n=299 according to stromal S100A9 expression. For the purpose of analysis, patients were catagorised as having high or low S100A8 and S100A9 counts based on the median cell count (≤23 versus >23 cells S100A8, ≤65 versus >65 cells S100A9). No survival difference was observed for either protein (Figure 3.30A(i/ii). On analysis of patients with loss of Smad4 expression in the primary tumour (n=38), high S100A8 counts were associated with poorer survival at 36 months, (76% versus 39%, p=0.02 Mantel-Cox), but not at 96 months (*P*=0.29, Mantel-Cox), figure 3.30B(i). A similar pattern was seen for S100A9 counts, high S100A9 in the stroma corresponded with poor early survival (73% versus 41% *P*=0.02, Mantel-Cox) but no difference was seen in the long term (*P*=0.36, Mantel-Cox), Figure 3.30B(ii). In patients with Smad4 positive tumours, neither S100A8 nor S100A9 counts in the stroma appeared to predict overall short, or long-term survival, (*P*=0.37 and *P*=0.80 respectively), Figure 3.30C(i/ii).

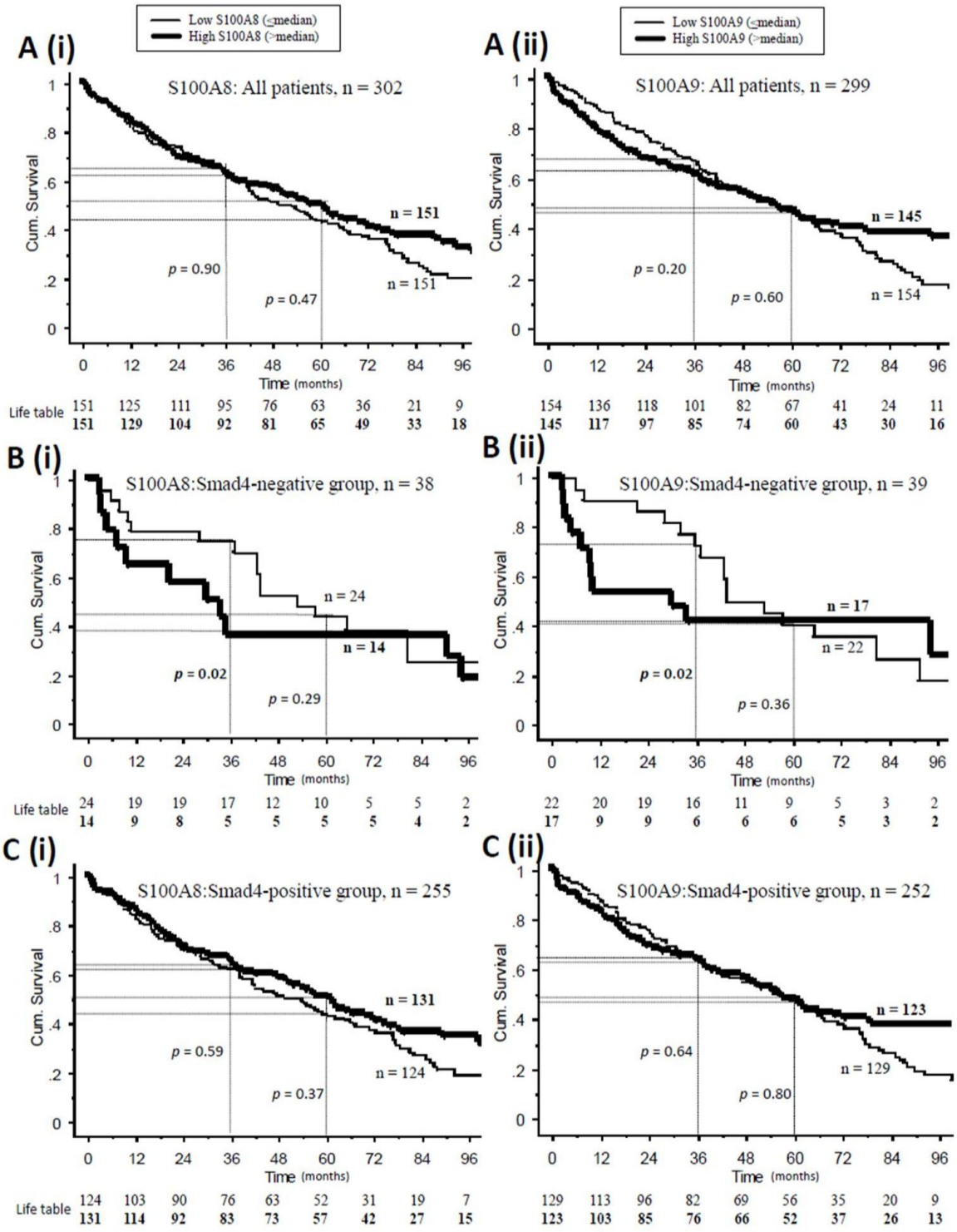


Figure 3.30: Cancer specific survival according to stromal S100A8 and S100A9+ cells. Kaplan-Meier curves with p-values (Mantel-Cox). A: Survival according to stromal S100A8+ cells (i) and S100A9+ cells (ii). B: Smad4 negative tumours; survival according to S1008+ cells (i) and S100A9+ cells (ii), C: Smad4 positive tumours; survival according to S1008+ cells (i) and S100A9+ cells (ii)

3.8.7 LOSS OF SMAD4 EXPRESSION PREDICTS FOR POOR SURVIVAL IN SOME PATIENT GROUPS

Loss of Smad4 expression has been mooted as a poor prognostic marker in colorectal cancer, we planned to establish whether this was the case in our cohort of patients. Kaplan-Meier curves were constructed to assess the impact of Smad4 on overall survival in colorectal cancer. Survival analyses were performed on 259 patients after excluding 37 patients with non-curative tumor resections (positive tumor resection margins) and 7 patients who died within 30 days of surgery. Patients with node-negative tumors had significantly better survival compared to those with node-positive tumors (P<0.0001, Figure 3.31A). Smad4 loss was not associated with survival (P=0.09) when the entire cohort of 259 patients was examined (Figure 3.31B). However, when the node-negative (Stage I and Stage II) and node-positive (Stage III) cancer patients were analyzed separately, it was observed that patients in the node-negative cancer group who had lost Smad4 expression had poorer survival compared to patients in that group who had retained Smad4 expression (n=156; P=0.019, Figure 3.31C). In fact, the survival of patients with node-negative tumors who had lost Smad4 expression was similar to that of patients with nodal metastases (P=0.542, Figure 3.31D). There was no association between Smad4 expression and survival in the node-positive cancer group (n=103; P=0.925, Figure 3.31E).

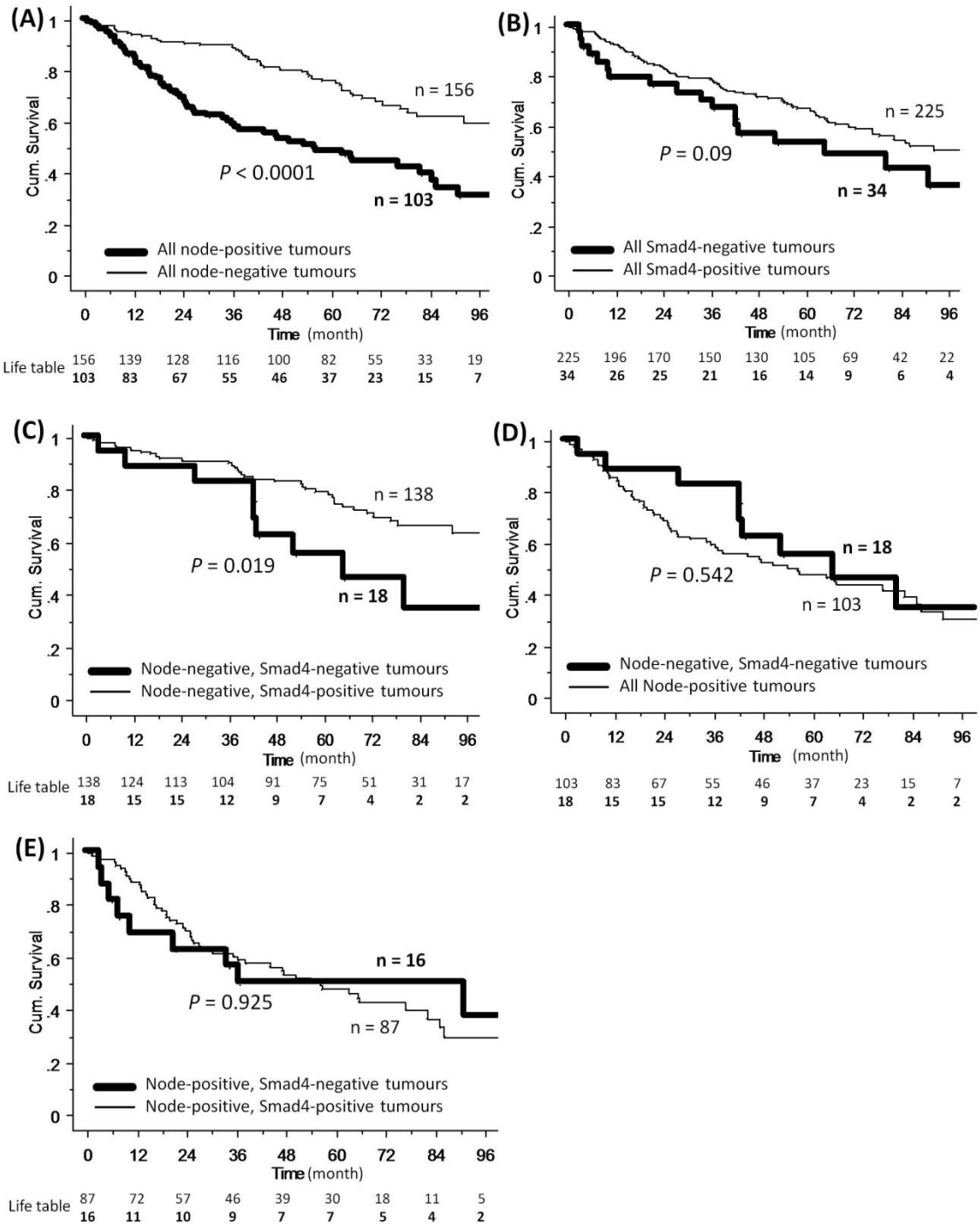


Figure 3.31: Association between Smad4 status and cancer-specific survival. Kaplan-Meier curves with p-values (Mantel-Cox). A: Nodal status, B: Smad4 expression, C: N0 tumours; survival according to Smad4 status, D: N+ tumours; survival according to Smad4 status, E: N0, Smad4 negative tumors versus all N+ tumours

4. DISCUSSION

The key findings of this MD are summarized as follows:

1. Laser-capture microdissection of frozen colorectal tissue was successfully performed and sufficient material generated to produce 19 good quality (10 tumour and 9 normal epithelium), silver-stained 2D gels using SDS-PAGE.

2. 2D gels were analysed for variations in protein spot pattern between tumours of different morphologies; depressed-type, flat-type and polypoid-type. Six proteins were successfully identified using in-gel trypsin digestion and tandem mass spectrometry; these were redox proteins peroxiredoxin 2, peroxiredoxin 6 and SH3 binding glutamic acid-rich protein-like 3; and cytoskeletal protein cofilin1. Also identified were the anti-apoptotic protein heat shock protein 27 and inflammatory protein S100A8, which had been previously identified in 2D gel analysis of undissected colorectal cancer in our Institution (n=12 gels).
3. Heat shock protein 27 expression was evaluated in a large cohort of n=404 paraffin-embedded colorectal cancer tissue, including n=98 samples previously validated in a small cohort of paraffin-embedded colorectal cancers in our Institution. High HSP27 levels were strongly associated with poor cancer-specific survival in rectal cancer (n=205, *P*=0.0063) but not colon cancer; (n=199, *P*=0.7385). Multivariate Cox regression confirmed nodal metastases (*P*=0.0001) and HSP27 expression (*P*=0.0233) as independent markers of survival in rectal cancer.

4. S100A8 and related proteins S100A9 and Smad4 were similarly evaluated in a large cohort (n=313). S100A8 expression co-localised with a subset of S100A9-positive monocytes. S100A9 was co-expressed with CD14 in tumour-associated monocytes, but not with CD68 in tissue macrophages. Smad4 was expressed in the tumour cytoplasm of 262/304 (14%) tumours. Loss of Smad4 expression correlated with a reduction in the stromal S100A8-positive, but not S100A9-positive cell count, (*P*=0.034, Mann-Whitney U test) and was associated with a poorer overall survival in patients with stage I-II disease, but not stage III disease.
5. Antibodies to cofilin1 and cofilin-phospho(ser3) were assessed in colorectal cancer cell and tissue lysate and found to be specific on 1D and 2D western blot. Cofilin antibody did not show sufficient variability of staining when used for immunohistochemistry in colorectal cancer tissue to be useful for accurately assessing and scoring expression of the cofilin protein.

Further discussion of these findings is detailed in the following sections.

4.1 PROTEINS IDENTIFIED USING LASER CAPTURE AND 2D GEL ANALYSIS

Laser-capture microdissection and 2D gel electrophoresis for proteomic analysis was first described in 1999.(319) Numerous groups have successfully combined laser capture with 2D SDS-PAGE,(239, 320-324) DNA analysis,(325-329) and mRNA analysis.(330, 331) The advantage of this methodology is that it allows specific selection of cell populations from human colorectal cancers in situ, without resorting to cell line culture. Normal colonic epithelium, not amenable to culture as a cell line, can be also analysed using this technique.(188, 332) Our results confirm that laser capture

microdissection and 2D gel proteomic analysis is feasible in frozen colorectal adenocarcinoma and benign colonic epithelium. The gels produced are readily amenable to interpretation visually and electronically to highlight differentially-expressed protein spots, and in this study the technique led to the identification of proteins that appear to be variably expressed both between different morphologies of colorectal cancer and between cancer tissue and benign epithelial tissue. A recent meta-analysis of proteomic analysis in colorectal cancer tissue found 98 proteins reported as variably regulated in more than one study. They included high abundance proteins (serum albumin, HLA class I and kerritin type II cytoskeletal 8); cytoskeletal proteins (annexin A5, A4, A3 and A1, tropomyosin beta chain, actin, coronin 1C, ezrin, vimentin and gelsolin); inflammatory proteins (S100A9, macrophage capping protein, leukocyte elastase inhibitor); redox proteins (peroxiredoxin1, glyceraldehydes-3-phosphate dehydrogenase, glutathione s-transferase P, thiosulfate sulfurtransferase); cellular stress proteins (heat shock protein beta1, heat shock protein 75, endoplasmin, heat shock protein 90); synthesis/degradation proteins (elongation factor 2, apolipoprotein A1, UDP-glucose dehydrogenase, serpin B6, eukaryotic initiation factor 4A); cell to cell interaction proteins (thrombospondin 1, lamin-B1).(332)

4.2 HEAT SHOCK PROTEIN 27

We report the identification of the low molecular weight heat shock protein, HSP27 as differentially expressed in colorectal cancer using proteomic techniques. It is interesting to note that HSP27 has been reported as over-expressed in approximately 30% of 2DE proteomic studies.(333) It is

consistently identified in investigative proteomic studies of colorectal cancer, both tissue and cell lines.(334, 335) Despite promising initial reports, this study was the first to undertake validation of the expression of this protein on a large scale in adenocarcinoma of the colon and rectum.

Tissue microarrays are a powerful tool for high-throughput evaluation of putative biomarkers, although concerns have been raised that lack of tumour heterogeneity could cause misleading results. Several studies have addressed this issue and reported the concordance between TMA and tumour sections when assessing one core is in the region of 80% and this increases to 95% when two cores are included.(336) Our TMAs were constructed using 2-6 cores per tumour and care was taken to include benign liver, kidney and colon tissue as internal control. Immunohistochemistry detection of HSP27 has been previously reported in formalin-fixed colorectal cancer using a variety of antibodies and scoring protocols. The findings have thus far been inconclusive; one group reported a strong relationship between HSP27 expression in the primary tumour and nodal status in n=40 patients,(334) whilst a second group found no such association in n=68 patients.(337) We devised a reproducible method of grading intensity of HSP27 staining by direct comparison to internal TMA control tissue (colon, liver and kidney); in addition to scoring the percentage expression in each core. In our cohort of n=404 patients we observed no correlation between any aspect of HSP27 staining in the primary tumour, (intensity, percentage or combined score) with histopathological features including nodal status. As we proceeded to analyse the association between aspects of HSP27 expression and overall survival we found the intensity rather than percentage staining to be prognostic, however an index which combined both the intensity and percentage scores was most strongly associated with survival.

We report the novel finding that high HSP27 expression is an independent marker of poor prognosis in patients with rectal cancer, but not colon cancer. This adds to accumulating evidence that rectal and colon tumours are distinct biologically entities and have a different pattern of genetic mutations.(338) The link between HSP27 expression and survival has been investigated in a multitude of human cancers.(271) Poor overall- and cancer-specific survival has been linked to elevated HSP27 expression in prostate cancer; it also correlates with various histological parameters including Gleason score and lymph node metastases.(244, 339, 340) Despite initial promising reports in breast cancer,(341) larger studies of node-positive and negative patients failed to demonstrate a difference in overall or cancer specific survival according to HSP27 expression.(342, 343) Numerous other small studies have identified high HSP27 as poorly-prognostic in ovarian cancer,(344) gastric cancer(345) and hepatocellular cancer.(346) We observed elevated HSP27 as predictive of lower five-year cancer-specific survival across all stages of rectal cancer, however there was a stronger association with stage III compared to stage I/II disease. However, utilising elevated HSP27 as a biomarker, we defined a poor prognostic group of stage II patients with a similar survival to that of stage III patients. This may be an area in which further work could aid clinical decision making in the management of this patient group. HSP27 expression was independently associated with survival in rectal cancer patients on multivariate analysis. We were interested to note that age and resection margin status, approached, but did not reach significance which was contrary to our expectations. We felt this reflects the lack of standardised pathological reporting of rectal cancer in our institution. The introduction of standard surgery in the form of total mesorectal excision (TME) and guidelines on the minimum pathological dataset were introduced in 1999.

We report the novel findings that radiotherapy treatment does not alter the expression of HSP27 between diagnosis and surgical resection of a rectal tumour. Variation, both up and down regulation, was observed in 19% of diagnostic biopsies compared to the primary tumour. This may reflect the small, superficial nature of some of the biopsy material or could be indicative of tumour biology. Our findings concord with the only published prospective study of HSP27 expression in relation to radiotherapy treatment of rectal cancer, which reported no consistent change in HSP27 expression identified after radiotherapy was administered.(347) We conclude that there is no evidence to suggest that radiotherapy treatment induces HSP27 expression in rectal cancer.

The mechanism by which HSP27 exerts its effects on survival in cancer is not fully understood. HSP27 may confer a gain of function for the tumour cell in terms of motility and invasion or increase tumour cell survival by inhibition of apoptosis and resistance to anti-cancer treatments. There is emerging *in vitro* evidence that HSP27 can promote motility and increase cancer cell invasion, in part by promoting secretion of metalloproteinase MMP2.(348) This is mediated by the TGFβ/p38 mitogen-activated protein kinase pathway, implying a role for the phosphorylated isoform of HSP27 in this phenomenon.(349) HSP27 has been associated with inhibition of NF kappaB in hepatocellular carcinoma cells. Knockdown of HSP27 in these cells resulted in increased concentrations of NF kappaB in the nucleus, suppressed cell migration and invasion and induced apoptosis.(350) Reducing the expression of HSP27 by siRNA interference has demonstrated an increase in apoptosis and cell death via capase-3 in prostate cancer cell lines.(351) A similar study conducted using human bladder cancer cells in murine models demonstrated that HSP27 knockdown resulted in reduced tumour growth and enhanced sensitivity to chemotherapy.(352)

The role of HSP27 in chemoresistance has been intensely studied and the protein has been implicated in 5-fluorouracil and irinotecan resistance in colorectal cancer(353-355) and more recently, pancreatic cancer.(321) Of particular interest in rectal cancer, as with prostate and bladder cancer is the role that HSP27 plays in resistance to radiotherapy; indeed there is *in vitro* evidence that HSP27 confers resistance to gamma-radiation in human cancer cell lines.(356) Combined treatment using anti-sense oligonucleotides against HSP27 and radiotherapy in mice with SQ20B radioresistant head and neck squamous cell carcinomas resulted in tumour shrinkage, reduced angiogenesis and increased survival.(357) Further work is required to establish a link between HSP27 expression, resistance to radiotherapy treatment and prognosis in rectal adenocarcinoma.

To conclude, elevated HSP27 is an independent marker of poor prognosis in rectal cancer. Expression of the protein is not altered by neo-adjuvant radiotherapy and further work is necessary to determine whether HSP27 has a role to play in resistance to adjuvant radiotherapy and chemotherapy *in vivo.*

4.3 COFILIN1

In this study an isoform of cofilin1 was identified as potentially differentially expressed on 2D gel analysis of laser-captured frozen colorectal cancer tissue and benign colonic epithelium. The colorectal cancers formed three distinct morphological groups, flat, depressed and polypoid types.

The protein spot identified appeared to be maximally expressed in polypoid tumours, with correspondingly lower levels in the matched benign tissue. In the flat and depressed tumours the level of the protein was lower than polypoid tumours, with correspondingly higher expression in the matched benign tissue. The small numbers of tumours utilised in this stage of the analysis led us to investigate the protein expression further using colorectal cancer cell lines and tissue. We undertook preliminary investigations of two commercially available antibodies to cofilin1 and its physiologically inactive isoform cofilin-phospho(ser3) in protein lysate and paraffin-embedded tissue in order to confirm the identity of the protein spot and assess specificity prior to validation. Using 1D western blotting we established both anti-cofilin1 and anti-cofilin-phospho(ser3) antibodies to be specific for a protein band of the appropriate molecular weight in colorectal cancer cell lysate and whole cancer tissue lysate. Cofilin-phospho(ser3) was variably expressed whereas cofilin1 had a similar expression levels between cell lines. 2D western blot analysis in whole tumour lysate and colorectal cancer cell lysate confirmed the protein spot of interest to be cofilin-phospho(ser3).

Immunohistochemistry for cofilin1 was performed using the same antibody in paraffin-embedded benign and malignant colorectal tissue to assess distribution of staining. We found cofilin1 to be expressed strongly in smooth muscle and endothelium in addition to normal colonic epithelium (particularly cells at the neck of crypts). Variation could be detected in the level of staining between tumours after staining of n=10 tumour sections and n=9 benign sections from the same tumour material subject to 2D analysis. This is consistent with data from the only paper reporting cofilin1 immunohistochemistry in tumour tissue which found variable expression in n=34 gastrointestinal

endocrine tumours.(304) Unfortunately, on increasing the numbers of colorectal adenocarcinomas stained to n=313, the results showed poor consistency of staining between cores from the same tumour and insufficient variation in staining between tumours for cofilin1 to be of value as a prognostic marker. Further cofilin1 staining was therefore abandoned in favour of the more variably-expressed cofilin-phospho(ser3), although evaluation is not yet complete. Accurate assessment of phosphorylated proteins is notoriously difficult due to 1. Lack of antibody affinity and 2. Degradation of phosphorylated proteins during tissue fixation. One paper reported that levels of phospho-proteins were unreliable in formalin-fixed tissue specimens larger than $2cm^3$ due to time-dependent de-phosphorylation of proteins following devascularization.(358, 359) Assessment of a large number of tumours is vital in order to obtain reproducible results.

4.4 S100A8

We have previously demonstrated that the calcium-binding proteins S100A8 and S100A9 are expressed in tumour-associated monocytes in pancreatic cancer stroma in differing quantities.(59) A number of other studies have used similar proteomic techniques to detect the differential expression of the S100 group of proteins in cancer material, including pancreas,(59, 360) breast,(361) prostate,(362) bladder (363) and colorectal.(364) In this study S100A8 was found expressed at higher levels in colorectal cancer tissue compared to benign epithelial tissue on 2DE gel analysis. Verification of our findings using immunohistochemistry demonstrated low-level expression of S100A8, but not S100A9 in all colorectal cancer tissue. The tumour stroma was found

to contain variable numbers of S100A8 and S100A9-positive infiltrating cells. Co-immunofluorescence indicated that S100A8 is expressed in a subset of S100A9 positive cells which is consistent with our finding that tumours contained greater numbers of S100A9- than S100A8-positive cells. S100A8 and S100A9 are reported to be expressed in circulating granulocytes and pro-inflammatory monocytes but not in tissue resident macrophages.(365, 366) They promote phagocyte migration by enhancing the polymerisation of microtubules and S100A8/S100A9 heterodimers (also known as calprotectin) are believed to recruit further monocytes to sites of inflammation. The expression and secretion of these proteins in the tumour microenvironment is likely to contribute to the host inflammatory response to the tumour.(367) Given that S100A9 positive cells did not co-express CD68, a marker of mature macrophages, but did co-localise with CD14, a monocyte marker, the cells we have observed are likely to represent tumour-associated monocytes or immature macrophages, which are characterised by a lack of or low expression of differentiation-associated macrophage antigens (reviewed in Lewis *et al.*(316)).

There has been a great deal of work on the influence of tumour-associated macrophages, (TAMs) in the stroma of colorectal cancer patients. Evidence suggests there is a spectrum of macrophage phenotypes in the tumour environment ranging from M1, which co-operate with T-cell and dendritic cells and have potent anti-tumour cytotoxic capacity; to M2, which promote stromal remodelling, angiogenesis and have immumnosuppressive capabilities.(368) Certainly, a dense CD86+ macrophage infiltrate around the tumour margin is associated with a good prognosis in colorectal cancer,(369, 370) conversely high intra-tumoural macrophages, (TAMs) have been found shown to predict for poor survival.(314) Less is known about the recruitment of monocytes from

the circulation into the tumour-associated stroma, the differentiation of these cells or the prognostic significance of such infiltrates. One recent report centred on the discovery of a subset of Tie2+ monocytes in the circulation which were attracted to the tumour both by hypoxia, and the expression of the receptor Angiopoietin-2 of the surface of the cancer cells.(371) These monocytes, on recruitment, were associated with areas of angiogenesis and high TAM infiltration within the stroma. We found that in this study, large colorectal tumours (≥60mm) were coupled with a profound increase in the S100A8 and particularly S100A9-positive cell counts in the stroma. This may indicate a role for these cells in supporting stromal vascularisation and tumour growth. In common with other studies and our own findings in pancreatic adenocarcinoma,(59, 372) we did not observe any differences in overall survival associated with the numbers of infiltrating S100A8 or S100A9-positive monocytes in the stroma in colorectal cancer patients .

Loss of Smad4 expression is observed in 10-15% of colorectal cancers and is reported to be a relatively late event in cancer development. We have ascertained the loss of Smad4 expression in 42/304 (14%) cases overall, which is consistent with previous reports.(58, 373) In our patient group we found no difference in the Smad4 expression according to the presence of lymph node metastases, but there was a relationship between loss of Smad4 and depth of tumour invasion. This is in contrast to a number of other studies which have observed that loss of Smad4 expression occurs with increasing frequency in tumours which have metastased to nodes or distant organs.(374-376) There is also conflicting evidence for the use of Smad4 as a prognostic marker in colorectal cancer, however most papers report poor prognosis in association with loss of Smad4 expression or deletion of 18q.(58, 373, 377) There has been recognition that loss of Smad4

expression is poorly-prognostic in earlier stage colorectal tumours in some studies.(60, 378) We found that loss of cytoplasmic Smad4 expression does predict poor survival in those patients with node-negative disease, whereas in those with node-positive disease there was no detectable difference.

Smad4 is an intracellular mediator of TGF-β signalling. While TGF-β functions as a tumour suppressor in early stages of carcinogenesis by potently inhibiting cell growth, cancer cells characteristically acquire resistance to this growth inhibition and frequently secrete high levels of TGF-β at later stages.(52) There is mounting evidence to suggest that loss of Smad4 expression and resistance to TGF-β are separate and unrelated events in carcinogenesis.(379) Re-introduction of Smad4 expression into Smad4-negative colorectal cancer cells did not restore TGF-β mediated growth inhibition, or impede proliferation of tumour cells *in vitro*. However, these clones showed a marked restriction of tumour growth *in vivo* in nude mice which was related to a reduction in angiogenesis. Smad4-negative clones were found to produce 2-3 fold more of the cytokine Vascular Endothelial Growth Factor, VEGF than clones which re-expressed Smad4.(380) A recent study has shown that knock-down of Smad4 rendered cancer cells resistant to TGF-β induced cell cycle arrest and migration but not to TGF-β induced epithelial-mesenchymal transition.(381) This indicates that cancers cells which have lost Smad4 expression respond differently to stimulation from exogenous cytokines such as TGF-β and this may be associated with a fundamental change in the behaviour and secretion profile of these cells.

Consistent with a recent report in primary pancreatic adenocarcinoma; in this study we observe the same association in colorectal cancer.(59) There are few reports to date of direct relationships

between the genetic status of tumour cells and the expression patterns of surrounding host cells. Our data thus provide important evidence of a direct relationship between cancer cells and host stromal response. The Smad4 status of cancer cells has been shown to directly influence the protein composition of the surrounding stroma; Smad4-negative clones have been shown to differentially secrete high levels of the matrix proteins SPARC (secreted protein, acidic and rich in cysteines) compared to clones which re-express Smad4.(382) SPARC has previously been characterized as an anti-adhesive and invasion-promoting protein which may have a role in facilitating tumour invasion.(383) Evidence suggests that loss of Smad4 also induces changes in the cytokine expression profile of the primary tumour which, in turn, changes the stromal environment.(384) We have postulated that these changes to the tumour microenvironment may promote recruitment of distinct sets of monocytes from the circulation or influence the rate or direction of differentiation of these cells. Support for this theory comes from a murine model of wound healing in which S100A8 expression was induced in fibroblasts by fibroblast growth factor-2 (FGF-2) and reduced by TGF-β.(385) More research is required into this area to fully understand the mechanisms behind this phenomenon.

We found that high levels of infiltrating S100A8 positive cells in the stroma of Smad4-negative colorectal cancer patients demonstrated poorer short-term survival than those with low levels of infiltrate, although there was no difference in survival in the longer term. This may indicate that Smad4-negative tumours have a decreased time to recurrence or distant metastases in the presence of high numbers of S100A8-positive monocytes. It is possible that Smad4-negative tumours with high S100A8 infiltrate develop early metastases and are not candidates for curative

resection of the primary tumour, which may explain the lower frequency of this phenotype in our cohort. This is supported by evidence from a murine model in which VEGF and other soluble chemokines released by the tumour induced production of S100A8 and S100A9 from recruited myeloid cells in the lung, promoting the subsequent development of metastases at the site, endorsing the theory that S100A8 and S100A9 are key to priming the pre-metastatic niche for tumour cells.(386)

Both S100A8 and S100A9 have been reported to have cytotoxic effects at high levels,(387) however recent reports using a number of human cancer cell lines suggests that S100A8/A9 may trigger intracellular signalling pathways in cancer cells by activating the multiligand receptor RAGE (receptor advanced glycation end-products).(310, 388) RAGE signalling induces an inflammatory signalling cascade inside the cell which activates Nuclear Factor-Kappa-Beta, NF-κβ. Several NF-κβ-regulated genes encode adhesion molecules, matrix metalloproteinases (MMP), serine proteases and chemokines (such as IL-6) which are known to be implicated in tumour invasion and metastasis.(389) It is clear that interaction between the cancer cell and its environment is one important factor in determining metastatic behavior.(390, 391) Changes to the tumour environment induced by loss of Smad4 may allow cancer cells to take advantage of S100A8/A9-induced signalling *in vivo*. This may explain the difference in survival observed in Smad4-negative, but not Smad4-positive tumours in response to high stromal S100A8 positive monocytic infiltrate.

To conclude, we have demonstrated that expression of S100A8 and S100A9 can be detected on infiltrating CD14+/CD68- monocytes or undifferentiated macrophages in the stroma. Smad4-negative colorectal cancers are associated with a change in composition of the surrounding stroma

demonstrating lower levels of S100A8-positive infiltrating monocytes. Loss of Smad4 expression is not predictive of overall survival except in those patients with node-negative disease, and those with high levels of infiltrating S100A8-positive stromal cells. Our findings underpin the importance of the interaction between cancer cells and their microenvironment to the malignant potential of colorectal tumours.

4.5 FINAL CONCLUSION

In conclusion, laser-capture microdissection combined with 2D gel analysis and tandem mass spectrometry allowed me to successfully compare protein-expression between colorectal cancer and normal tissue, and between different morphologies of colorectal cancer. In this small study it was not possible to identify all differentially-expressed proteins on the gels; however the most promising candidate spots according to Samespot software analysis and intra-observer visual analysis were identified. Validation of five candidate proteins was attempted and succeeded two proteins, owing to available antibodies with specific and reproducible staining on tissue immunohistochemistry. At the conclusion of this study, HSP27 as a prognostic marker in rectal cancer and S100A8 expression in the stroma of colorectal cancer tissue have been characterised and published. The aims of this study at the outset have not been met in entirety as there remain other differentially-expressed spots to identify and more proteins to validate; however a sound platform for further work in the area has been achieved.

4.6 AREAS OF FUTURE RESEARCH

Further areas of investigation are outlined as follows:

1. A second cohort of paraffin-embedded specimens was utilized (with kind permission from Dr Heike Grabsch) to futher validate expression of the HSP27 protein in rectal cancer patients using the immunohistochemistry protocol described in this thesis. The results were reported in the following publication; Low Molecular Weight Heat Shock Protein, HSP27 is a prognostic indicator in rectal cancer, but not colon cancer. Elizabeth Tweedle, Ilyas Khattak, Chin Wee Ang, Taoufik Nedjadi, Rosalind Jenkins, B. Kevin Park, Helen Kalirai, Andy Dodson, Bahram Azadeh, Monica Terlizzo, Heike Grabsch, Wolfram Mueller, Arthur Sun Myint, Peter Clark, Helen Wong, William Greenhalf, John P Neoptolemos, Paul S Rooney, Eithne Costello. *Gut. 2010 Nov;59(11):1501-10.*

2. The cellular response of colorectal cancer cell lines to S100A8/S100A9 was further investigated in our Institution on the basis of immunohistochemistry findings included in this thesis. The findings were reported in the following publication; Smad4 loss is associated with fewer S100A8-positive monocytes in colorectal tumors and attenuated cellular response to S100A8 in colorectal and pancreatic cancer cells. Chin W. Ang, Adnan A. Sheikh, Elizabeth M. Tweedle, Sarah Tonack, Taoufik Nedjadi, Rosalind E. Jenkins, Kevin Park, Irmgard Schwarte-Waldhoff, Ilyas Khattak, Bahram Azadeh, Andrew Dodson, Helen Kalirai, John P Neoptolemos, Paul S Rooney, Eithne Costello. *Carcinogenesis. 2010 Sep;31(9):1541-51. Epub 2010 Jul 9.*

3. Elevated HSP27 expression is postulated as a predictive marker for response to chemo-radiotherapy in rectal cancer patients. Tissue from patients recruited to phase II trials of chemoradiotherapy in Clatterbridge Centre for Oncology will be collated on a tissue microarray and HSP27 levels determined using the immunohistochemistry scoring protocol outlined in this work. Comparison will be made in histopathology features, tumour regression grade and patient survival.

4. HSP27 has been found at elevated levels in the tumour microenvironment of breast cancers, where it has been shown to influence phenotypic differentiation and activity of tumour associated macrophages. Future work will seek to investigate the association between HSP27 levels in the microenvironment of rectal cancers and the numbers and phenotypes of tumour-associated macrophages in the stroma.

5. Evaluation of oxidized peroxiredoxin 2 and SH3BGRPL3 expression in colorectal cancer tissue is planned when commercially available antibodies are available for use in immunohistochemistry.

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