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University of Dublin, Trinity College.

M.D. Thesis

Predictors of Response of Rectal Cancer to Neoadjuvant Radiochemotherapy

Fraser McLean Smith
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2006

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DECLARATION

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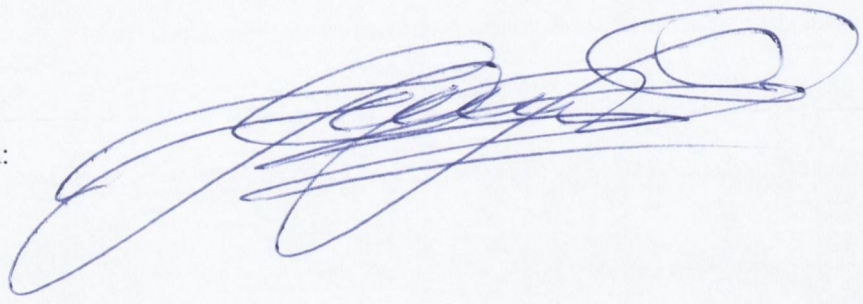
Signed: 

Let your hook always be cast; in the pool where you least expect it, there will be a fish.

Ovid

Publications to date from this thesis.

1) **Smith F.M.,** Stephens R.B., Kennedy M.J., Reynolds J.V. p53 abnormalities and outcomes in colorectal cancer: a systematic review. (Letter) **British Journal of Cancer** 2005 May 9; 92(9): 1813.

2) **Smith F.M.,** Reynolds J.V., Miller N., Stephens R.B., Kennedy M.J. Pathological and molecular predictors of response of rectal cancers to neoadjuvant radiochemotherapy. (Review) **European Journal of Surgical Oncology** 2006 Feb; 32 (1): 55-64

3) **Smith F.M.,** Reynolds J.V., Kay E., Crotty P., Murphy J.O., Hollywood D., Gaffney E.F., Stephens R.B., Kennedy M.J. COX-2 Overexpression in pretreatment biopsies predicts response of rectal cancers to neoadjuvant radiochemotherapy. **Int J Rad Oncol Biophys** 2006 Feb 1; 64 (2): 466-72

4) **Smith F.M.,** Fox E. Gallagher W.M., Stephens R.B., Gaffney E.F., Hollywood D., Whitely G., Petricoin E.F., Liotta L., Kennedy M.J., Reynolds J.V. Combination of SELDI-TOF-MS and Data Mining Provides Early-Stage Response Prediction for Rectal Tumours Undergoing Multimodal Neoadjuvant Therapy. **Annals of Surgery** 2007 Feb; 245(2):259-66.

Published abstracts

Smith F.M., Adida C., Kay E., Gaffney E.F., Crotty P., Stephens R.B., Kennedy M.J., Reynolds J.V. Level and intensity of IAP4 (survivin) staining in diagnostic biopsies do not predict response of rectal cancer to neoadjuvant radiochemotherapy. **Irish Journal of Medical Science** 2004 Sir Peter Freyer Supplement.

Smith F.M., Carton E., Stephens R.B., Keeling P.W.N., Kennedy M.J., Gaffney E.F., Reynolds J.V. Creating a colorectal 'biobank' at St James's Hospital. Practical considerations for 21st century research. **Irish Journal of Medical Science** 2004 Sir Peter Freyer Supplement.

Smith F.M., Stephens R.B., Gaffney E.F., Crotty P., Petricoin E., Liotta L., Kennedy M.J., Reynolds J.V. Exploring the proteome as a response predictor for rectal cancer undergoing neoadjuvant radiochemotherapy (RCT). **J Clin Oncol** 2005 23 (S16) 3573

Smith F.M., Miller N., Duggan S., Stephens R.B., Kennedy M.J., Reynolds J.V. Optimal storage and extraction methods for biobanked colorectal tissues. **Endoscopy** 2005; 37

Smith F.M., Kay E., Crotty P., Gaffney E.F., Stephens R.B., Kennedy M.J., Reynolds J.V. Evaluating COX 2 and apoptosis levels in pre-treatment biopsies as response predictors for rectal cancer undergoing neoadjuvant radiochemotherapy. **Colorectal Disease** 2005 7 (Suppl1) 6

Published abstracts Contd.

Smith F.M., Keoghan M., Carton E., Stephens R.B., Crotty P., Kennedy M.J., Reynolds J.V. pN but not pT may be accurately assessed by MRI after neoadjuvant radiochemotherapy (RCT) for locally advanced rectal cancer. **Colorectal Dis** 2005 7 (Suppl 1) 120

Smith F.M., Fox E. Gallagher W.M., Stephens R.B., Gaffney E.F., Hollywood D., Whitely G., Petricoin E.F., Liotta L., Kennedy M.J., Reynolds J.V. Combination of SELDI-TOF-MS and Data Mining Provides Early-Stage Response Prediction for Rectal Tumours Undergoing Multimodal Neoadjuvant Therapy. **Irish Journal of Medical Science** 2005 Sir Peter Freyer Supplement.

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Oral presentations (National)

Sir Peter Freyer Meeting Galway 2004

1) Creating a colorectal 'biobank' at St James's Hospital. Practical considerations for 21st century research.

2) Level and intensity of XIAP 4 (survivin) staining in diagnostic biopsies do not predict response of rectal cancer to neoadjuvant radiochemotherapy.

Irish Society of Gastroenterology, Dublin Castle 2004

Do spontaneous apoptosis and COX 2 predict response of rectal cancer to neoadjuvant radiochemotherapy?

Irish Association of Gastroenterology, Kilkenny 2004

Lymph node status may accurately predict rectal tumour downstaging after multimodal neoadjuvant therapy.

Irish Society of Coloproctologists, Londonderry 2004

Pre operative MRI as a predictor of response of rectal cancer to neoadjuvant radiochemotherapy

Sylvester O'Halloran Scientific Meeting, Limerick 2005 Plenary session

1) Non-invasive monitoring and response prediction for rectal cancer undergoing neoadjuvant radiochemotherapy

2) Evaluating COX2 and apoptosis levels in pre treatment biopsies as response predictors for rectal cancer undergoing neoadjuvant radiochemotherapy.

Sir Peter Freyer Meeting, Galway, 2005 Plenary session

Combination of SELDI-TOF-MS and Data Mining Provides Early Stage Response Prediction for Rectal Tumours Undergoing Multimodal Neoadjuvant Therapy

Oral Presentations (International)

Association of Surgeons of Great Britain and Ireland, Glasgow, 2005.

Exploring the proteome as a response predictor for rectal cancer undergoing neoadjuvant radiochemotherapy (RCT).

Tripartite Colorectal Meeting, Dublin, 2005.

1. Exploring the proteome as a response predictor for rectal cancer undergoing neoadjuvant radiochemotherapy (RCT).
2. Evaluating COX 2 and apoptosis levels in pre-treatment biopsies as response predictors for rectal cancer undergoing neoadjuvant radiochemotherapy.

Poster presentations (National)

Irish Cancer Society Belfast 2004

Creating a colorectal biobank in St James's Hospital. Practical considerations for 21st century research.

Irish Society of Gastroenterology, Dublin Castle 2004

Optimal storage and extraction methods for surgically acquired and biobanked colorectal tissues.

Sir Peter Freyer Meeting, Galway, 2005.

pN but not pT may be accurately assessed by MRI after neoadjuvant radiochemotherapy (RCT) for locally advanced rectal cancer.

Poster Presentations (International)

ESH-EBMT First Euroconference on Biobanking, Dublin, 2005.

Creating a colorectal biobank at St James's Hospital. Practical considerations for 21st century research.

American Society of Clinical Oncology (ASCO) Orlando, Florida, 2005.

Exploring the proteome as a response predictor for rectal cancer undergoing neoadjuvant radiochemotherapy (RCT).

Summary of Thesis

Rectal cancers staged as cT3/T4 +/- node positive routinely receive neoadjuvant radiochemotherapy (RCT) followed by total mesorectal excision (TME). By this approach tumours can be both downstaged and downsized resulting in increased rates of complete tumour resection and sphincter preservation. Oncologically, patients with tumours that demonstrate a good histological response to RCT, characterised by complete or near-complete replacement of tumour by radiation-induced fibrosis, have lower rates of local recurrence and show improved overall survival compared to those with poor response. Furthermore, a recent seminal study found improved overall outcomes in patients with a complete clinical response to RCT managed conservatively with observation alone compared to patients who underwent surgery.

The main clinical problem with RCT currently is that only the minority of patients achieve a good response. As there are no existing means to predict response to treatment it must be given empirically meaning that the majority of patients will derive sub-optimal benefit. This is a particular problem as RCT is expensive, time-consuming and increases both peri-operative morbidity and radiation-induced neoplasia. Efforts are now being made in order to identify biomarkers in biological specimens from patients that may allow for response prediction. The work contributing to this thesis aims to augment existing clinical research into response prediction

In chapter 3, staining with a panel of immunohistochemical (IHC) markers known to mediate radioresistance *in vitro* and in animal models was performed.

Here, archival, pre treatment biopsy tissues from a historical cohort of 49 patients who had previously undergone RCT followed by excisional surgery were studied. Markers assessed were COX 2, survivin, apoptosis and proliferation. The level of staining of each marker was compared to the degree of histological tumour response for each patient. Our study found that both low levels of spontaneous apoptosis and overexpression of COX 2 in pre treatment biopsies independently predicted a poor response to RCT. Neither survivin expression nor tumour proliferation was able to predict response with statistical significance.

In chapter 4, serum proteomic analysis using surface-enhanced laser desorption/ionisation incorporating time-of-flight mass spectrometry (SELDI-TOF-MS), was evaluated as a means to predict tumour response to RCT. Prospectively collected serum samples from 20 patients taken before, during and after treatment were analysed. Generated mass spectra were compared between responsive and non-responsive patients at each timepoint. We conducted this study in collaboration with the Clinical Proteomics Reference Laboratory, National Cancer Institute/Food and Drug Administration, National Proteomics Program in Gaithersburg, Maryland, USA. In total, 230 proteomic spectra were generated representing all available timepoints run in duplicate. Support vector machine (SVM) analysis of spectra was used to generate a predictive algorithm for each timepoint based on proteins that were maximally differentially expressed between good and poor responders. The greatest classification accuracy was achieved using serum sampled 24 to 48 hours into treatment, where prediction of response could be performed with a sensitivity of 87.5% and a specificity of 80%. This was achieved using a 14-peak classifier comprising 7 positive and 7 negative markers of good response to RCT at each timepoint identified 14 key protein differences.

In chapter 5, SELDI-TOF-MS was evaluated as a non-invasive disease-screening tool for colorectal cancer. Although this deviated somewhat from the main objective of the thesis, this study was prompted by our NCI collaborators' recent work, which had shown that the technique could correctly identify blinded serum samples from patients with or without ovarian cancer with 100% sensitivity and 95% specificity. We therefore attempted to replicate their study in CRC given the limitations of existing screening methods and the fact that there were no existing reports of using SELDI-TOF-MS in this manner. Serum from 46 healthy patients, 23 with benign adenomas and 56 with CRC was analysed. All samples were prospectively collected over the course of a year from patients undergoing elective endoscopy or cancer surgery at St James's Hospital. Although several proteomic differences were observed between patients with or without cancer, disappointingly these could not be used to correctly classify patients with clinical significance.

In chapter 6, gene expression microarray was performed on extracted RNA from 9 fresh pre treatment biopsies taken from patients before undergoing RCT followed by surgery. Specimens from 4 from good responders and 5 from poor responders were available for study. At the time of starting this thesis, there were no existing reports where microarrays had been used for this purpose. Parallel expression analysis of expression profiles between good and poor responders was then performed using data-mining software and a list of genes differentially up or down regulated was generated. Using a p value of <0.01 and a 2-fold difference in expression, a total of 49 genes were identified which were differentially expressed. Using an on-line database, the identities and molecular and biological functions of these genes were revealed. They represented multiple different proteins involved in many different aspects of cellular functioning.

In chapter 7, the accuracy of clinical staging obtained by post RCT MRI scanning was performed. At the time of starting this thesis, all patients undergoing RCT in St James's Hospital underwent post RCT MRI scans to assess whether they had responded to treatment. No formal audit, however, had been performed to determine whether these MRI scans could accurately detect downstaging after RCT. Our study found that MRI was 100% inaccurate in determining changes in T stage after treatment but its accuracy was slightly higher in detecting change in nodal stage (66%). As well as simply correlating tumour and nodal stage correlation between post RCT scans and pathological reports, however, we also aimed to investigate whether features of post RCT MRI staging could be correlated with a good response to treatment. We found that node negative status on MRI predicted a good response to RCT with 75% sensitivity and 83% specificity. In addition, comparison of pathological node negative status and TRG showed that absence of nodal metastases predicted a favourable TRG with 78% sensitivity and 91% specificity. Whilst MRI may not yield sufficient clinical accuracy in the prediction of nodal status *per se*, node negative status on post RCT MRI may be an adjunctive means to predict patients who have undergone a large biological response.

In conclusion, the work contributing to this thesis has shown that using a combination of traditional and translational approaches, biomarkers capable of predicting response of rectal cancers to RCT in the early stages of treatment may be found. These studies suggest that response to RCT is a dynamic, multi-factorial phenomenon; therefore future research should focus on translational approaches capable of assessing multiple markers concurrently rather than trying to identify single predictive markers.

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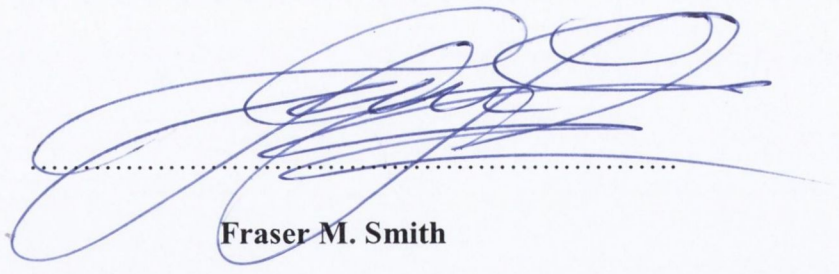
Mr Calvin Coffey

Declaration

I declare that all work described in this thesis, including statistical analysis, was carried out by myself unless otherwise stated in the text. This work has not been submitted for a degree at any other university. I agree that this thesis may be loaned at the discretion of the librarian.

Signed: 

Fraser M. Smith

Table of Contents

Chapter 1 Contd...

Chapter 3 Contd...

Chapter 3 Contd...

Chapter 4 Contd...

Chapter 5 Contd...

Chapter 6 Contd...

Chapter 1: Figures and Tables

Chapter 3: Figures and Tables

Chapter 4: Figures and Tables

Chapter 5: Figures and Tables

Chapter 6: Figures and Tables

Chapter 6: Figures and Tables Contd...

Chapter 7: Figures and Tables

Chapter 1

Introduction

1.1 History and evolution of radiation and chemotherapy in the management of rectal cancer.

Over the last few decades, increasing emphasis on the combination of radiotherapy and chemotherapy in addition to radical surgery has had a marked effect on both local recurrence and surgical management of patients with rectal cancer (Table 1.1.).

The first major publication that evaluated the addition of adjuvant radiation or chemotherapy after surgery for rectal cancer was by The Gastrointestinal Tumor Study Group in 1985(1). This study demonstrated that both local recurrence and 5-year survival were maximally improved if patients with locally advanced tumours received post-operative radiochemotherapy after surgery. Whilst numbers in this study were small, its results were subsequently confirmed by Krook et al in 1991, using larger patient groups(2). This prompted a National Institute of Health recommendation that combined postoperative adjuvant therapy be used in all patients with adverse prognostic factors(3).

The Swedish Rectal Cancer Trial, published in 1997, prompted a switch from postoperative to preoperative therapy, so-called neoadjuvant therapy(4). This study was the first randomised trial to show that short course radiation given prior to surgery could decrease local recurrence and improve survival compared to surgery alone. The rationale for using neoadjuvant radiation was compounded by its additional clinical advantages such as low toxicity, its ability to shrink and downstage tumours before surgery allowing resection of previously inoperable tumours and increased rates of sphincter preservation(5-7).

Concurrent with the advances in radiation and chemotherapy, advances in surgical technique were also being made. The most significant of these, which first came to light in the early 1980s, was the technique of Total Mesorectal Excision (TME), first described by Heald et al(8, 9). This technique stressed the importance of meticulous, sharp dissection in the plane between the visceral fascia of the mesorectum and the parietal fascia of the pelvis based on the concept that cancer spread was likely to be confined to the tissue of its embryological origins, namely the visceral hindgut mesentery.

TME was so effective that reported rates of local recurrence using this technique alone were comparable to the best results obtained with surgery plus adjuvant therapy regimes in surgical clinical trials(10). Comparison of TME alone vs neoadjuvant radiation followed by TME was therefore needed. As surgery was not standardised in the Swedish Rectal Cancer Trial, The Dutch Colorectal Cancer Group assessed outcome between TME alone compared with TME after short course radiotherapy. This study demonstrated that the rate of local recurrence at two years was 2.4 percent in the radiotherapy-plus-surgery group and 8.2 percent in the surgery-only group (11).

Both The Swedish Rectal Cancer Trial and the study from The Dutch Colorectal Cancer Group, which used short course neoadjuvant radiation, included all patients with adenocarcinoma of the rectum.

Table 1.1

Summary of the main publications influencing the development of neoadjuvant multimodal therapy in rectal cancer

Ref No	Year	Study	Groups	Patients per group	5 year local recurrence (%)	% alive at 5 years
1	**1985**	Gastrointestinal Tumour Study Group	Surgery alone	58	24	36
			Adjuvant RTx	50	27	46
			Adjuvant CTx	48	20	46
			Adjuvant RCT	48	11	56
2	**1991**	North Central Cancer Treatment Group	Adjuvant RTx	100	25	48
			Adjuvant RCT	104	13	58
4	**1997**	Swedish Rectal Cancer Study	Surgery alone	553	27	48
			Neoadjuvant RTx + surgery	557	11	58
11	**2001**	Dutch Colorectal Cancer Group	TME	924	9*	82*
			Neoadjuvant RTx + TME	925	2*	82*
5	**2004**	German Rectal Cancer Study	Neoadjuvant RCT	405	6	76
			Adjuvant RCT	394	13	74
21	**2004**	Habr-Gama et al	Stage 0 + surgery	22	17	88
			Stage 0 + observation	71	8	100

RTx= Radiotherapy, CTx=Chemotherapy, RCT=radiochemotherapy, TME=Total mesorectal excision, *= 2 year data.

For patients with rectal tumours staged as T3/T4 +/- node positive, however, long-course neoadjuvant radiochemotherapy (RCT) has become the recommended standard therapy. Recently, the results of a large, randomised controlled trial by The German Rectal Cancer Study Group showed that this approach, followed by standardised TME, not only improves local control when compared to adjuvant therapy but is also associated with lower rates of patient toxicity(5).

In summary, randomised controlled clinical trials have established that neoadjuvant radiotherapy followed by TME is the optimal treatment for patients with rectal cancer. For tumours that are locally advanced, combination of long-course neoadjuvant RCT and TME is the optimal treatment (5, 11).

1.2 The importance of response to RCT.

Within multimodality protocols, the best surrogate marker of an excellent prognosis after treatment is the attainment of a complete pathological response (pCR), characterised by sterilisation of all tumour cells(12-18). This phenomenon, however, is only observed in approximately 10-30% of cases (12, 13). Remaining patients demonstrate a spectrum of residual disease ranging from microscopic foci of tumour cells on a background of radiation-induced fibrosis to no response at all(19).

Increasing data now confirm that a complete or near complete response to treatment confers both a decreased risk of local relapse and improved overall survival compared to patients with poorly responsive tumours (Table 1.2).

Table 1.2 Main studies assessing impact of post RCT pathological downstaging on local recurrence and recurrence.

Author	Reference No	Year	No Patients	Median follow up (mths)	Improved Local Recurrence	Improved Overall Survival
Guillem et al	7	2005	297	44	Yes	Yes
Vecchio et al	20	2005	144	72	Yes	Yes
Habr-Gama et al	21	2005	260	60	Yes	Yes
Wheeler et al	18	2004	65	39	Yes	Yes
Reerink et al	13	2003	66	54	Yes	Yes
Theodoropoulous et al	14	2002	33	33	Yes	Yes
Ruo et al	16	2002	69	69	Yes	No

We sourced 7 studies assessing outcome in relation to the degree of tumour response to RCT (7, 13, 14, 16, 18, 20, 21). The largest of these studies published to date assessed 297 patients who received RCT followed by TME with a median follow up of 44 months. In this study by Guillem et al multivariate analysis found that a >95% pathological response conferred a highly significant improvement in both recurrence-free and overall survival(7). Another large study by Vecchio et al assessed 144 patients treated with RCT followed up for a median of 72 months(20). This study again found a 2% vs 17% local failure rates and 86% vs 54% metastasis-free survival in tumours with a complete/near complete pCR compared with poorly responding tumours. Interestingly, Vecchio et al also found that tumours which demonstrated a high degree of biological response to RCT, had a higher chance of being node negative when compared to poor responders(20).

This finding is mirrored in several other published reports(22-24) and suggests that a reciprocal relationship exists between the primary tumour and its nodal metastases, the combination of which may explain the favourable outcome in good responders.

1.3. Introducing the concept of non-operative management of good responders to RCT

In a recent seminal publication, Habr-Gama et al provided the first evidence that patients who underwent a complete clinical response could be safely managed non-operatively(21). Their study included 265 patients with distal rectal carcinoma sited 0-7cm from the anal verge. All patients underwent standard RCT regimens and underwent clinical assessment 8 weeks after the final dose of radiation. This consisted of endoscopic and histologic assessment. Patients who had either residual tumour or ulceration seen endoscopically or those with positive biopsies were deemed incomplete responders and underwent radical surgery. Those without any of these features were deemed complete responders and were followed up by clinical observation alone.

In total, 71 patients underwent a complete clinical response. They were followed up for a mean of 57.3 months having a monthly examination examination consisting of digital rectal examination, proctoscopy +/- biopsies and serum CEA assessment. They also underwent abdominal and pelvic CT scanning every 6 months. There were 3 systemic recurrences in this group, of which all patients were still alive at the time of publication. There were also 2 endoluminal recerrences, one of which was treated with transanal full thickness excision and the other was treated with salvage brachytherapy.

The remaining 194 patients with an incomplete clinical response underwent radical surgery and routine follow up for a mean of 48 months. Histopathological assessment of the resected tumour specimens revealed that 22 patients (8.3%) deemed incomplete responders, in fact demonstrated pCR at the time of histopathological analysis. Of these, 9 were treated with APR and 13 with sphincter saving surgery, 7 of which had diverting loop ileostomies in order to protect the coloanal anastomosis. Cumulatively, this meant that of the 22 patients with pCR treated with surgery, 72.7% ended up with stoma, which was either permanent or temporary. Overall there were 2 deaths in this group at 21 and 24 months as a result of metastatic disease.

Overall comparison found 100% vs 88% overall survival between non-operative and operative management of pCR patients respectively (P=0.01) (Fig 1.1). There was no difference in local recurrence between groups(21). As such, in this study, non-operative management was superior to operative management in terms of outcome and had the further advantage of obviating operative morbidities such as perioperative sepsis, stomas and sexual/urinary dysfunction.

Figure 1.1. Comparison between overall and disease free survival after observational versus surgical treatment post RCT (Habr-Gama et al).

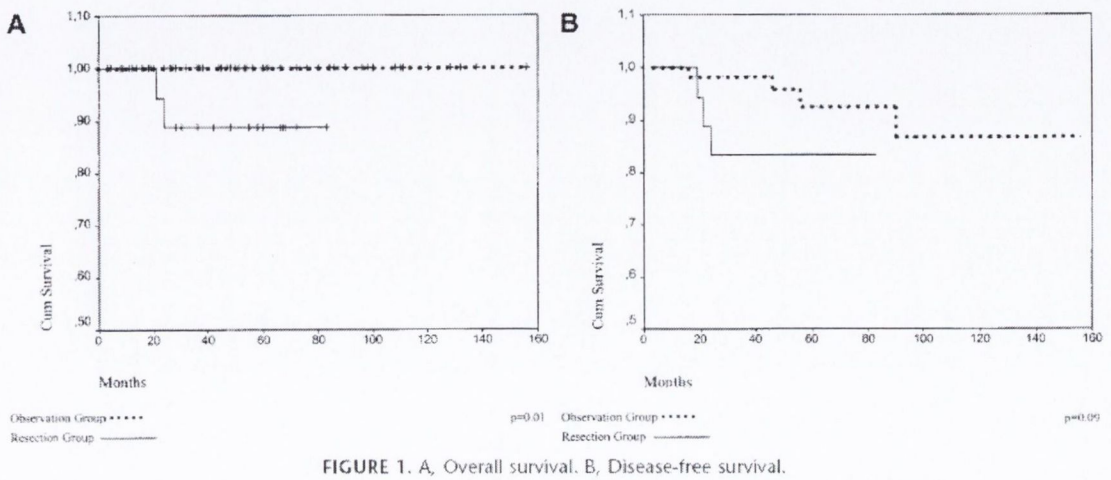


FIGURE 1. A, Overall survival. B, Disease-free survival.

1.4. Predictors of response

Although the concept of observational management after RCT is certainly novel in the setting of rectal cancer, it has long been the standard of care in patients who present with invasive squamous cancer of the anus, thus obviating the need for APR and a permanent colostomy(25, 26). The main limitation in implementing this approach is that the ability to predict the degree to which a patient will respond to RCT using conventional clinical indices does not currently exist either pre- or post- treatment. Consequentially, both RCT and surgery must be administered empirically. A focus of contemporary research, therefore, is to elucidate whether factors within pre-treatment tumour biopsies can predict a patient's sensitivity to RCT on an individual basis. This would be of clinical advantage for several reasons. Firstly, RCT is time-consuming, expensive and increases perioperative morbidity (27-29). Secondly, by gaining an understanding of the factors mediating radioresistance, the ability to develop alternative treatment strategies that could be encompassed in phase II trials of novel biologic agents may be realised.

1.5. Histological quantification of Tumour Response to RCT

In the existing literature, two methods have been commonly used to measure response. These are clinicopathological downstaging and tumour regression grade

Clinico - pathological downstaging (CPD) is where the most accurate T and N stage before treatment determined clinically e.g. by magnetic resonance imaging (MRI) or trans rectal ultrasound (TRUS), is compared with the pathological T and N stage in the resected specimen(30, 31). This is a commonly used means of assessing response, but the accuracy of

this technique may be flawed by limitations in these imaging modalities. For example, in a recently published study of 1184 patients, TRUS was found to have a staging accuracy of only 69% (32, 33). This was due to its limited depth of acoustic penetration preventing adequate assessment of local tumour extent in bulky T3 and advanced rectal cancers. Similarly, the accuracy of MRI staging using a body coil is reported to range from between 59-88%(33). Here, most staging failures occur due to difficulties in the accurate discrimination of T2 and T3 lesions because of over staging due to desmoplastic reactions and difficulties encountered in discriminating between spiculation of the peri-rectal fat caused by fibrosis alone as opposed to tumour. Interpretive inaccuracies may therefore effect the validity of CPD being used to measure response.

Tumour regression grade (TRG), although originally described for tumours of the oesophagus by Mandard et al (Fig 1.2), has been adapted to rectal cancer(19) Regression grading stratifies response based on the biological effect of radiation on tumours, dividing it into 5 different grades based on the ratio of fibrosis to tumour. The value of TRG is that it can highlight those tumours demonstrating large degrees of biological response to radiation but not necessarily undergoing a change in T stage (Fig 1.3 a-d). This is important as evidence now shows that patients demonstrating marked tumour regression may have similar outcomes to those showing complete responses (15, 16, 18).

Figure 1.2.

5 – point tumour regression grading system originally proposed by Mandard et al(19)

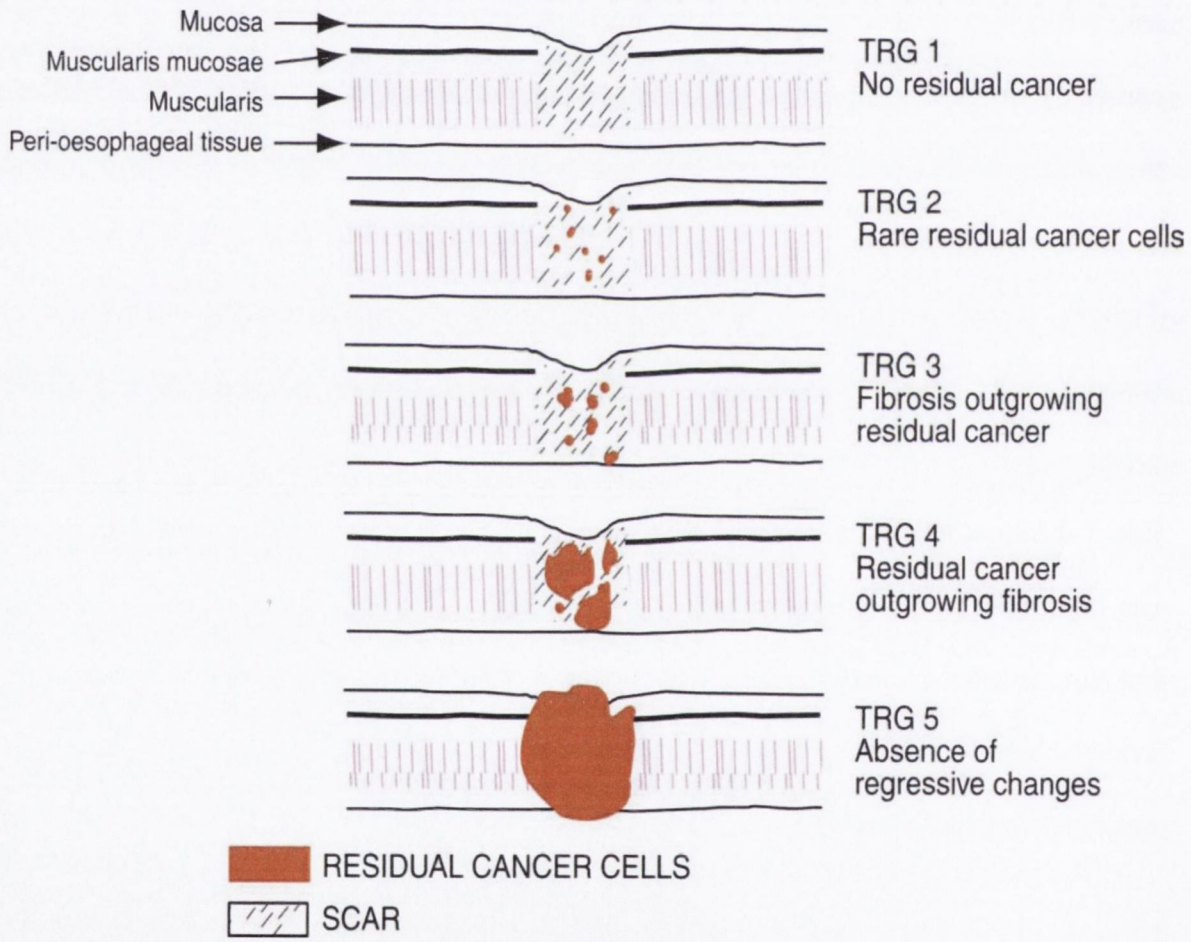


Figure 1.3 a-d

Representative photomicrographs of different degrees of histological tumour response.

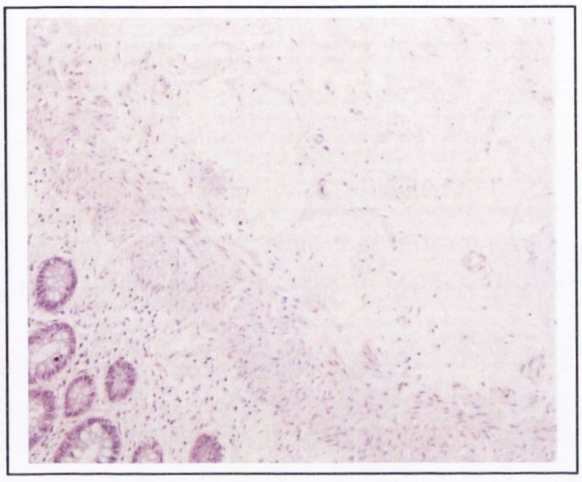


Fig 1.1a

Complete response TRG 1 (H+E, X10) Here no residual tumour cells remain. They have been replaced by radiation-induced fibrosis.

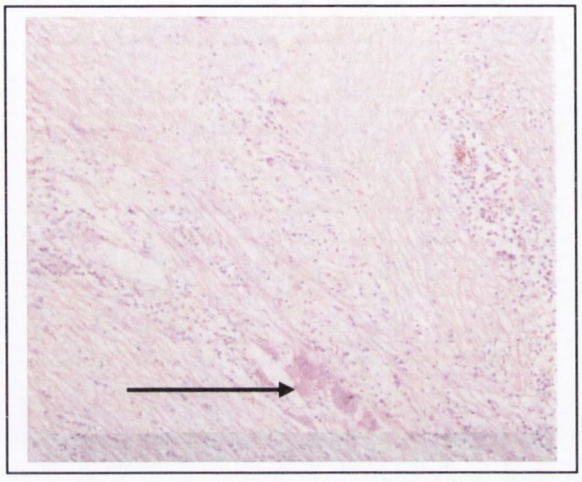


Fig 1.1b

Near-complete response TRG 2 (H+E X20) Here the vast majority of tumour cells have disappeared with only one or two remaining scattered amongst radiation-induced fibrosis

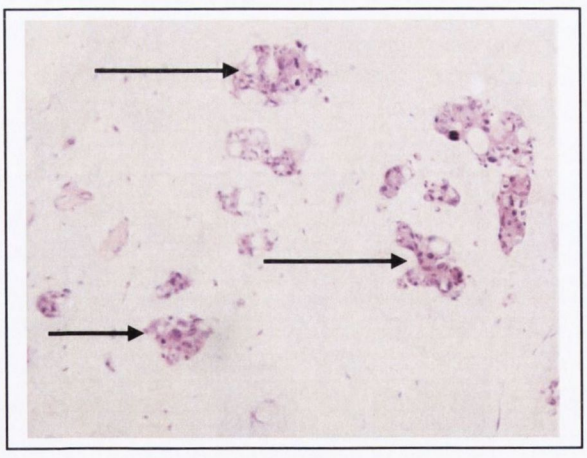


Fig 1.1c

Poor response TRG 3 (H+E X20) Here residual tumour cells are common (arrow) but still radiation-induced fibrosis predominates.

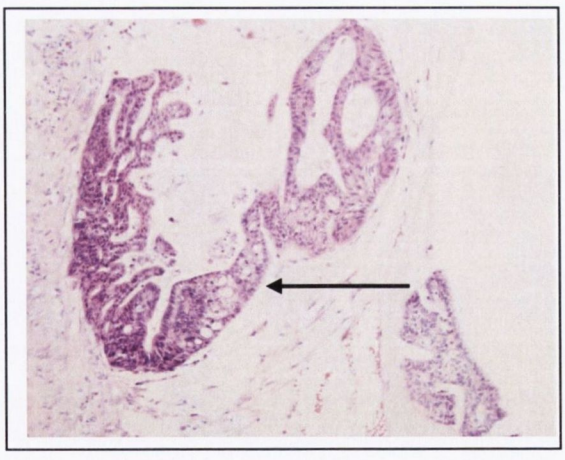


Fig 1.1d

Poor response TRG 4 (H+E X20) Here large islands of radioresistant tumour are seen throughout the specimen (arrow).

1.6. Conventional clinical and histological indices as response predictors: Pre RCT

The ability of conventional patient and tumour indices to predict response before RCT is administered has been addressed by multivariate analysis in 4 studies assessing molecular response predictors. These studies all concluded that pre treatment T stage, N stage, grade, differentiation, and age and gender could not predict histological response to RCT (30, 34-36). Whilst conventional factors may have no influence over tumour radiosensitivity, they may however influence rates of local recurrence. Myerson et al identified that tumour location <5cm from the anal verge, circumferential lesions, obstruction and tethered/fixed tumours were all independent risk factors for local recurrence(37).

1.7. Conventional clinical and histological indices as response predictors: Post RCT

Radiological assessment is the mainstay of staging patients prior to RCT in rectal cancer. Its ability to accurately restage patients following RCT has been assessed in several studies. Vanagunas et al assessed the use of endoscopic ultrasound (EUS) in 82 pts after RCT(38). They found that T stage could only be accurately classified in 48% of patients with 38% of the remainder being overstaged and 14% being understaged. In contrast it was able to detect nodal stage with 77% accuracy. Overall, however, this study concluded that the routine use of EUS for restaging after RCT should be discouraged.

MRI has also been assessed as a post RCT staging modality. The first published report, which assessed 35 patients, was by Hoffman et al(39). This study found that both T and N stage could only be predicted with 54% accuracy with 9 of 20 responsive tumours being overstaged and 15 non-responsive tumours being understaged. They concluded that MRI was

an inaccurate means of restaging tumours. Since then, several other reports have appeared in the literature. Chen et al assessed 50 patients and found that T stage could only be predicted with 52% accuracy, with 38% and 10% of patients being over and understaged respectively(40). N stage was assessed with 68% accuracy with 24% and 8% of patients being over and understaged respectively. Kuo et al also assessed the ability of MRI to predict response to RCT in 36 patients(41). Again, T stage prediction was very poor at only 47%, with the majority of patients being overstaged and N stage was correctly predicted in 64% of patients. Finally, Kim et al assessed 112 patients and compared MRI-derived tumour volume to both pathological stage and the degree of tumour regression(42). Although this study found a significant relationship between a reduction in tumour volume and a decrease in histological stage, volumetric analysis could not differentiate between patients with pCR and those with residual disease.

Less routine radiological methods have also been assessed as response predictors. Dzik-Jurasz et al assessed diffusion weighted MRI in 14 patients undergoing RCT(43). They found that there was a strong correlation between mean pre treatment apparent diffusion coefficient and percentage change in tumour size post RCT. Unlike Kim et al, however, they did not equate decrease in size to the presence or absence of residual disease and so the significance of this study is uncertain. Furthermore, in a separate study, Yang et al equated tumour volume reduction to downstaging in 30 patients and found that all tumours showed volume reduction after RCT to some degree but that the downstaging benefits were not in proportion to the change in size(44).

FDG-PET scanning has also been assessed in one study by Calvo et al(45). This group assessed a series of 25 patients by performing pre and post RCT scans. This group found that

the change in maximised standard uptake value (SUVMAX), representing the tumour: tissue metabolic ratio, was significantly associated with T-level downstaging. As yet their results have not been evaluated in further studies.

To date, we could only source one study that has formally assessed the ability of clinical examination post-RCT to evaluate tumour response. This study, by Guillem and colleagues in Memorial Sloan Kettering, prospectively assessed 94 patients who underwent RCT using a combination of digital rectal examination (DRE) and endoscopy(46). They found that DRE underestimated pathological response in 78% of cases. Furthermore it only correctly identified 21% of complete pathological responders. Similarly, the endoscopic appearance of tumours was inaccurate in predicting response, a finding mirrored post RCT in oesophageal tumours(47).

1.8. Molecular response predictors

As neither clinical nor radiological means of response assessment currently appear to be accurate in the setting of rectal cancer undergoing RCT, alternative predictive means are being actively sought. As such, there is an increasing interest in assessing the molecular profiles of pre treatment tumour biopsies in order to determine whether response may be predicted at the molecular level.

The vast majority of these studies have been performed by using immunohistochemical analysis of pre treatment tumour biopsies. A summary of studies performed and their findings can be seen in table 1.3.

1.8.1. P53

To date, p53 is the most studied response predictor in rectal cancer. It acts by maintaining the G1/S and G2/M cell cycle checkpoint(48, 49) where it detects damaged or mutated cells which are attempting to replicate and initiates a pathway which causes them to be eliminated via apoptosis(50-52)(Fig 1.3). As such, p53 is often referred to as 'a guardian of the genome'(53).

To date, 22 studies assessing the ability of p53 status to predict histological response have been reported. These studies utilised a variety of techniques including immunohistochemistry (IHC)(13, 30, 31, 34-36, 54-69), single strand conformational polymorphism (ssCP) analysis(54, 57, 61, 63) and direct p53 gene sequencing analysis(30, 31).Eighteen studies utilising IHC were sourced. Of these, only 4 (18%) found that p53 could be used to significantly predict response (35, 58-60). In contrast, and with similar sample size and pathological end-points, the remaining 14 IHC based studies (82%) found no association between overexpression of the p53 protein and treatment response.

Single strand conformational polymorphism (ssCP) analysis screens for genetic mutations based on the principle that the electrophoretic mobility of DNA molecules will differ if their conformations differ(70), and can therefore differentiate between the mutant and wild type p53 gene, which is not possible with IHC. To date, this technique has been assessed in 3 studies using pre treatment biopsies, and in no study did it predict response to treatment.

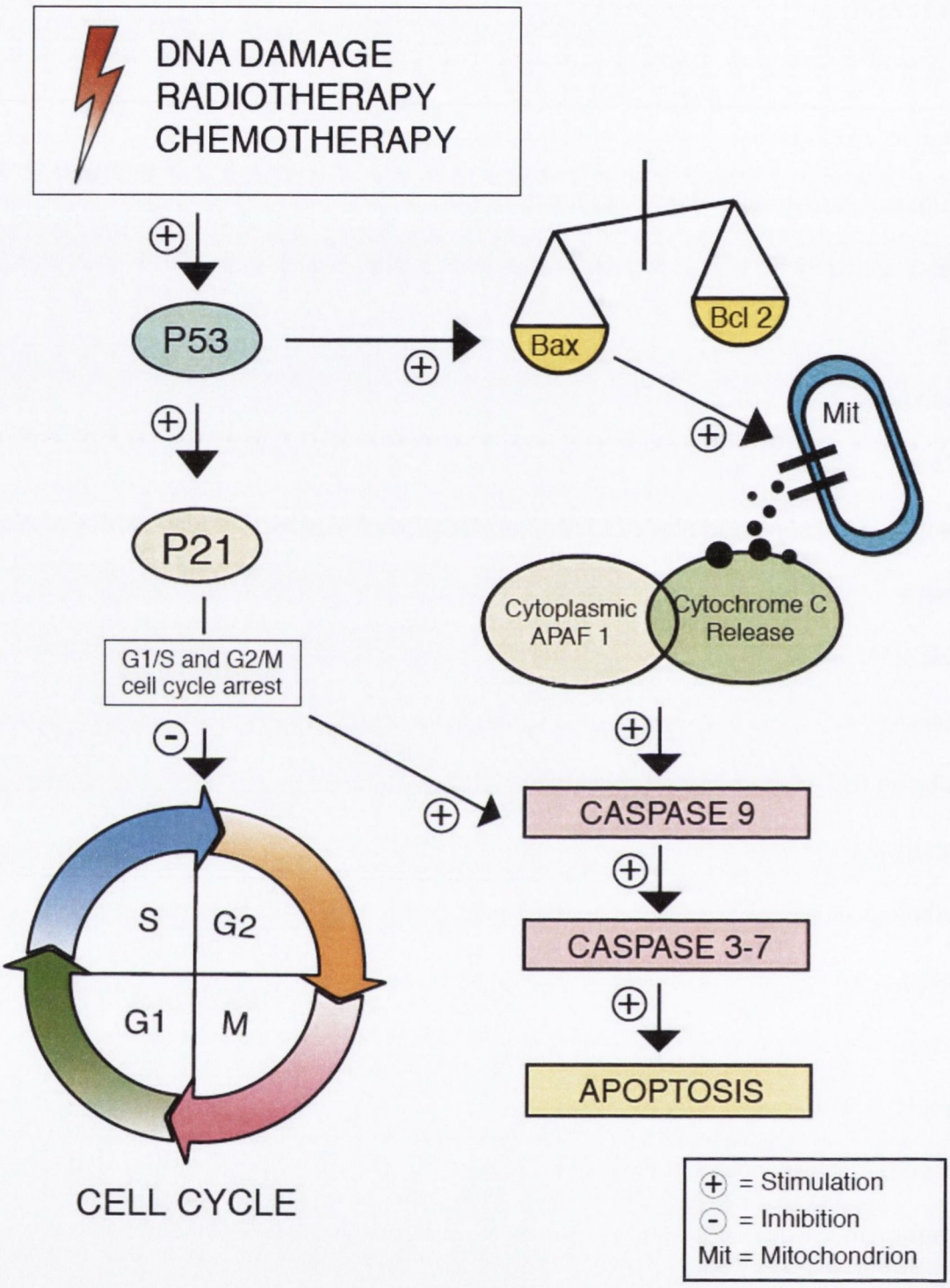


Figure 1.4. Flow diagram of the p53 pathway. Constituents of this pathway are the most commonly assessed predictive markers in rectal cancer.

Direct sequence analysis of the p53 gene appears to hold greater promise as a predictive marker. Kandioler et al studied pre treatment biopsies from a series of 64 patients receiving short course neoadjuvant radiotherapy. They assessed for p53 mutations by complete direct sequencing of exons 2 – 10. Using a reduction in T stage as an outcome measure, mutant p53 genotype was significantly associated with radioresistance ($p<0.0001$).

Rebischung et al studied pre treatment biopsies from 86 patients receiving short course neoadjuvant radiotherapy by direct gene sequencing, and reported that p53 gene mutations was significantly ($p<0.01$) higher in non-responders.

1.8.2. P21

P21 protein is a key member of the p53-signalling pathway (Fig 1.3). It is transcriptionally activated by p53 after DNA damage by ionising radiation(71, 72) which in turn causes cell cycle arrest and apoptosis(73, 74). It has been studied as a response predictor because of disruption of regulatory networks, in particular those involved in cell death signalling, may be a causative factor of radioresistance. Endogenous p21 expression has been assessed in 4 IHC-based studies(13, 34, 35, 54), 3 of which indicated an association with response. Moreover, Rau et al not only reported that p21 expression in pre-treatment biopsies correlated to response but also showed that levels changed during treatment. Tumours demonstrating a rise in levels after treatment had worse long term outcomes compared to those showing no rise. The inclusion of p21 analysis in the design of prospective neoadjuvant studies in rectal cancer would appear warranted.

1.8.3. Bcl 2 and Bax

The Bcl 2 family (Fig.1.3), of which Bcl 2 and Bax are members, regulate caspase activation and caspases in turn are at the centre of the cell's decision to live or die in response to an apoptotic signal, including cytotoxicity and ionising radiation (75, 76). Bcl 2 and Bax are pro-survival and pro-death proteins respectively.

The primary function of Bcl 2 is to maintain the mitochondrial outer membrane integrity. Bax, on the other hand, acts to breach mitochondrial outer membrane integrity and can be activated by pro apoptotic stimuli or p53 (77, 78). Bcl 2 negatively regulates this process. Once the membrane has been breached, pro-apoptotic proteins such as cytochrome C are released which cause caspase activation and cell death. Due to the critical role of Bcl 2 family members in regulating apoptosis in response to cytotoxic insults, both Bcl 2 and Bax have been studied as molecular biomarkers. To date, a total of 8 studies have assessed Bcl 2 expression and 3 have assessed Bax expression as predictive markers using IHC. 7 studies have assessed Bcl 2 alone (34, 36, 64-66, 68, 69), 2 studies have assessed Bax alone (54, 79) and 1 study has assessed Bcl 2 and Bax in combination (80).

Of the 7 studies that have assessed Bcl 2 alone, only one found that Bcl 2 in pre treatment biopsies was an independent predictor of response. All other studies, however, have found Bcl 2 to have no value in predicting response. Similarly, of the 3 studies assessing Bax as a response predictor, none found that it was associated with either downstaging or outcome. It therefore seems unlikely that these will prove to be clinically useful response predictors.

1.8.4. EGFR

The epidermal growth factor receptor (EGFR) is expressed in between 50 –70% of cancers, and therapies targeting this and related pathways are increasingly being developed for translational trials(81). Upregulation of EGFR is associated with a more aggressive tumour growth, poor prognosis and resistance to radiation (82). Despite this theoretical relevance to response prediction, it has only been evaluated in a solitary study. Giralt et al assessed pre treatment biopsies from 45 patients, using a decrease in T stage as an endpoint(81). Although EGFR was unable to predict tumour or nodal status after treatment in the majority of patients, only one of 7 tumours demonstrating CPR expressed EGFR (P=0.003). EGFR may therefore be a potentially useful marker in predicting complete responders.

1.8.5. Microsatellite instability and mismatch repair proteins.

DNA damage induced by radiation and chemotherapy is a key mechanism of cell death. Accordingly the relationship between intrinsic cellular radiosensitivity and endogenous DNA repair mechanisms may be of considerable importance in predicting response or resistance(83-85). Tumours demonstrating MIS have been found to have a better prognosis compared with tumours with intact repair systems. In addition, MIS tumours have been found to have an altered response to radiotherapy *in vitro*(86). The effects of MIS and mismatch repair on rectal cancer patients undergoing RCT has been evaluated in 3 clinical studies(34, 54, 87). Qui et al screened only for MIS(34) using polymerase chain reaction method from tissue DNA samples. The other 2 assessed IHC overexpression of the mismatch repair proteins HMSH 2(54, 87) and HMLH 1(54) in pre-treatment biopsies. Neither MIS nor deficits in MMR protein expression could predict response to treatment.

Komuro et al using IHC on pre- treatment biopsies from 111 patients studied the expression of Ku70, a heterodimer known to play an important role in double stranded break repairs (88), reported that it could predict response, particularly when combined with mutant p53 status (59).

1.8.6. Tumour hypoxia.

Tumour hypoxia can lead to radiation and chemoresistance by depriving cells of oxygen essential for the cytotoxic activities of these agents(89). Moreover, tumour hypoxia promotes upregulation of angiogenic and tumour cell survival factors resulting in increased proliferation, radioresistance and angiogenesis. Only one marker for hypoxia, vascular endothelial growth factor (VEGF), has currently been assessed as a response predictor in rectal cancer. This has been assessed in 2 separate studies. Qui et al assessed expression of VEGF in pre treatment biopsies of 72 patients undergoing long-course neoadjuvant radiotherapy; however, they found that histological response was unrelated to pre treatment VEGF status(34).

George et al assessed serial levels of serum/plasma VEGF in a series of 16 patients undergoing RCT (90), combined with serial dynamic contrast-enhanced MRI (DCE-MRI), a non- invasive measure of vessel permeability. Whilst this study showed no predictive ability of circulating VEGF levels in predicting response, it did show that those tumours with a higher pre treatment permeability on DCE-MRI responded significantly ($p=0.03$) better to RCT than those with low permeability.

1.8.7. Spontaneous apoptosis.

Apoptosis is a cellular death program under physiological control (91) and as such it is often referred to as 'programmed cell death'(92). Apoptosis is mediated by the caspase family after a specific death stimulus for the cell has been given, and it is essential for tissue homeostasis in health and disease(93) (Fig 1.2). The ability to evade apoptosis is thought to be central in both tumorigenesis(94) and resistance to cytotoxic drugs and radiation (95-98). *In vitro* studies suggest high levels of spontaneous apoptosis in tumour cell lines may correlate with their inducible apoptotic rate and determine radiosensitivity (99, 100). As such, a total of 8 studies have investigated whether levels of spontaneous apoptosis in rectal tumours may act as a response predictor to neoadjuvant therapy (36, 55, 57, 64, 65, 67, 69, 101). In 7 of these studies pre treatment biopsies were assessed. In one study only the resected specimen was assessed (57). Patients in all studies received long course neoadjuvant RCT, and hyperthermia regimens were included in two (55, 57).

In 6 of the 7 studies assessing pre treatment biopsies, those with a high spontaneous apoptosis index were found to be significantly more likely to undergo a good response than in those where it was low (36, 55, 64, 65, 69), and this is consistent with patterns observed for other tumour types(102).

1.8.8. Tumour proliferation.

After ionizing radiation, cell death typically occurs at the next attempt of mitotic division (103, 104). Proliferation is upregulated in hypoxic tissues. Proliferation in pre treatment biopsies has hence been studied as a predictive marker in 4 IHC-based studies. (13, 36, 54, 68), 3 of which found no association. In contrast, Kim et al, in a study of 23 patients found that a high proliferative index could predict non-responders from complete or partial responders(68). As this study was conducted in a small cohort, and conflicts with the conclusions from the other reports, it appears unlikely that the proliferation index predicts response.

Table 1.3 Comparison of currently assessed predictive markers.

Marker	Total studies	Detection technique	Predictive value	No predictive value
p53	18	IHC	4	14
p53	4	ssCP	0	4
p53	2	Gene sequencing	2	0
p21	4	IHC	3	1
Bcl 2	8	IHC	1	7
Bax	3	IHC	0	3
EGFR	1	IHC	1	0
COX 2	1	IHC	1	0
MIS	1	PCR	0	1
MMR	2	IHC	0	2
Ku70	1	IHC	1	0
VEGF	1	ELISA	0	1
VEGF	1	IHC	0	1
Apoptosis	8	TUNEL	6	2
Proliferation	4	IHC	1	3

Key: IHC, immunohistochemistry; ssCP, single strand conformational polymorphism analysis; ELISA, enzyme-linked immunosorbant assay; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling; MIS, microsatellite instability; MMR, mismatch repair.

1.9. Biomarker discovery using proteomic analysis of serum.

As serum constantly perfuses through the cells of the body, it is potentially endowed with an archive of histological information consisting of peptides and proteins released into the bloodstream from diseased, dead or dying cells(105). In recent years, the technique of surface-enhanced laser desorption/ionisation incorporating time-of –flight mass spectrometry (SELDI-TOF-MS) proteomic analysis of serum has allowed the large-scale analysis of these proteins and has emerged as a powerful and exciting means of discovering potential disease biomarkers.

This technique employs commercially available ProteinChips® (Ciphergen, CA, USA) which are comprised of a metal bar containing eight 'spots' (Fig 1.5). Each spot on a ProteinChip® has a selective charged surface onto which biological samples, such as serum or urine, can be directly applied. As the spot surface of chips is selectively charged, this allows the selective capture of only a fraction of proteins from a complex mixture. After a sample is applied to the ProteinChip®, a series of wash steps follow that serve to remove non-adherent proteins and any other impurities. An acidic matrix, which is capable of being photoactivated, is then applied to each spot on the chip and allowed to dry. Chips are then loaded into a vacuum chamber and each spot is pulsed with a laser beam (Fig 1.6). This causes activation of the matrix and a resultant desorption of the sample-derived proteins on each spot. Desorbed proteins, now transformed into charged ions, accelerate down the vacuum chamber towards a charged detector plate. The time-of-flight (TOF) of each ion, measured from the ionisation source to the detector plate, is analysed in order to determine its mass to charge (m/z) ratio.

Figure 1.5 - ProteinChips® laid out prior to use. The 8 test spots on each chip are clearly visible (arrow).

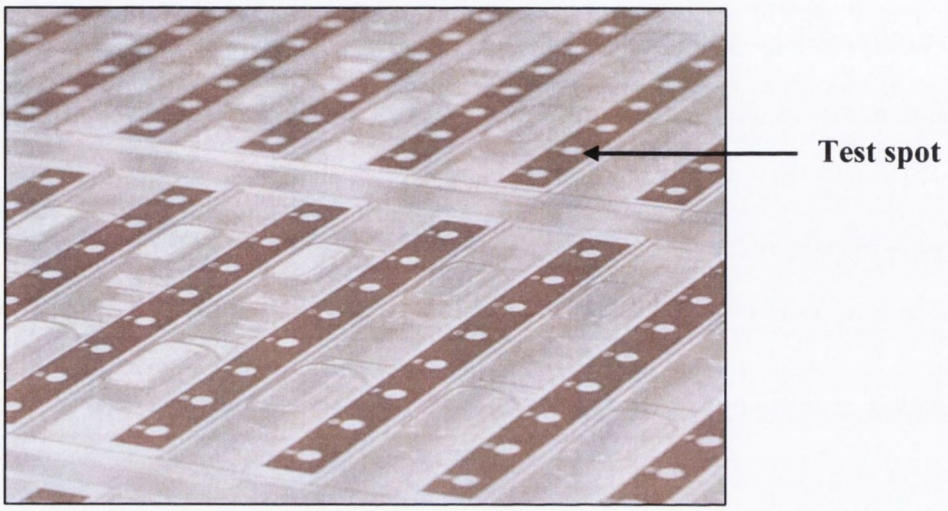
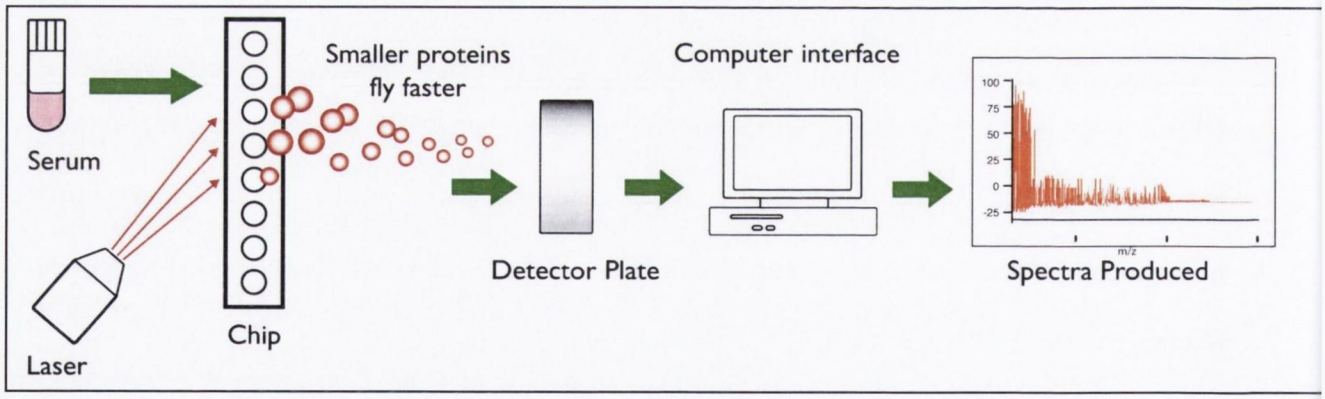


Figure 1.6 Overview of the proteomic process



The prepared ProteinChip is placed inside a vacuum tube and pulsed with a laser. This causes the proteins bound to each spot on the chip to be ionised and accelerate towards the detector plate. The mass and intensity of the ions hitting the detector plate as well as their time of flight are used to generate a spectrum that is representative of the proteins/peptides in each sample.

The m/z of all detected ions in a sample and their relative intensities are fed into a computer workstation and are displayed graphically as a spectrum where each peak represents a protein or peptide (Fig 1.6). Using complex biostatistical software, differences in generated spectral profiles, can be compared between groups of interest e.g. cancer and non-cancer. During this process, characteristic expression differences are sought in order to generate a predictive algorithm. In theory, this algorithm can then be used to prospectively characterise samples in the clinical setting.

1.9.1. History of proteomic analysis

The father of mass spectrometry was Joseph John Thompson, The Cavendish Professor of Experimental Physics at The University of Cambridge in the early parts of of the last century (Fig 1.7). In the years before developing mass spectrometry, he completed a study of cathode rays, which culminated in his discovering the electron in 1897 and in 1906 he received the Nobel Prize for Physics. This was promptly followed by a knighthood in 1908. It is thought that he first discovered the technique of mass spectrometry in 1912 when he developed a method for separating different kinds of atoms and molecules in a vacuum tube using 'positive rays'(106).

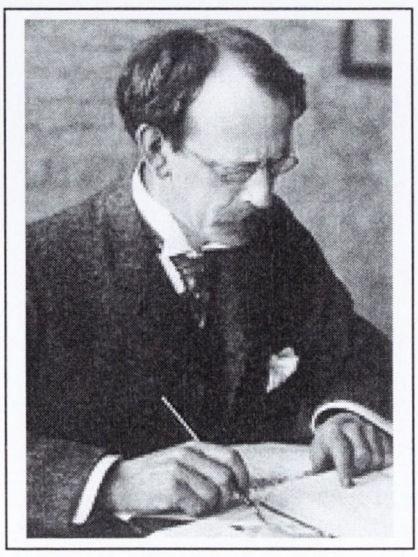


Figure 1.7

J.J. Thompson, the father of modern proteomics at his desk.

For many years, this technique was only used to analyse minerals and small molecules as larger molecules such as proteins and peptides were unable to withstand the harsh ionisation methods available to create the ions.

It was only in more recent years that developments in mass spectrometric techniques allowed the 'soft ionisation' of large biomolecules found in biological samples such as proteins and peptides to be developed. This represented the foundation stone of the emergence of mass spectrometry as a modality with which to investigate biological samples as it allowed principal ions to be assessed without too much fragmentation. Work performed in the 1980s focussed on two main soft ionisation techniques: electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI)(107). Surface enhanced laser-desorption/ionisation incorporating time of flight mass spectrometry (SELDI-TOF-MS) was commercially developed in the early 1990s and has become the mainstay of the 'new-wave' of serum proteomic based biomarker research. It is advantageous over previous methods as it is fast, reproducible, relatively inexpensive, simple to perform, versatile and sensitive to the femtomolar range(107, 108). The potential of the technique to revolutionise modern medicine was recognised in 2002 when John B. Fenn and Koichi Tanaka won the Nobel Prize for Chemistry in recognition of their work in developing modern proteomic methods.

1.9.2. Clinical applications of serum proteomics for biomarker discovery.

An ideal disease biomarker should be measurable in a readily accessible body fluid such as serum, urine or saliva allowing non-invasive and repeatable sampling. As such, in the last few years, there has been a huge amount of interest in the developing field of serum proteomics.

In particular, serum is an attractive biomarker as blood constantly perfuses throughout the tissues of the body and so is potentially a vast repository of cell-derived proteins. An added benefit of this technique is that proteomic methods allow the generation of a 'signature'of response consisting of differential expression of multiple markers. This supersedes the generally unsuccessful earlier studies where the 'one-at-a-time' approach to biomarker identification was used in an attempt to detect a 'key' marker that was pathognmonic for a particular disease(109).

The seminal study, which highlighted the potential power and utility of serum proteomics was published in The Lancet by Petricoin *et al* in 2002(110). This study ran a preliminary 'training set' of proteomic spectra from 50 women with ovarian cancer and 50 women with either benign ovarian disease or no disease. All generated spectra were analysed using an iterative searching algorithm that identified a proteomic pattern that completely discriminated between cancer and non-cancer. This algorithm was then used to classify an independent 'test set' of 116 masked serum samples: 50 and 66 from women with or without ovarian cancer respectively. They found that the algorithm correctly classified all cases of cancer and correctly classified all but 3 samples without cancer. The sensitivity and specificity of their algorithm was 100% and 95% respectively.

Since the publication of this study, there has been an exponential increase in interest in serum proteomics as a tool for biomarker discovery. This is reflected in the increasing numbers of studies assessing the technique as a cancer-screening tool for multiple malignancies including breast, lung, prostate and colorectal. (111-115). Similar to the initial study by Petricoin et al, the majority of these studies have found that serum proteomic patterns provided an excellent platform for cancer screening, generally being able to correctly classify

disease with around 90-95% sensitivity and specificity. At the time of starting this thesis no study had assessed serum proteomics either as a screening methodology for colorectal cancer or as a means of predicting response to RCT.

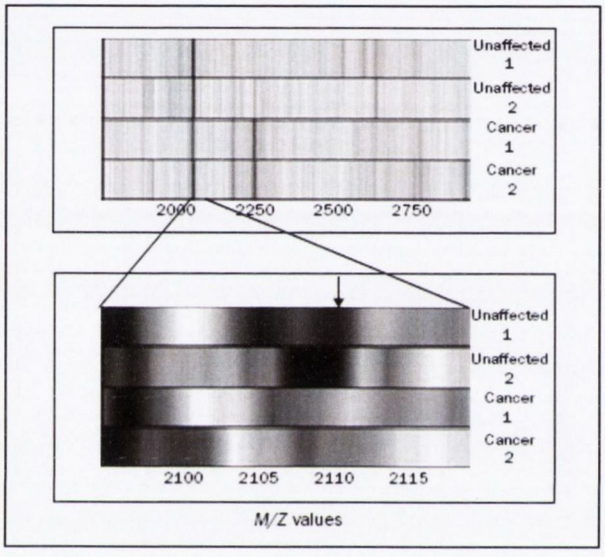


Fig 1.8 – Figure from The Lancet publication by Petricoin et al, 2002.Here differential peak expression between women with ovarian cancer and healthy controls can be clearly seen.

	Classification by proteomic pattern		
	Cancer	Unaffected	New cluster
Unaffected women			
No evidence of ovarian cysts	2/24	22/24	0/24
Benign ovarian cysts <2·5 cm	1/19	18/19	0/19
Benign ovarian cysts >2·5 cm	0/6	6/6	0/6
Benign gynaecological disease	0/10	1/10	9/10
Non-gynaecological inflammatory disorder	0/7	0/7	7/7
Women with ovarian cancer			
Stage I	18/18	0/18	0/18
Stage II, III, IV	32/32	0/32	0/32

Table 2: **Classification of serum samples from masked validation set by proteomic pattern**

Table 1.4 – Table from The Lancet publication by Petricoin et al, 2002. Here classification into disease type can be seen based on proteomic pattern profiling.

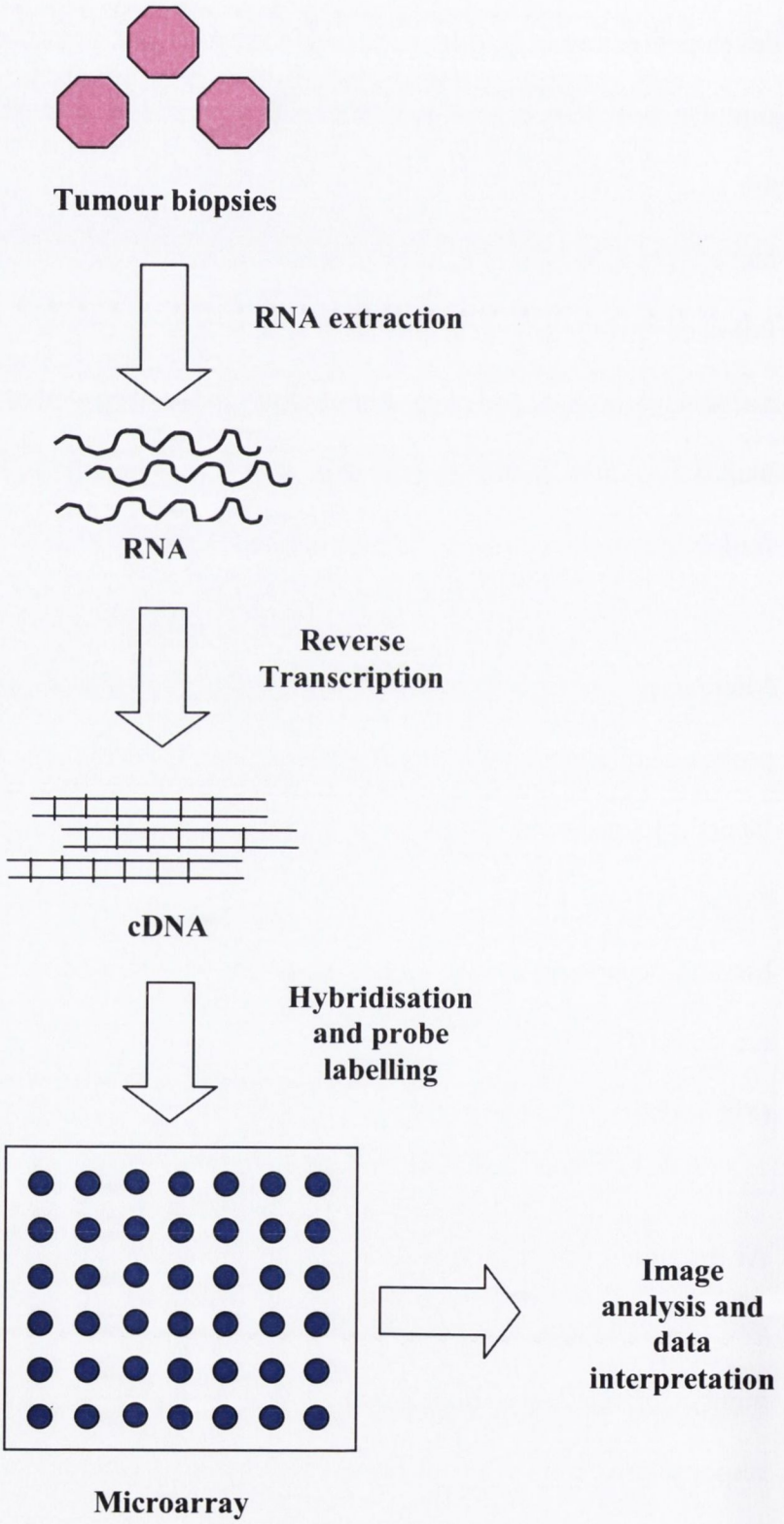
1.10 Global gene expression profiling using microarray chips.

Global gene expression profiling using microarray chips is a powerful and recently developed research tool. In comparison to more traditional molecular techniques that allow simultaneous analysis of only single or small numbers of markers, microarray analysis allows the assay of the complete transcriptional activity in a biological sample across the known human genome(116). In cancer research, parallel comparison of gene expression profiles followed by sophisticated biostatistical analysis has been embraced as a means to identify differences between sub-sets of tumours or how they respond to cytotoxic therapies. By identifying transcriptional differences in this manner, differentially expressed genes can be further targetted as putative disease biomarkers.

Microarrays themselves consist of an orderly framework of tens of thousands of cDNA probes or oligonucleotide targets placed onto a silicone, glass or nylon 'chip' in a series of 'spots'. In order to identify gene expression profiles, high quality RNA must first be extracted from tumours, converted to cDNA and then hybridised onto the chips. Here, binding occurs to specific targets based on base-pairing rules and relative bound quantities are detected using either fluorescence or chemiluminescence using image analysis software (Fig 1.9).

At the time of starting this thesis, gene expression profiling using cDNA microarrays was still in its infancy. Although the clinical utility of microarrays had been demonstrated in a number of relatively small but high profile clinical studies, it had not been assessed as a response predictor for rectal cancers undergoing RCT.

Fig 1.9. Flow diagram outlining the steps required for microarray analysis.



One of the first reports showing the huge clinical potential of microarrays in biomarker research was in hereditary breast cancer by Hedenfalk et al in 2001(117). This group compared the gene expression profiles of 7 carriers of the BRCA1 mutation, 7 carriers of the BRCA2 mutation and 7 cases of sporadic breast cancer. They found that each of the 3 groups had significantly different gene expression profiles and that 176 genes were differentially expressed between patients with BRCA1 mutations when compared to BRCA2 mutations. They concluded that heritable mutations influenced the gene expression profiles of tumours.

In a much larger study in 2002, Rosenwald et al used gene expression profiling to identify predictors of survival after chemotherapy for patients with diffuse large-B-cell lymphoma(118). This study included samples of 240 patients. This was split into a training set of 160 patients on which a predictive algorithm was generated. This was then validated on the further 80 patients not included in the original analysis. The study found 3 gene expression subgroups able to differentiate between germinal-centre B-cell-like, activated B-cell-like and type 3 diffuse large-B-call lymphomas respectively. In addition, 2 common oncogenic events in diffuse large-B-cell lymphome, bcl-2 translocationa and c-rel amplification, were detected only in germinal-centre B-cell-like subgroup. Patients in this subgroup had the highest 5-year survival rate. They also went on to classify tumours based on the differential expression profiles of 17 genes and were able to construct an independent predictor of overall survival.

Following on from this work, the first study that identified the potential for global gene expression changes to occur during cytotoxic therapy was by Buchholz et al in 2002(119). Here, comparative microarray analysis was performed on core biopsy specimens from tumours obtained before and 24 and/or 48 hours after initiation of neoadjuvant chemotherapy

for primary breast cancer treatment from 21 patients. Although the sample size of this study was too small to reach statistical significance, it found that in patients who ultimately had a favourable pathological response after chemotherapy, gene expression patterns that clustered distinctly from poor responders were identified in pre treatment biopsies. Another interesting finding in this study was that that significant transcriptional responses could be identified in tumours after the onset of chemotherapy.

At the time of starting this thesis, there were no published reports of using gene expression microarrays to predict response to RCT in rectal cancer. It was therefore our aim to develop the technique to identify gene expression differences between good and poor responders to RCT in order to identify response predictive biomarkers. During the course of this research, however, Ghadimi et al published the first such study(120). Pre -treatment tumour biopsies from 30 patients who were randomised to receive RCT as part of the German Rectal Cancer Study(121) were studied. Response to treatment was measured both by TRG and T stage change. Using class comparison analysis between gene expression profiles from an initial run of 23 responsive or non-responsive tumours, 54 genes that were differentially expressed at significant levels ($p<0.01$) were identified. The ability to use these gene expression profiles to predict response was then assessed using leave one out cross validation. This generated an algorithm that allowed tumour behaviour to be predicted with 78% sensitivity and 86% specificity.

This algorithm was then further verified using a further 7 tumour samples hybridised on an alternative cDNA microarray platform which was able to correctly predict the response of 6 of 7 patients.

Of note, this study found that gene expression differences were only apparent between tumours that underwent a decrease in T stage, but no significant differences were observed for tumours with different TRGs.

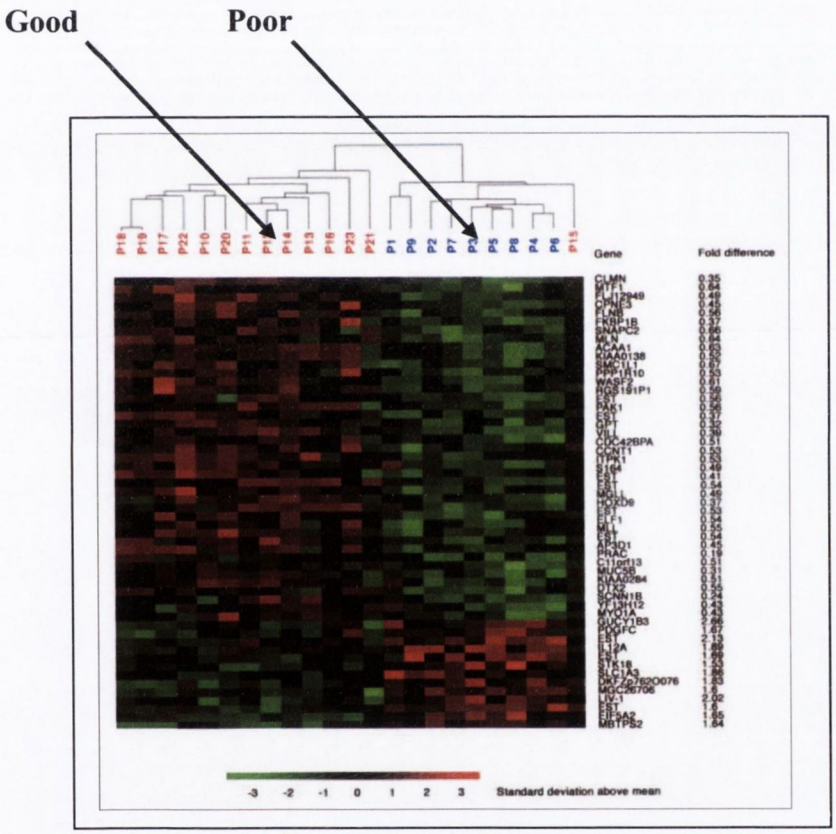


Figure 1.10. Pictoral representation of hierarchical cluster analysis of 23 patients.

Based on the 54 most significantly changed genes (P<0.001) taken from Ghadimi et al. Red indicates increased expression and green indicates decreased expression. Clear gene clustering can be seen between response groups.

Chapter 2

Aims and objectives

2.1 Overview of aims of thesis

The aim of the work contributing to this thesis was primarily to develop the existing means to predict response of patients with rectal cancer to RCT. One of the main emphases of this thesis, however, was the development of translational research methods, which at the time of starting this work were unassessed in rectal cancer. One aspect of the thesis veered away from response prediction. Here we assessed the potential for serum proteomic analysis to be used as a minimally invasive population-screening tool for colorectal cancer. Although this was not directly linked to the principal aims of this thesis, the opportunity to perform this study at the NCI arose and its huge clinical potential prompted us to pursue it.

2.2 The specific aims of this thesis were:

Chapter 3:

To assess the expression of COX 2, survivin, Ki67 and apoptisis in archival pre treatment rectal cancer biopsy tissues as response predictors to RCT.

Chapter 4:

To analyse serial serum profiles from patients with rectal cancer undergoing RCT using SELDI-TOF-MS and employ biostatistical analysis to identify diagnostic proteomic patterns capable of predicting response.

Chapter 5:

To collect serum from a large cohort of patients with no colonic disease, adenomatous polyps and colonic malignancy. To then analyse this serum using SELDI-TOF-MS and employ biostatistical analysis to identify diagnostic proteomic patterns capable of discriminating cancer from non-cancer.

Chapter 6:

To collect fresh, pre treatment biopsies from patients who were to undergo RCT for analysis using gene expression microarrays. To then perform parallel expression analysis in order to identify differential gene expression profiles between responsive and non-responsive patients

Chapter 7:

To determine the staging accuracy of post RCT MRI scanning and determine whether an association between MRI staging and tumour regression existed.

Chapter 3

Immunohistochemical analysis of response predictors for rectal cancer undergoing neoadjuvant radiochemotherapy.

3.1 Introduction

Current research is focussed on the identification of inherent molecular differences between pre treatment tumour biopsies of responders and non-responders to treatment. To date, most research has focussed on constituents of the p53 pathway but despite an increasing number of studies, it is still undecided whether or not these may be used to predict response(122, 123). Molecular markers representing alternative pathways are therefore being sought. Two such molecular markers, as yet unassessed in the setting of rectal cancer, are cyclo-oxygenase 2 (COX 2) and survivin.

3.1.1 COX 2 as a response predictor

COX 2 is an important mediator of tumour invasiveness and metastasis and is one of 3 known isoforms of the enzyme which catalyses the conversion of arachidonic acid to prostaglandins (PG). It is known that tumour cells can use COX 2 to produce PGs, especially PGE2, after exposure to radiation. They are then able to use these compounds as survival factors to protect against radiation-induced cell death (124). Recent *in vitro* and *in vivo* studies have demonstrated that the addition of COX 2 inhibition, in conjunction with radiation, can significantly enhance tumour response by blocking prostaglandin release(125). In addition, clinical studies in laryngeal (126) and cervical cancer (127, 128) have assessed whether endogenous COX 2 expression in PTBs may be indicative of how they respond to radiation and chemotherapy. These studies all found that COX 2 expression was associated with a poor response to treatment and an associated unfavourable prognosis.

3.1.2 Survivin as a response predictor

Survivin is a structurally unique member of the inhibitor of apoptosis (IAP) family. It is expressed in embryonic and foetal development but not in fully differentiated adult tissues except for the thymus and placenta(129). It blocks apoptosis by inhibiting the activation of caspase-3 and –7 and regulates the cell cycle in the G2/M phase(129). The protein is prominently re-expressed in several tumour types including those of the lung, breast, prostate and colon/rectum(130). As a result of its selective expression in malignant cells but not normal adult tissues, survivin has generated much interest as a pharmacological target for modulating tumour apoptosis.

Rodel et al assessed survivin protein expression by Western blotting and survivin mRNA expression by quantitative TaqMan reverse transcription polymerase chain reaction in 3 different colorectal cancer cell lines of different intrinsic radiosensitivity. They found that in radiosensitive lines there was higher spontaneous and radiation-induced apoptosis as compared to the radiosensitive cell lines. This corresponded to a higher basal and radiation induced levels of survivin protein in radioresistant cell lines whereas survivin levels were low when untreated and not increased after irradiation in radiosensitive cell lines(131).

Knutsen et al found that patients with tumours where >50% of cells expressed survivin protein had poorer overall survival after neoadjuvant radiotherapy compared to patients with <50% staining(129). Rodel et al found a similar trend in 54 patients treated with neoadjuvant RCT(64). Here low survivin expression was significantly related to an increased rate of spontaneous apoptosis in pre treatment biopsies, increased disease-free survival rate and to a

decreased risk for distant metastases. Neither of these studies, nor any other study to date has assessed the effect of survivin overexpression on histological tumour response.

3.1.3 Apoptosis and proliferation as response predictors

The levels of apoptosis and proliferation in a tumour represent cell death and regeneration respectively. Both of these indices are therefore commonly assessed in studies assessing putative response predictor proteins in an attempt to relate their effects on baseline tumour growth characteristics(132).

3.2 Aims

1. To assess whether endogenous COX 2 and survivin expression in pre treatment tumour biopsies could be used to predict pathological response in rectal cancers undergoing neoadjuvant RCT.

2. To assess whether tumour growth kinetics measured by apoptosis and proliferation could be used to predict pathological response in rectal cancers undergoing neoadjuvant RCT.

3. To assess whether COX 2 and survivin expression were inter-related.

4. To identify whether overexpression of COX 2 or survivin influenced tumour apoptosis or proliferation.

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3.3 Patients and methods

3.3.1 Patient selection and procurement of research tissue

Before any aspect of this study began, written ethical approval was obtained from the local ethics committee. As the ethics jurisdiction was shared between both St James's Hospital (SJH) and the Adelaide and Meath incorporating the National Children's Hospital (AMNCH) all patients who had received neoadjuvant radiochemotherapy in both institutions were sourced. This was done using a colorectal database.

Once identified, original diagnostic H+E stained biopsy slides were obtained from the histopathological archives. After ensuring adequate tumour for study was present in diagnostic biopsy slides, archival wax-embedded blocks were retrieved and 4μm sections were cut from these onto silane-coated slides (cutting done by FMS). These were placed in an oven at 50 degrees Celsius overnight and then refrigerated at four degrees Celsius until ready for use.

3.3.2 Pre and post treatment staging

All patients included in this study were staged according to the TNM classification. Pre treatment staging consisted of a CT scan of chest, abdomen and pelvis in all patients followed by local staging using pelvic MRI scanning or transrectal ultrasonography (TRUS) either alone or in combination.

In order to grade the degree of pathological response, diagnostic slides from resected specimens of all patients were retrieved. These included no less than three sections of the irradiated area per patient. A single consultant pathologist blinded to patient identifying characteristics reassessed all sections.

A TRG of 1-5 based on scoring system previously described by Mandard(19) was given to each based on the relative amounts of residual tumour and fibrosis (Fig 1.2). For analysis in this study, patients with a TRG of 1 or 2 were deemed to have demonstrated a good response, those with a TRG 3 a moderate response and those with TRG 4 and 5, a poor response.

3.3.3 Neoadjuvant regimen

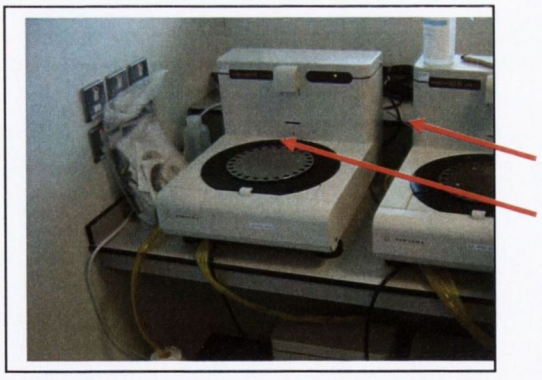
Before receiving RCT, all patients had been discussed at a weekly multi-disciplinary meeting. All but one patient was staged as cT3/4 ± lymph node positivity. The patient staged as T2 included in this study was found to have a fixed tumour at the time of surgery. The initial operation was therefore cancelled and the patient referred for RCT.

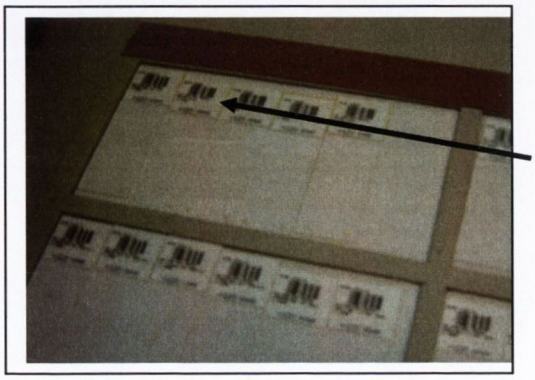
All patients were treated at St Lukes's Hospital, Rathgar, Dublin. The treatment regimen received by all patients consisted of 40-45Gy radiotherapy delivered on five days for five weeks. Continuous IV 5- fluorouracil 225mg/m^2/day was given concurrently with radiotherapy via a syringe driver. Surgery was performed approximately six weeks after the last dose of radiation had been given.

3.3.4 Immunohistochemical staining for COX 2

Dewaxing, rehydration and antigen retrieval was performed using Trilogy Solution (Cell Marque, Hot Springs, AK) at 1:20 dilution in a pressure cooker for 15 minutes. Endogenous peroxidase activity was blocked using 0.1% hydrogen peroxide (Sigma-Aldrich, St Louis, MO) in industrial strength methylated spirits solution for 30 minutes. Non-specific binding was blocked using an avidin-biotin blocking step (Vector Blocking Kit, Vector Labs, Burlinghame, CA). The remainder of staining was performed using a Ventana Nexes I-view machine (Ventana medical systems, Tucson, Az) (Fig 3.1)

Figure 3.1 - Nexes I-View automated staining machine, equipment and barcode labelled slides.





All slides were incubated with 1:200 mouse anti-COX 2 monoclonal (Zymed, San Francisco, CA) for 1 hour at room temperature. Antibody binding was visualised using DAB (Fast-tab, Sigma Aldrich). Sections were lightly counterstained in haematoxylin and then dehydrated through a series of alcohols and xylene before being mounted under a cover slip. Positive and negative controls were run with each batch of staining. These consisted of squamous cell carcinoma of skin and omission of the primary antibody respectively.

3.3.5 Immunohistochemical staining for survivin

Tissue sections were taken to water by immersing in 3 changes of xylene for 20 minutes. They were then placed in 95% alcohol for 5 mins, 70% alcohol for 5 mins then tap water for a further 5 mins. Endogenous peroxidases were blocked in 0.1% hydrogen peroxide in industrial methylated spirits (IMS) for 30 minutes (Sigma-Aldrich, St Louis, MO, 30% W/W solution). Sections were then immersed in fresh IMS for a further 10 mins followed by immersion in tap water for 10 mins. Antigen retrieval was performed by boiling sections at full pressure for two minutes in a stovetop pressure cooker using Antigen Unmasking Solution (Vector Labs, Burlinghame, CA). Sections were then cooled in running tap water. Non-specific binding was blocked using 1% BSA (Dako Cytomation, Glostrup, Denmark) for 10 minutes and normal goat serum in 1:10 dilution (Dako Cytomation) for 20 minutes. Primary antibody used was the polyclonal anti survivin antibody Ab469 (Abcam, Cambridge, UK). This was applied in a ratio of 1:1000, diluted in phosphate-buffered saline (Sigma-Aldrich, St Louis, MO). This was left at room temperature for 2 hours then placed in a refrigerator overnight. The following day, sections were washed 3 times in PBS (Sigma-Aldrich, St Louis, MO) for 5 minutes each.

Secondary antibody biotinylated rabbit anti goat (SCBT, Santa Cruz, CA) was then applied at a concentration of 1:800 for 30 minutes. Antibody binding was visualised using DAB Fast-tab (Sigma-Aldrich, St Louis, MO). Sections were lightly counterstained in haematoxylin and then dehydrated through a series of alcohols and xylene before being mounted under a cover slip. Positive and negative controls were run with each batch of staining. These consisted of a colonic tumour known to express survivin and a section of normal colonic mucosa that did not express survivin.

3.3.6 TUNEL staining for apoptosis

Tissue sections were dewaxed in three changes of xylene then rehydrated through a series of graded alcohols. Sections were incubated with 20μg/ml proteinase K (DAKO, Carpinteria, CA) for 15 minutes then rinsed in distilled water. Endogenous peroxidases were blocked in 0.1% hydrogen peroxide (Sigma-Aldrich, St Louis, MO) in industrial strength methylated spirits (IMS) for 30 minutes. Samples were then incubated with equilibration buffer for 30 seconds before the application of TdT enzyme for 1 hour in a humidified chamber. Slides were then put into working strength stop/wash buffer at room temperature for 10 minutes and incubated with antidigoxigen-peroxidase for 45 minutes. Each step was separated by careful washings in PBS (Sigma-Aldrich, St Louis, MO). Antibody labelling was viewed using DAB. All sections were counter-stained using methyl green. Positive control was provided in the kit and was derived from a rat mammary gland.

3.3.7 Immunohistochemistry for Ki67 (proliferation)

Tissue sections were dewaxed in three changes of xylene then rehydrated through a series of graded alcohols. Endogenous peroxidases were blocked in 0.1% hydrogen peroxide (Sigma-Aldrich 30% W/W solution) in industrial strength methylated spirits for 30 minutes.

Antigen retrieval was performed by pressure-cooking sections at full pressure for two minutes in Antigen Unmasking Solution (Vector Labs, CA). They were blocked with 1% BSA and 1:250 dilution of primary antibody applied for one hour at room temperature. Antibody binding was localised using DAB. Sections were lightly counterstained in haematoxylin and then dehydrated through a series of alcohols and xylene before being mounted under a coverslip. Positive and negative controls were provided by normal colon and omission of primary antibody

3.3.8 Scoring systems for immunohistochemical staining

3.3.8a COX 2

Sections were scored by assessing the quantity and intensity of staining. Brown, granular, cytoplasmic staining was deemed positive. Interpretation was performed by a single observer (FMS) and equivocal cases reviewed with a consultant pathologist (EWK). Intensity of staining was graded semi-quantitatively in four grades 0: nil, 1: weak, 2: moderate, 3: strong. Extent of staining was divided into four groups; (0: nil, 1: <10%, 2:10-50%, 3: >50%). These were multiplied to give a raw score for each case. Based on this, sections were divided into four groups of incremental COX 2 staining. Nil (0/9), Light (1-3/9), Moderate (4-6/9) and strong (7-9/9).

This scoring system was based on those used in previously published studies(133, 134). For the purposes of analysis, only cases with strong COX 2 expression were deemed to have overexpression.

3.3.8b Survivin

Sections were scored by counting both nuclear and cytoplasmic survivin staining. Nuclear staining was expressed as the percentage of positively staining cells per 1000 cells using high power light microscopy. Cytoplasmic staining was expressed in the 4 grades: 1- Nil, 2-Light, 3-Medium, 4-Strong. For the purposes of analysis and based on previous studies, an arbitrary value of over 15% nuclear staining was chosen above which cases were deemed to be positive for survivin staining(135). This allowed the division of all cases stained into two approximately equal groups.

3.3.8.c TUNEL

An apoptotic index (AI) was ascribed to each section by counting the percentage of apoptosomes per 1000 cells using high power light microscopy. Apoptotic cells were counted on the basis of their morphology as well as positive DAB staining.

3.3.8.d Proliferation/Ki67

A proliferative index (PI) was ascribed to each section counting the percentage of positively staining cells per 1000 cells using high power light microscopy. (102).

3.3.9 Statistical analysis

Statistical analyses were performed using SPSS Version 6 (SPSS, Chicago, IL). Only p values of <0.05 were considered significant.

3.4. Results

3.4.1. Pathological demographics and tumour response rates.

A total of 49 patients had pre treatment biopsies and corresponding tumour specimens available for analysis. All had RCT followed by surgery with curative intent. Complete pathological response pCR (TRG 1) characterised by complete absence of residual tumour, was observed in 5 (10%) of patients and near pCR (TRG2), was observed in a further 16 (33%) patients. This gave an overall good response rate of 43% in this study. Of the remaining 57% of patients, 11 (22%) showed moderate response (TRG 3) and 17 (35%) patients showed poor response TRG 4 (Table 3.1). Of note, no patient was found to have a TRG of 5.

3.4.2 Results from COX 2 immunohistochemistry

Most tumours (94%) expressed COX 2 to some degree. Within tumour cells, the most common pattern of COX 2 immunoreactivity was diffuse, granular, cytoplasmic staining (Fig 3.2). A total of 70% of tumours expressed COX 2 at a moderate to strong level, consistent with other published literature in colorectal cancer (134). For purposes of analysis, however, only those patients demonstrating strong expression were classified as having COX 2 overexpression, again consistent with previously published studies (128).

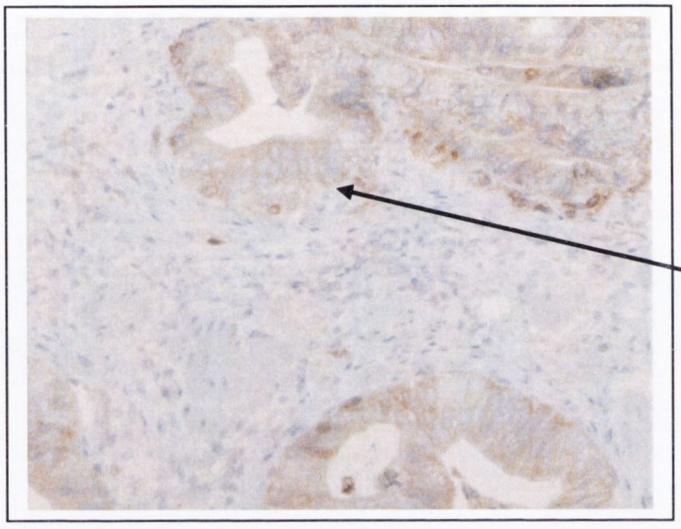
In the radiosensitive group (TRG 1+2) only 5 of 21 (24%) of patients overexpressed COX 2. In comparison, 5 of 12 patients (41%) with intermediate response (TRG 3) and 10 of 21 (48%) patients with poor response (TRG 4) showed COX 2 overexpression. On statistical analysis, pre treatment biopsies where COX 2 was overexpressed were found to be more likely to demonstrate a poor response to RCT (p=0.026 Chi Square) (Fig 3.3)

Table 3.1. Patient and tumour characteristics in IHC analysis study group.

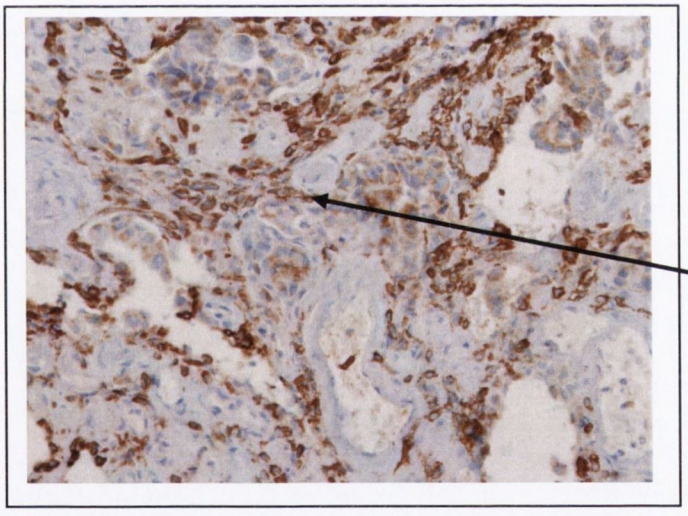
	Category	Number of patients
Patients	Male	31
	Female	18
Investigations	CT	49
	MRI	29
	TRUS	24
Pretreatment T stage	2	1
	3	43
	4	5
Pretreatment N stage	0	14
	1	34
	2	1
Operation type	AR	39
	APR	10
pT	0	5
	1	4
	2	7
	3	26
	4	6
pN	0	32
	1	11
	2	5
TRG	1	5
	2	16
	3	11
	4	16
	5	0

Abbreviations: APR = abdominoperineal resection; AR = anterior resection; CT = computed tomography; MRI = magnetic resonance imaging; TRUS = transrectal ultrasound; TRG = tumor-regression grade.

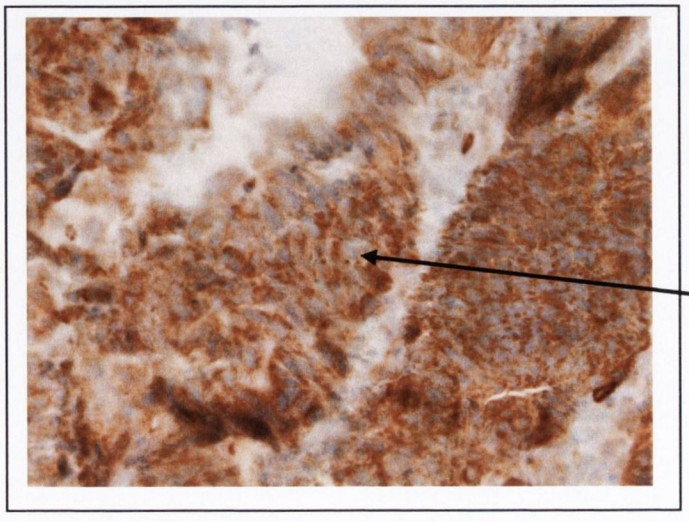
Figure 3.2. Photomicrographic examples of COX 2 staining in pre-treatment rectal cancer biopsies.



Weak cytoplasmic COX 2 staining (X20)

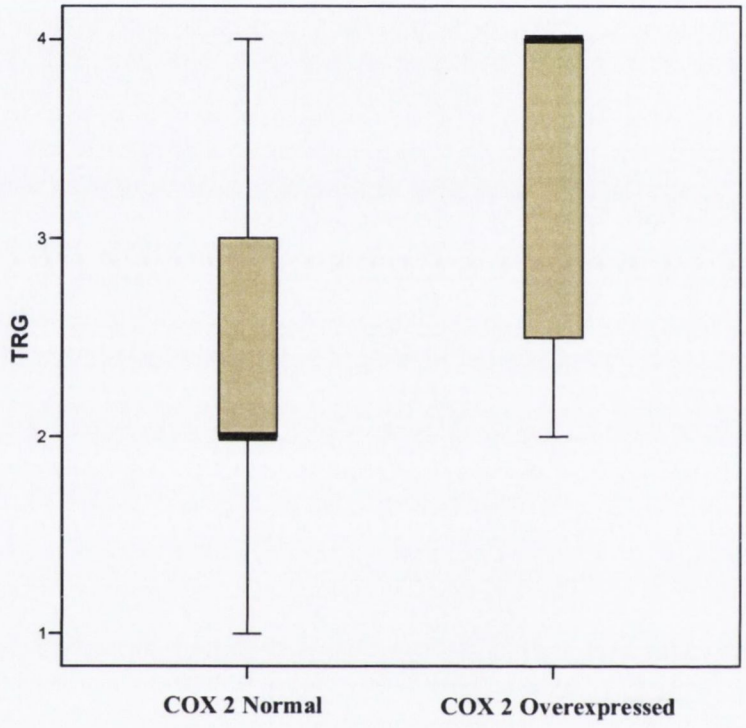


Moderate cytoplasmic staining (X20)



Intense cytoplasmic staining (X20)

Fig 3.3. Boxplot comparing COX 2 status with TRG. Note that TRG is significantly poorer in tumours overexpressing COX 2.



P=0.026

3.4.3 Results from survivin immunohistochemistry

Initially, we encountered several difficulties in optimising the survivin stain. Firstly, we could not get any positive staining from the original monoclonal antibody that we had selected (Survivin-D8 (SC-17779) SCBT, Santa Cruz, CA) so we contacted a local group who had successfully used a polyclonal goat anti-survivin antibody(136). We found that this antibody initially worked well but we experienced a large amount of non-specific background staining.

After titrating the dilution of both the primary and secondary antibodies and experimenting with different primary antibody incubation periods, we finally found that the stain worked well. Because of the initial difficulties encountered with this stain, however, only a small pilot study consisting of tissue from 22 randomly selected patients was initially performed.

Positive nuclear survivin staining was seen as dark brown stained nuclei that were easily distinguishable from adjacent unstained nuclei (Fig 3.4). All tumours (100%) expressed nuclear survivin staining ranging from 3-50% of cells. There was neither significant differences nor apparent trends in nuclear survivin expression between different response categories ($P>0.05$) (Fig 3.5). As a result of this disappointing pilot study, no further survivin IHC was performed.

Fig 3.4. Photomicrographic example of survivin staining (x20)

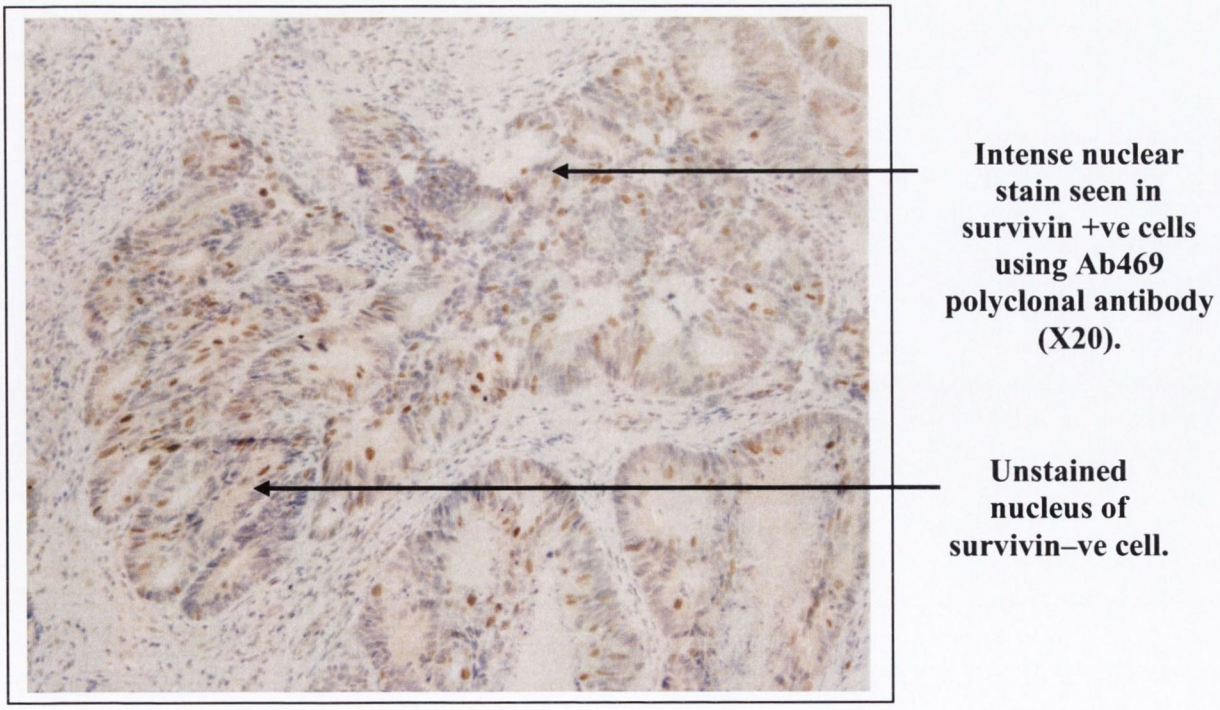
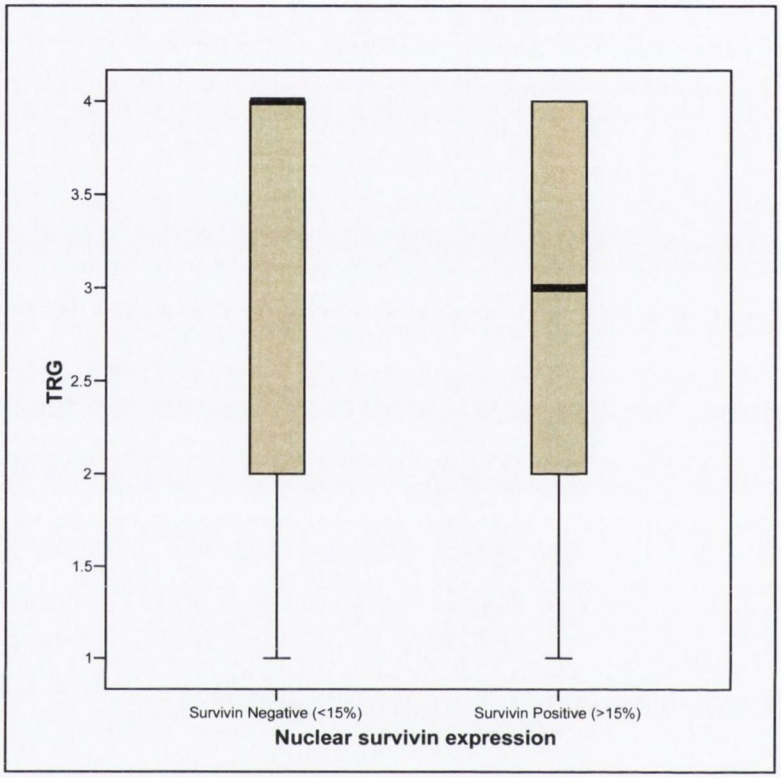


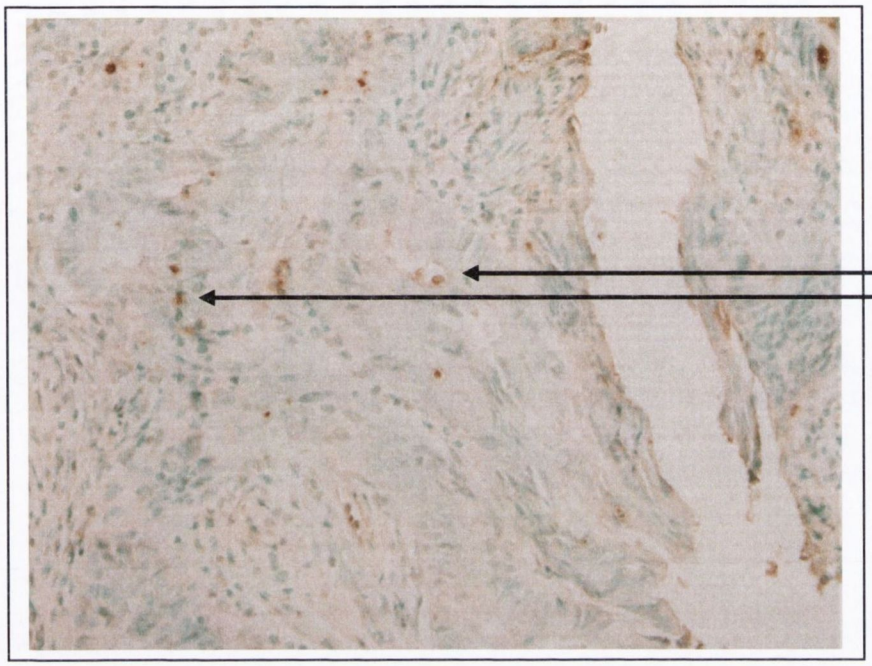
Figure 3.5. Boxplot comparing nuclear survivin staining to TRG. No significant difference was seen between groups.



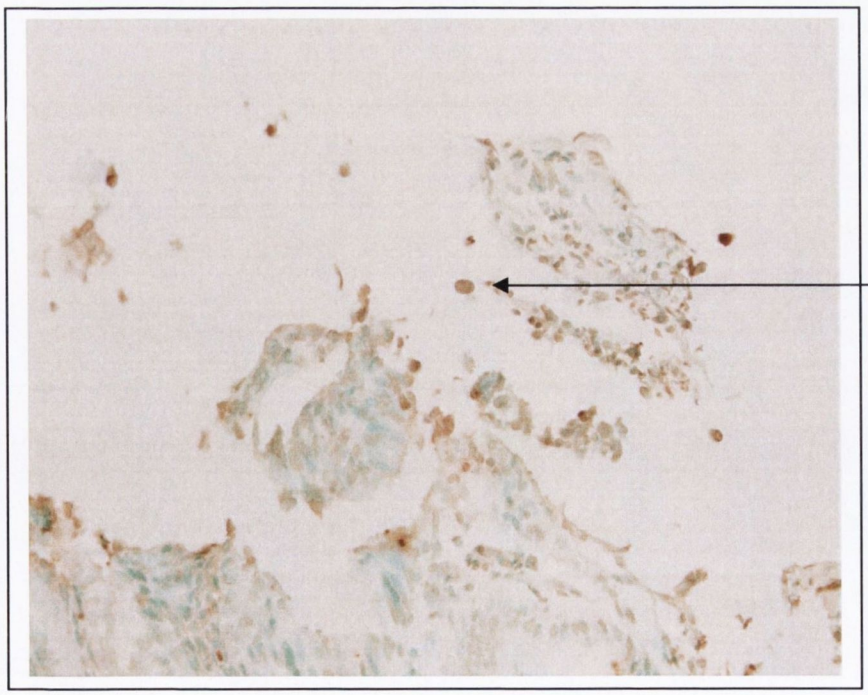
3.4.4 Results from TUNEL staining for apoptosis.

Apoptotic cells were found in all pre treatment biopsies. Histologically, these were seen as shrunken, pyknotic cells which stained intensely with DAB (Fig 3.6a). Of note, apoptotic cells in or close to areas of necrosis were not counted to avoid the risk of false positive staining (Fig 3.6b). Between TRG categories, a wide variability in the range of AI was seen (Table 1). Tumours demonstrating pCR had the highest rates of AI whereas those with TRG 4 had the lowest rates. This was confirmed on statistical analysis where a strong statistical difference in AI even across individual TRG categories was found (p=0.0007). (Fig 3.7).

Figure 3.6 a and b - Photomicrograph demonstrating *in situ* apoptosis detection using T.U.N.E.L. stain.

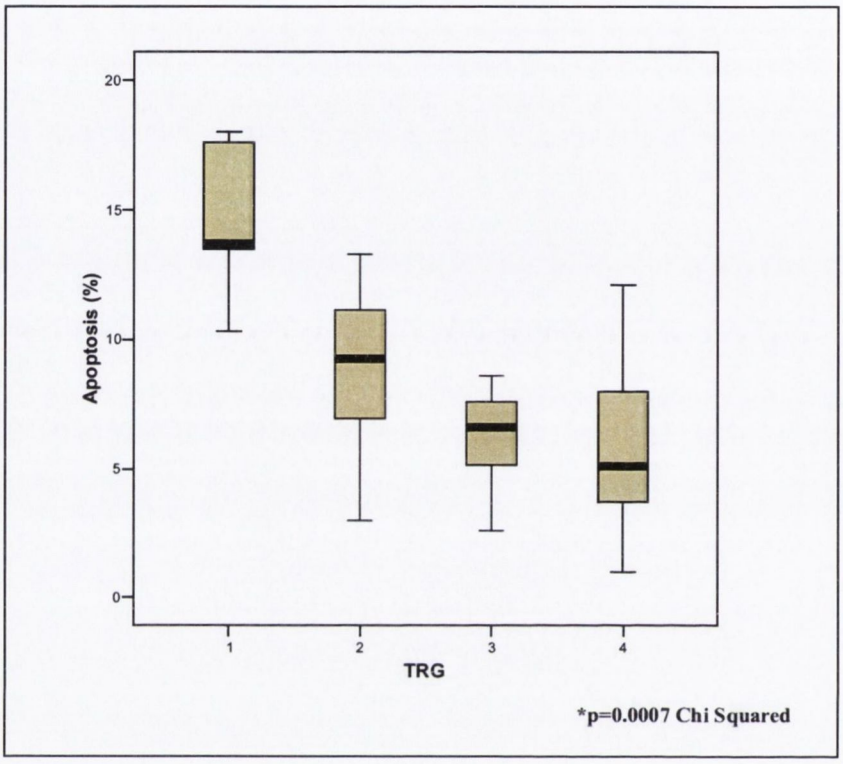


T.U.N.E.L. positive cells showing intense nuclear staining (X20)



Example of artefactual T.U.N.E.L. positivity at the edge of tissue. These cells were excluded from all counts.

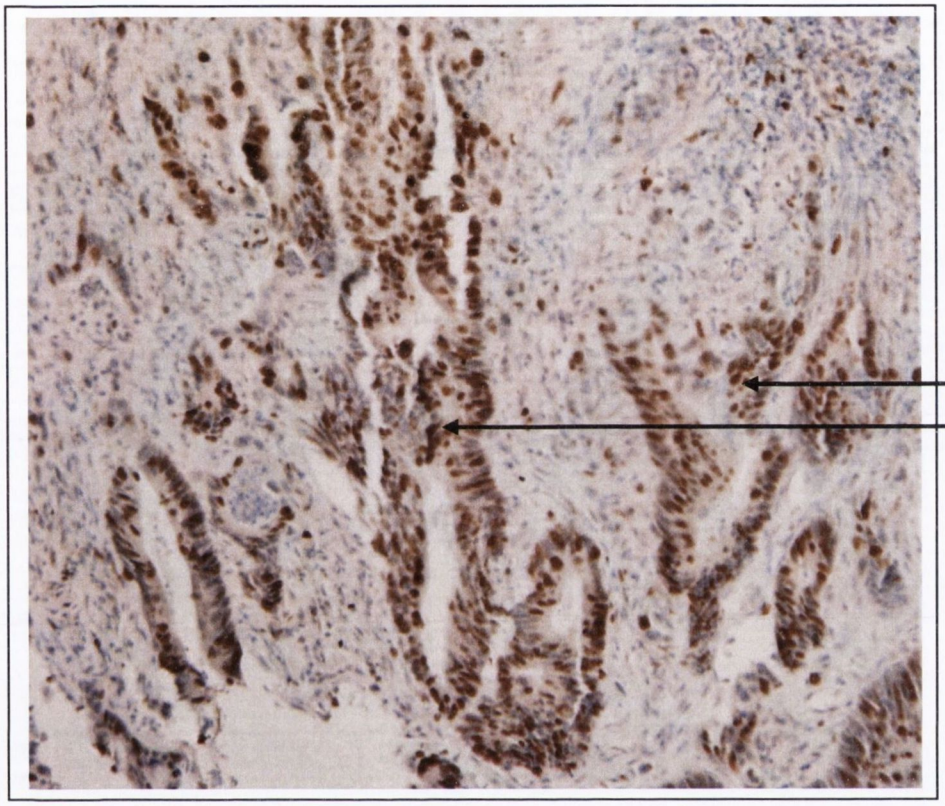
Figure 3.7. Boxplot of TRG vs % apoptosis. There was a strong statistical association where high levels of apoptosis were associated with a good response.



3.4.5 Results from Ki67 staining for proliferation

Ki67 was detected by an intense brown nuclear stain (Fig 3.8). It was expressed in all PTBs and varied little between TRG categories. Statistical analysis was performed to assess whether proliferative index (PI) could be used to predict response. This found that there was no statistical difference in PI between different TRG categories. ($P>0.05$ Chi Square).

Figure 3.8. Photomicrograph of Ki67 (Proliferation) stain.



3.4.6 Interrelation of COX 2, Survivin, TUNEL and Ki67.

To identify whether overexpression of COX 2 or survivin influenced tumour apoptosis or proliferation, statistical analysis was performed. This showed that there was no interrelation between any of these variables (P>0.05 Chi square) (Tables 3.2 and 3.3)

Table 3.2. Table of COX 2 staining, apoptosis and proliferation separated by TRG. (49 patients)

TRG	No cases	COX 2 over expressed (Cases)	COX 2 Normal (Cases)	Median (min-max) apoptosis	Median (min-max) Ki 67
1	5	0	5	13.7 (10.3-18.0)	47.0 (44.0-64.0)
2	16	5	11	9.5 (3.0-17.5)	56.0 (32.0-90.0)
3	12	4	7	6.7 (2.6-15.4)	52.5 (23.0-74.0)
4	16	10	7	5.1 (1.0-16.0)	52.0 (16.0-66.0)
5	0	N/A	N/A	N/A	N/A

P=N.S

Table 3.3. Table of survivin staining, apoptosis and proliferation separated by TRG. (22 patients)

TRG	No Cases	Survivin Nuclear (%)	Survivin Cyto (Average Intensity)	Median (min-Max) apoptosis	Median (Min-Max) Ki67
1	2	14.0(10.0-18.0)	1.5	15.1 (13.5-18.0)	44.5 (44.0-45.0)
2	6	23.0(3.0-50.0)	1.6	10.2 (5.4-17.5)	52.3 (35-70)
3	4	16.5(15.0-19.0)	1.3	5.4 (3.3-6.0)	59.5 (50-74)
4	10	13.1(3.0-25.0)	1.2	5.8 (2.0-11.7)	57.6 (44-74)
5	0	N/A	N/A	N/A	N/A

P=N.S

3.5 Discussion

Molecular differences between the pre treatment biopsies (PTB) of good and poor responders are being actively sought in order to explain the variability in response seen after RCT. This study of 49 patients is the first to assess whether endogenous COX 2 and survivin staining may be used to predict histological response to RCT in patients with rectal cancer. In this study, COX 2 but not survivin overexpression was significantly associated with a poor response to RCT, a finding that may be clinically important for several reasons.

Because there is currently no means of predicting response to treatment, all patients must undergo empirical RCT and surgical treatment. Being able to identify patients with adverse molecular tumour characteristics before treatment would allow them to be selected for alternative treatment regimens aimed at improving their response. COX 2 expression is therefore an ideal response predictor, as COX 2 inhibition already exists as a therapeutic modality with comparatively cheap and widely available inhibitors already in the marketplace. Although there have recently been concerns over the long term safety of COX 2 inhibition, there are no data to suggest that short term useage is harmful(137). Accordingly, there may be therapeutic rationale in using COX 2 inhibitors either before or during treatment in patients found to over express COX 2 in an attempt to improve overall rates of response. This makes COX 2 a considerably more attractive response predictor in comparison to previously studied predictors such as components of the p53 pathway as they are not amenable to pharmacological manipulation.

High levels of spontaneous apoptosis were also associated with a good response to radiation in this study, a result mirrored in other published reports(132). Statistical differences were

found even between individual TRGs. Whether levels of spontaneous apoptosis alone in PTBs could be used to withhold empirical treatment of patients is uncertain as our data highlight that analysis of individual cases reveals that equally high levels of apoptosis were found in some biopsies from patients who were TRG 1 and TRG 4 (Fig 3.7). This high degree of variability therefore precludes measurement of spontaneous apoptosis alone from being useful in the clinical setting. Discovery of molecules regulating spontaneous apoptosis in tumours before treatment, however, may help elucidate pathways of response. To this end, we hypothesised that COX 2 overexpression might influence spontaneous apoptosis in tumours but statistical analysis of our data showed that no such relationship existed.

This result may be explained by previously published work by Kishi et al(125). In a murine model they showed that addition of a specific COX 2 inhibitor significantly enhanced tumour radioresponsiveness, but its effects were independent of radiation-induced apoptosis. Instead they found that COX 2 inhibition caused decreased tumour vascularisation, which was associated with tumour growth retardation. More recently, Davis et al have further explored this phenomenon in another murine model(138). Here they used contrast enhanced MRI to directly assess vascular function in experimentally induced tumours undergoing radiotherapy with and without COX 2 inhibition. They found that animals receiving COX 2 inhibition demonstrated increased vascular permeability 24 hours after radiation compared to those that did not. They propose that this represents greater damage to the tumour neovasculature and that subsequent maintenance of COX 2 inhibition may impair tumour neovascularisation and regrowth after treatment.

Whilst it is of biological and clinical interest that COX2 over expression is able to predict a poor response to RCT, it is unlikely that this observation alone will prove rigorous enough to

guide clinical management. More likely it represents an important, but not exclusive mechanism in the cellular response to radiation. In fact, the concept of using any single marker is unlikely to reflect the multiplicity of pathways controlling radiation response(139) This is exemplified by the fact that other predictive markers such as p53 gene mutations(30, 31) and p21 expression(54) have also been shown to predict radioresistance in rectal cancer with statistical significance. Furthermore, the first report of gene expression profiling in rectal cancer has recently demonstrated that a combination of 54 differentially expressed genes was required to differentiate responders from non-responders(121). Thus there is a need for further molecular targets to be assessed in rectal cancer to delineate pathways mediating response.

Surprisingly, no significant difference in survivin expression was found between pre treatment biopsies from all response groups analysed. Additionally it was unrelated to both apoptosis and proliferation. There is now increasing evidence in the literature that survivin expression is proportional to rates of spontaneous apoptosis in colorectal carcinoma(36, 140). Furthermore, compelling *in vitro* evidence links both baseline and radiation-induced survivin levels to the degree of cellular radiosensitivity. It is unclear why our study failed to demonstrate a link between survivin expression, apoptosis or radiosensitivity. We were fortunate enough to have our survivin staining protocol guided and viewed by Ms Collette Adida, an international authority in survivin and a member of the first group to ever describe survivin(130). Throughout we were confident that our staining was genuine and that our quantification methods accurate. We are therefore confident that our result represents a true finding, which casts doubt on the utility of survivin as a predictive clinical marker.

Summary

Our study has shown that COX 2 overexpression and increased levels of spontaneous apoptosis represent independent markers for response prediction in rectal cancer undergoing RCT. Nuclear or cytoplasmic survivin expression, however, do not serve as clinically useful response predictors. As COX 2 inhibitors are widely available with no reported short-term toxicity, their incorporation into neoadjuvant regimes may warrant assessment.

Chapter 4

Exploring the proteome as a response predictor for rectal cancer undergoing neoadjuvant radiochemotherapy.

4.1 Introduction

The power of proteomic analysis of the low molecular weight region of the serum proteome using SELDI-TOF-MS has recently been demonstrated as a minimally invasive means of cancer biomarker detection. Characteristic differences in spectral patterns generated by this technique can be identified using data-mining software, allowing discrimination between patients with or without cancer. In this role, pilot studies have verified high sensitivity and specificity in multiple cancer subtypes (110, 113, 114, 141).

Despite its widespread adoption as a potential cancer-screening tool, no study to date has assessed its ability to predict response to radiation-based therapies. It may be ideally suited to this purpose as in contrast to tissue-based methods, it is minimally invasive and may be repeated at multiple timepoints through treatment. In this study, we aimed to investigate whether SELDI-TOF-MS analysis of serum could be used to predict and monitor tumor response of patients with rectal cancer throughout the course of neoadjuvant RCT.

4.2 Aims

1. To identify whether serum proteomic differences between patients undergoing good and poor responses to neoadjuvant radiochemotherapy were identifiable during treatment.

2. To identify the optimal timepoint through treatment to identify proteomic differences.

3. To identify whether response to treatment could be predicted using serum derived proteomic patterns.

4.3 Materials and methods

4.3.1 Patients and ethics

Before any aspect of this study began, written ethical approval was obtained from the local ethics committee. In addition, our study was conducted in accordance with current ICH-GCP guidelines. From July 2003-October 2004, 20 consecutive patients were prospectively recruited. The inclusion criteria were that all patients had locally advanced disease and were to undergo neoadjuvant radiochemotherapy followed by excisional surgery. There were no exclusion criteria. All patients gave fully informed consent for their participation (Appendix 2).

4.3.2 Clinical (pre-treatment) and pathological staging

Pre treatment staging consisted of 1) thoracic and abdominal CT scanning and 2) pelvic MRI scanning in all patients. A subset of patients underwent transrectal ultrasound (TRUS) evaluation in addition to MRI to further define local disease. Any discrepancies between MRI and TRUS were discussed and a final pre treatment stage was assigned to each patient according to the TNM classification.

In order to grade pathological response to treatment, diagnostic slides from resected specimens of all patients were retrieved. These included no less than 3 sections of the irradiated area per patient. A single pathologist blinded to patient identifying characteristics reassessed all sections. Response to RCT was assessed using the scoring system previously described by Mandard et al(19). This system grades response on a 5-point scale according to

the relative amounts of residual tumour and fibrosis (Fig 1.2). For analysis purposes in this study, patients with a TRG of 1 or 2 were deemed to have demonstrated a good response and those with a TRG of 3-5 a poor response.

4.3.3 Neoadjuvant regimen

All patients were deemed suitable for RCT after discussion at a weekly multi-disciplinary meeting. Each received 45-50 Gy of radiotherapy, given in 25 fractions delivered on 5 days for 5 weeks. Continuous IV 5-Fluorouracil (225mg/m^2/day) was given concurrently with radiotherapy via a syringe driver. Surgery was performed approximately 6-8 weeks after the last fraction of radiotherapy had been given.

4.3.4 Serum sampling

Serum sampling was done in accordance with standard operating procedures of the NCI/FDA Clinical Proteomics Program, Gaithersburg, MD. Briefly, 12ml of serum (2x 6ml tubes) was collected from patients using Z-clot activator serum tubes (Greiner Bio-One, Gloustershire, UK). Importantly, all blood was drawn immediately before irradiation from a fresh vein using the 'Vacutainer' system (Becton Dickinson, NJ, USA).

Once blood was taken, a minimum of 45 minutes was allowed to elapse for clotting to occur. Between 45 minutes and 2 hours of being taken, centrifugation at 3000 r.p.m. for 10 minutes was performed which visibly separated clot from serum. Serum tubes were then removed from the centrifuge and placed into a storage rack under a laminated flow hood.

The serum fraction was aliquotted into 4 pre-labelled cryotubes (Nunc, Rochester, NY) using a sterile, disposable pipette and then stored at –80°C in a designated surgical tissue biobank.

Time points sampled through treatment were: pre-treatment, immediate/early (24/48hrs), then at 1 week, 2 weeks, 3 weeks and 5 weeks (before their final fraction of radiation). An additional sample was taken from patients the evening before surgery was performed.

4.3.5 Transportation of serum aliquots to NCI laboratory

All proteomic analysis was performed by FMS with the assistance of a laboratory technician in the laboratories of the NCI/FDA Clinical Proteomics Program, Gaithersburg, MD. As it was necessary that all samples remained frozen until immediately prior to analysis, it was necessary to ship samples to the USA packed in dry ice.

In order to ensure that this process occurred smoothly, we firstly had to send the samples on a Monday or Tuesday to ensure that they didn't arrive at the weekend (when there would be nobody there to collect them). We also had to order in a 20kg slab of dry ice, which had to arrive a few hours before the couriers came to collect the samples. Individual samples were placed into freezer boxes, which in turn were placed into a large insulated polystyrene box that was densely packed with dry ice. As dry ice is classed as a 'dangerous good', relevant documentation was necessary to ensure that it could be handled by the couriers and cleared customs. Immediately after sending a shipment, the tracking number was e-mailed to the NCI to inform them that it was due to arrive. In addition, we e-mailed an anonymysed Excel spreadsheet detailing the nature of each sample.

4.3.6 Preparation of Q10 ProteinChip arrays

Before performing each run, a Virtual Notebook, which linked relevant sample information with the spectrophotometer, was prepared using 'Ciphergen Express Data Manager' (Ciphergen Biosystems, CA, USA). Here, barcodes from the reverse side of 12 X Q10 chips were individually scanned using a hand-held scanner. This generated an on-screen computerised image of the 96 individual spots thus created. Of note, when handling chips, forceps were used and great care was taken not to touch the test surface (Fig 4.1).

Each serum sample to be analysed was then assigned an individual spot on a chip. This was done by selecting each of the 96 spots in turn on the on-screen computerised image and inputting the relevant information (Fig 4.1). Of note, for each batch run on the bioprocessor, normal, cancer and control serum were run concurrently in random order to avoid inter-run bias.

4.3.7 Sample preparation

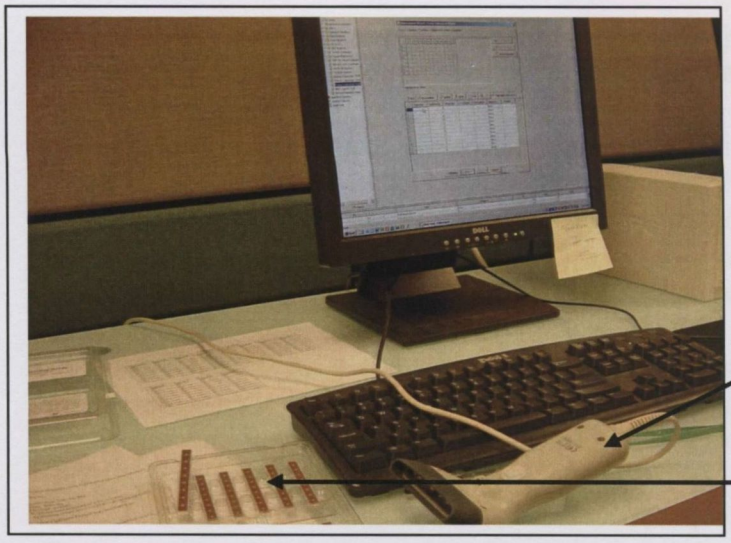
Before performing each run, raw serum samples to be included were removed from -80^0C freezer and allowed to thaw on ice at room temperature (Fig 4.2). Once thawed, all samples were briefly vortexed and 10 microlitres of serum from each was pipetted into a new, labelled cryogenic vial. Next they were diluted in a 1:10 concentration with 100M sodium phosphate buffer, pH 7 (Sigma-Aldrich) (Fig 4.2).

4.3.8 Assembly of the Bioprocessor

Once all 12 chips had been loaded into the ProteinChip cassette and placed into the base clamp assembly (Fig 4.3), the reservoir was placed over them (ensuring that the holes were aligned with the spots on the underlying chips) and the securing clamps closed on either side (Fig 4.3). This created a 96-well plate allowing containment of individual samples on each spot as they underwent preparatory sample application and wash steps. The fully loaded bioprocessor unit was then placed into the correct position on a liquid handling workstation (LHW) (Microlab STAR, Hamilton Company, Reno, NV).

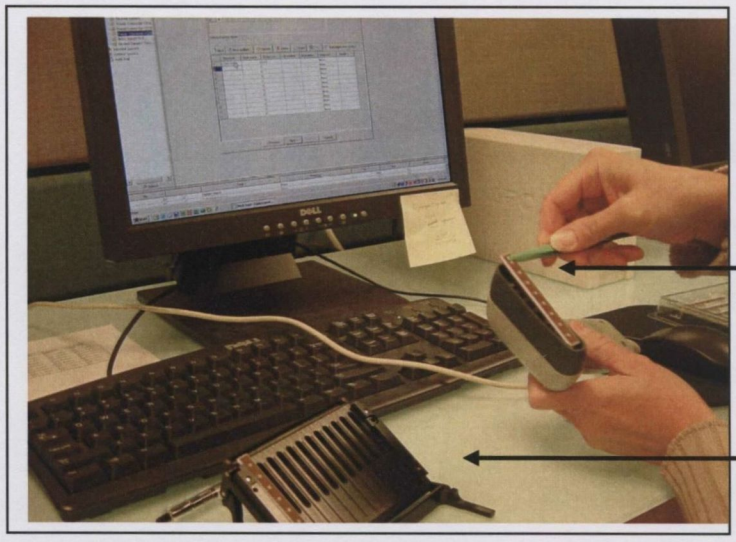
A series of pre-treatment wash steps were now performed by the LHW. Firstly, 100μ of 100mM Sodium Phosphate Buffer pH 7 was added to each well. This was left at room temperature for 5 minutes. After this time, buffer was removed by shaking the bioprocessor over a sink and blotting off excess buffer using a paper towel. A second wash of buffer was then applied for a further 5 minutes and removed again in an identical manner to the first wash.

Figure 4.1. Scanning Protein Chips into Ciphergen Virtual Notebook.



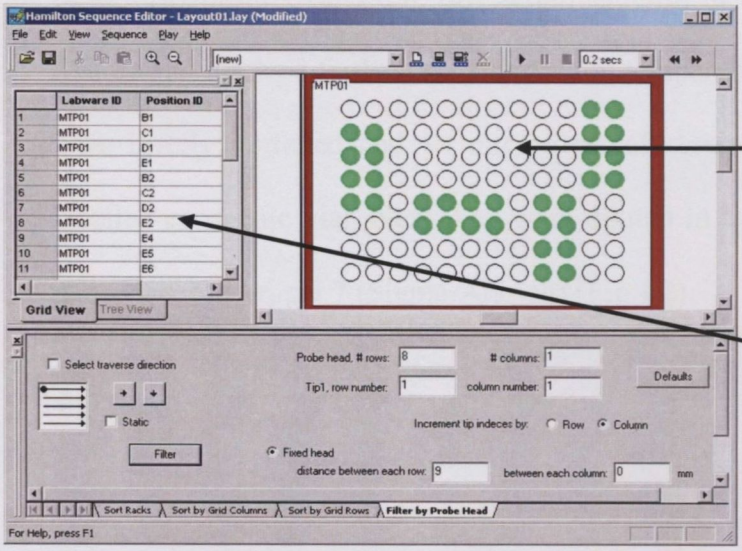
Handheld barcode scanner

ProteinChips in factory packaging



ProteinChips being scanned into software. Note handling with forceps so as spot surfaces were not contaminated.

Scanned chips on ProteinChip cassette component of bioprocessor.



Computerised image of 96 wells of bioprocessor. Spots in green have been designated serum samples.

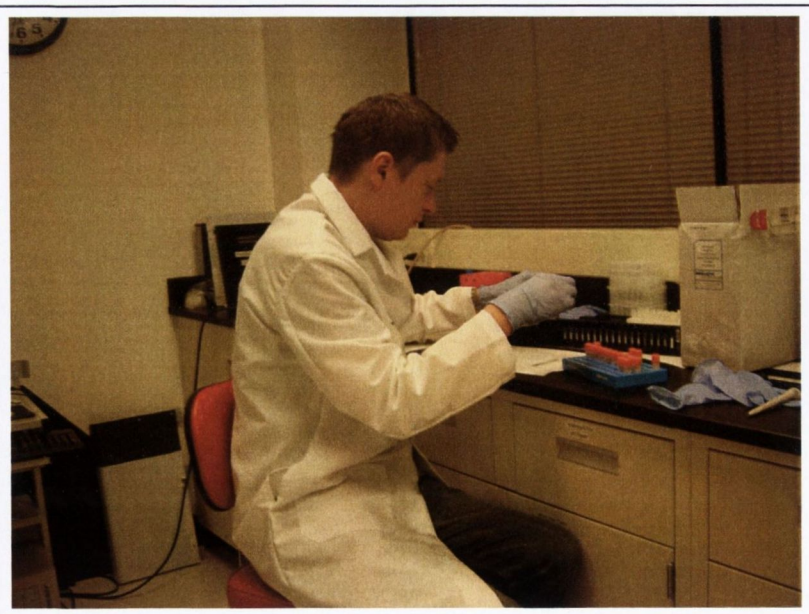
Tabulated patient and sample characteristics

Screenshot of Ciphergen Virtual Notebook.

Figure 4.2

Preparation of serum for analysis.

Serum samples in cryogenic biobank storage at –80° C. Samples to be used were taken from freezer boxes and thawed on ice immediately prior to use.



Photograph of raw serum samples being diluted with buffer in the NCI laboratories (FMS). Once diluted, samples were placed in an autoloader in the corresponding order to their location in the Virtual Notebook.

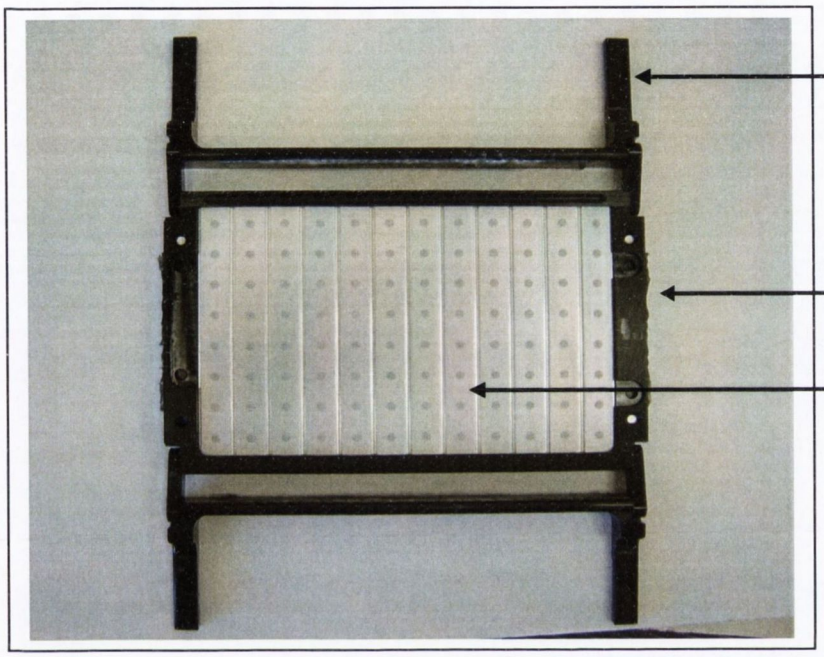
4.3.9 Addition of Pre-diluted Test Sample

A 25µl volume of each individual serum sample was then added to each spot on the bioprocessor using the LHW. This was done according to the placement map that had previously been generated using the 'Virtual Notebook'. Once this process was completed for all spots on the bioprocessor, careful examination for bubbles generated during pipetting was made under a light source. As bubbles can cause uneven sample application to each spot surface if left unchecked, any seen were burst with a sterile pipette tip. The samples were then left to incubate on the benchtop at room temperature for 30 minutes to allow protein binding to occur between serum samples and each test spot.

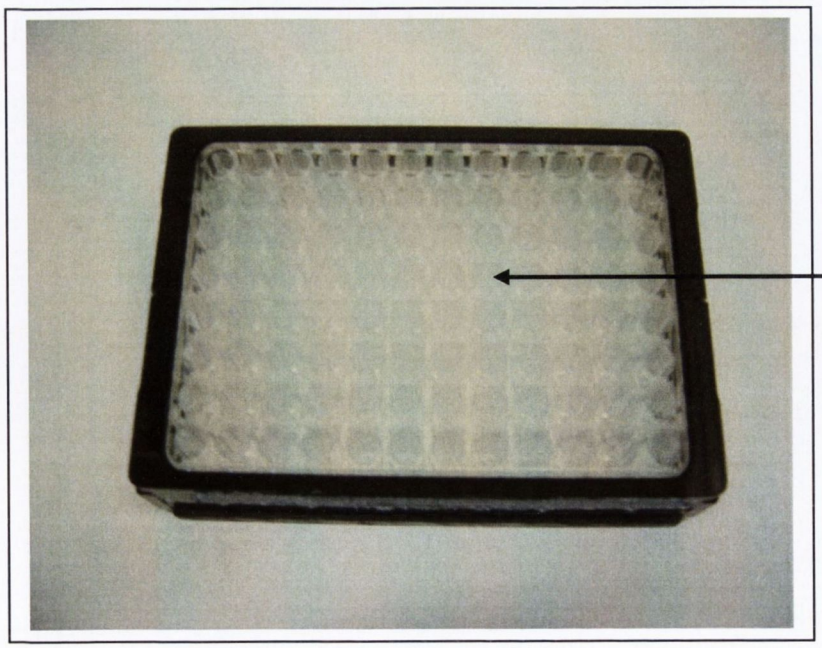
4.3.10 Buffer Washes

Once the 30 minutes had elapsed, chips were subjected to a series of 4 washes with 100µl of Sodium Phosphate Buffer. This was applied to each well for 5 mins per wash using the LHW. Chips were then washed with 150µl of deionised water for 1 minute and the bioprocessor was shaken over a sink and blotted with paper towel to remove all liquid. Chips were then dried for 15 mins at room temperature.

Figure 4.3. Assemply of the Bioprocessor



12 ProteinChips loaded onto base clamp assembly. At this stage the securing clamps are lying open.



Fully assembled bioprocessor. The 96 well reservoir has been placed over the chips and the securing clamps applied

4.3.11 Addition of Sinapic Acid Matrix to Spots

Before each run, a fresh batch of sinapic acid (SPA) matrix was prepared. Firstly, matrix diluent was made in the manner outlined in appendix 1. This was done under a laminar flow hood. Next 1.0µl of SPA matrix solution was added to each spot using the LHW using a fresh tip for each spot. This was then left to dry for 15 minutes at room temperature before a second application of 1.0µl of SPA matrix solution was applied. Again this was left to dry for 15 minutes at room temperature. The chips were now fully prepared and ready for analysis.

4.4 Chip detection

All ProteinChips were detected on the Protein Biological System II (PBS-II) Chip Reader System (Ciphergen Biosystems, CA, USA) (Fig 4.4). Here high mass was set at 50000 Daltons (Da) and optimised from 2750-20000 Da. Laser intensity was set at 185 with a starting detector sensitivity of 7. The mass deflector was set at 20000Da with a data acquisition parameter of 20. Warming positions were set with 2 shots at intensity of 195 and warming shots were excluded. Before runs were performed, the system was calibrated using protein standards.

Figure 4.4 - Ciphergen PBS II mass spectrometer used in our analyses.

4.5 Spectral normalisation and true-peak selection

After each run, spectra were examined by naked eye to ensure that they were comparable between duplicate samples and that the intensity of peaks was comparable between spectra (Fig 4.5). Samples whose spectra visibly differed between duplicates or those with poor intensity were repeated in further runs.

Good quality spectra were then normalized according to the total ion current of mass-to-charge ratios (*m/z*) between 2,000 and 40,000Da. Qualified mass peaks (signal-to-noise ratio>3) with *m/z* between 2,000 and 20,000 were automatically detected (Fig 4.6). Peak clusters were completed with second-pass peak selection (within a 0.3% mass window) and estimated peaks were added. These analyses were performed using Protein Chip Software 3.2.1. and Ciphergen Express Software (Ciphergen Biosystems).

4.6 Superficial spectral analyses

Spectra between good and poor responders were initially analysed using Ciphergen Express (Ciphergen Biosystems). Here, differences between generated spectral profiles of good and poor responders were analysed in order to determine whether differences in individual peaks were present. In addition time course analyses were performed to determine whether the intensity of individual peaks altered throughout the course of treatment.

Figure 4.5 Example of serum-derived spectra.

This image shows an example of spectra derived from the same sample but run on different spots. The near-identical similarity both in intensity and generated pattern profiles between them can be seen.

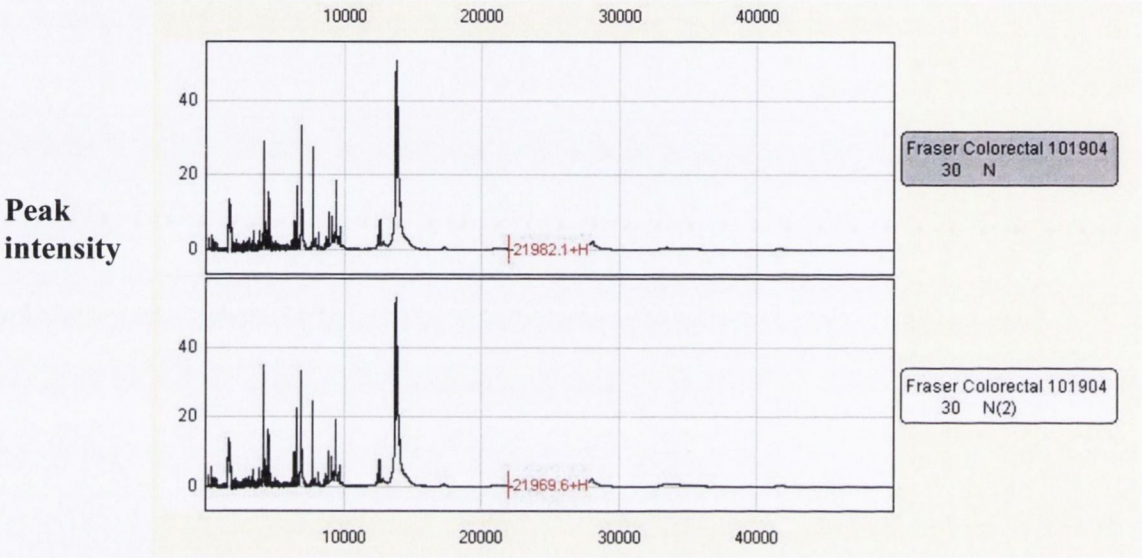
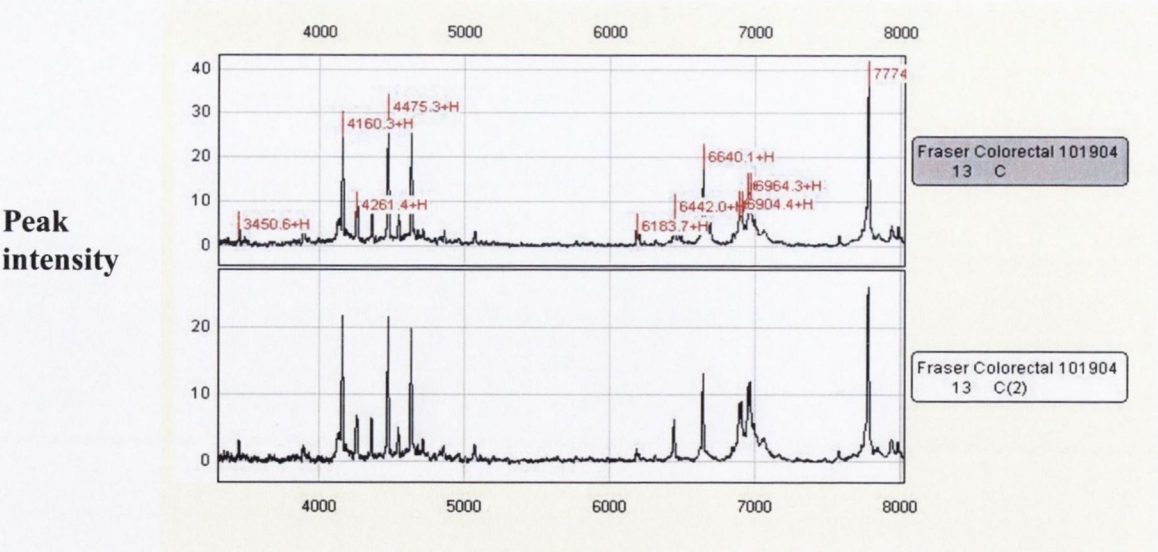


Figure 4.6. Close-up view of duplicate spectra showing identified peaks.



4.7 Bioinformatic analysis and generation of a predictive algorithm.

Support vector machines (SVMs) were applied to binary-labelled training data (i.e. responders and non-responders) and were used to perform classification on median normalised, averaged duplicate spectra at each timepoint sampled used to perform classification on median normalised, averaged duplicate spectra. (112, 142). The SVM method was chosen over other classification methods, such as decision tree based approaches, by virtue of the fact that it classifies data based on their maximal separation using a 3-D hyperplanerather than finding the highest training accuracy. This process therefore minimises the risk of chance error(143) and overtraining data and has been used in other studies involving serum proteomics(144). The mis-classification rates of the classifier developed for each timepoint was then tested using a leave-one-out cross-validation strategy using Genespring 7.22 (Silicon Genetics, Redwood City, CA). The predictive strength for each peak was then evaluated for all peaks in the classifier. This is a measure of the association between class (i.e. good/poor responder) and expression level of the peak and is the negative natural log of the peak's p-value (p-value derived by Fisher's Exact Test). The greater the predictive strength of a peak, the greater its ability to discriminate one class from another. This reflects the fact that not all peaks discriminate between the two groups equally but that they are collectively required to achieve optimal separation between groups.

4.8 Results

4.8.1 Patient, tumour and surgical characteristics

Sequential serum samples from 20 consecutive patients were available for study. Patient and tumour characteristics, pre- and post- treatment TNM staging as well as the surgical procedure performed can be seen in table 4.1. All patients included underwent full course RCT followed by surgical excision of their tumor.

4.8.2 Results of superficial analyses

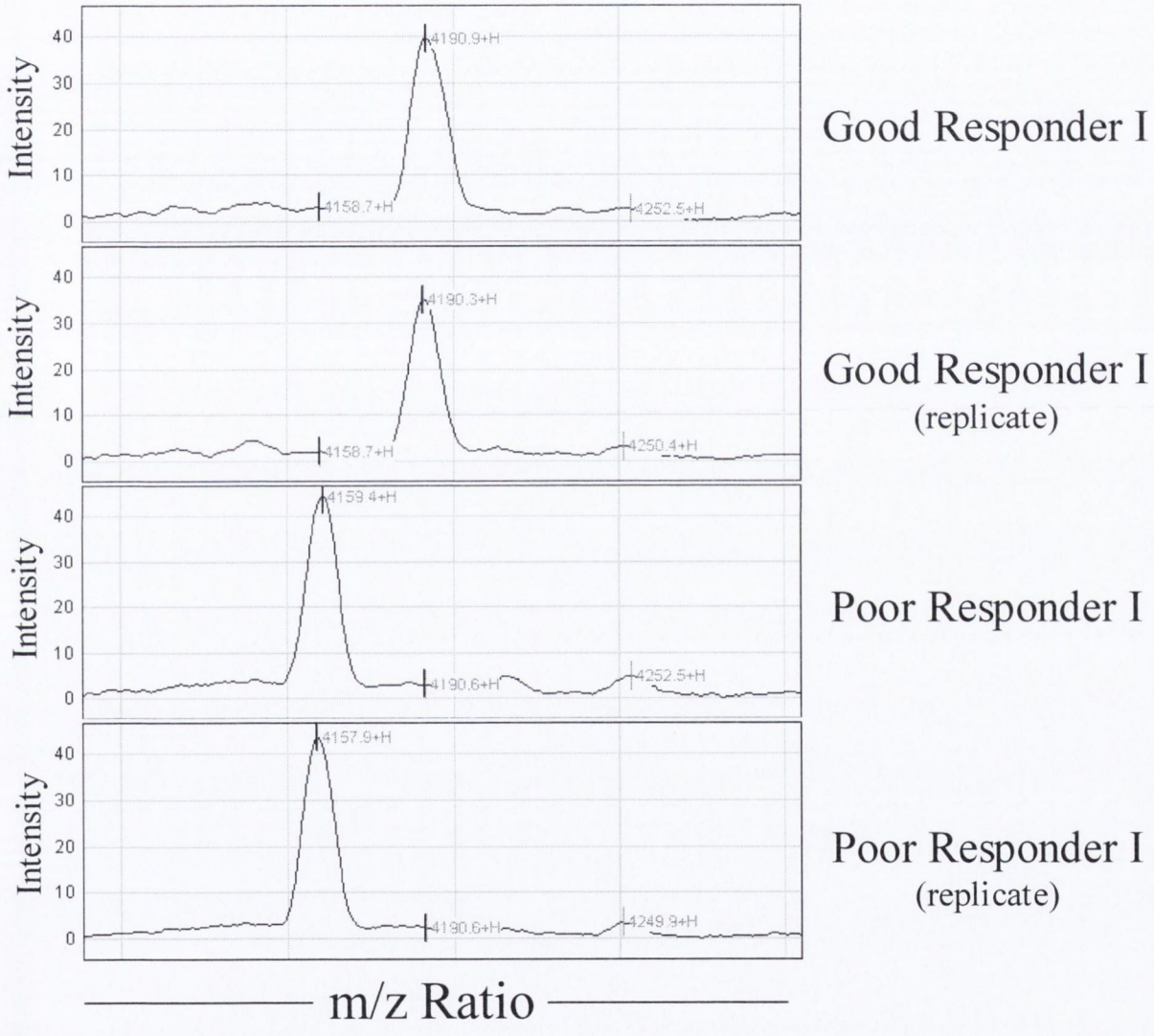
Comparative analysis at individual time-points demonstrated several peaks that were significantly differentially expressed between good and poor responders ($p<0.05$). This was even apparent in pre treatment serum (Fig 4.7).

Alterations in peak intensity over time were then examined using Biomarker Wizard (Ciphergen Biosystems). Analyses were performed by F.M.S. and Mr Edward Fox. Multiple trends in peak up and down regulation were apparent that could be directly related to RCT. These included peaks that were initially upregulated and were then down regulated (Fig 4.8) and peaks that were gradually upregulated through treatment and then fell back to pre treatment levels after RCT was stopped (Fig 4.9). There were also many examples of peaks that were unaffected by RCT, remaining constant throughout treatment (data not shown).

Table 4.1. Patient and tumour characteristics, pre- and post- treatment TNM staging and surgical procedure performed for all patients analysed.

Patient	Sex	Age (Yrs)	Anal Verge(cm)	Pre RCT Stage (MRI)	Pre RCT Stage (TRUS)	Pre RCT M Stage (CT)	Surgery performed	Post RCT Path Stage	TRG
1	M	79	1	T2N0	T4N0	0	AR	T1N0	2
2	F	57	11	T3N1	N/A	0	AR	T3N0	3
3	F	51	3	T3N1	N/A	0	AR	T3N1	3
4	M	52	7	T3N1	T2N0	0	AR	T3N1	4
5	M	55	10	T3N1	N/A	0	AR	T3N1	3
6	M	61	11	T3N1	N/A	0	AR	T3N0	1
7	F	72	8	T3N0	N/A	0	AR	T3N0	2
8	M	66	8	T3N1	T3N1	1	AR	T3N2	2
9	M	74	3	T3N0	T3N0	0	APR	T3N0	2
10	M	46	5	T3N1	N/A	0	AR	T3N1	4
11	M	63	7	T3N1	N/A	0	AR	T4N0	4
12	F	67	7	T3N1	T3N1	0	AR	T3N2	4
13	M	77	2	T3N0	T3N0	0	AR	T3N0	3
14	M	69	5	T3N1	T3N0	0	AR	T3N1	4
15	F	73	5	T2N0	N/A	0	AR	T0N0	1
16	F	66	2	T3N0	T3N0	0	APR	T3N0	3
17	M	53	8	T3N1	N/A	0	AR	T3N0	4
18	M	75	6	T3N1	T3N1	0	AR	T2N0	2
19	M	70	8	T3N1	T3N1	0	AR	T0N0	1
20	M	62	3	T3N1	T3N1	0	APR	T3N1	2

Figure 4.7. Close-up view of spectra showing differential peak intensities between good and poor response (24 hour timepoint)



P<0.05

Figure 4.8. Example of differential protein expression over time (early up-regulation). Time-course profile of peak of 4160m/z mapped from an individual patient (adapted from a screenshot generated using Ciphergen Biomarker Wizard). Note the sharp upregulation of this particular protein in the initial 1-2 weeks followed by steady down regulation that persists even after RCT is stopped.

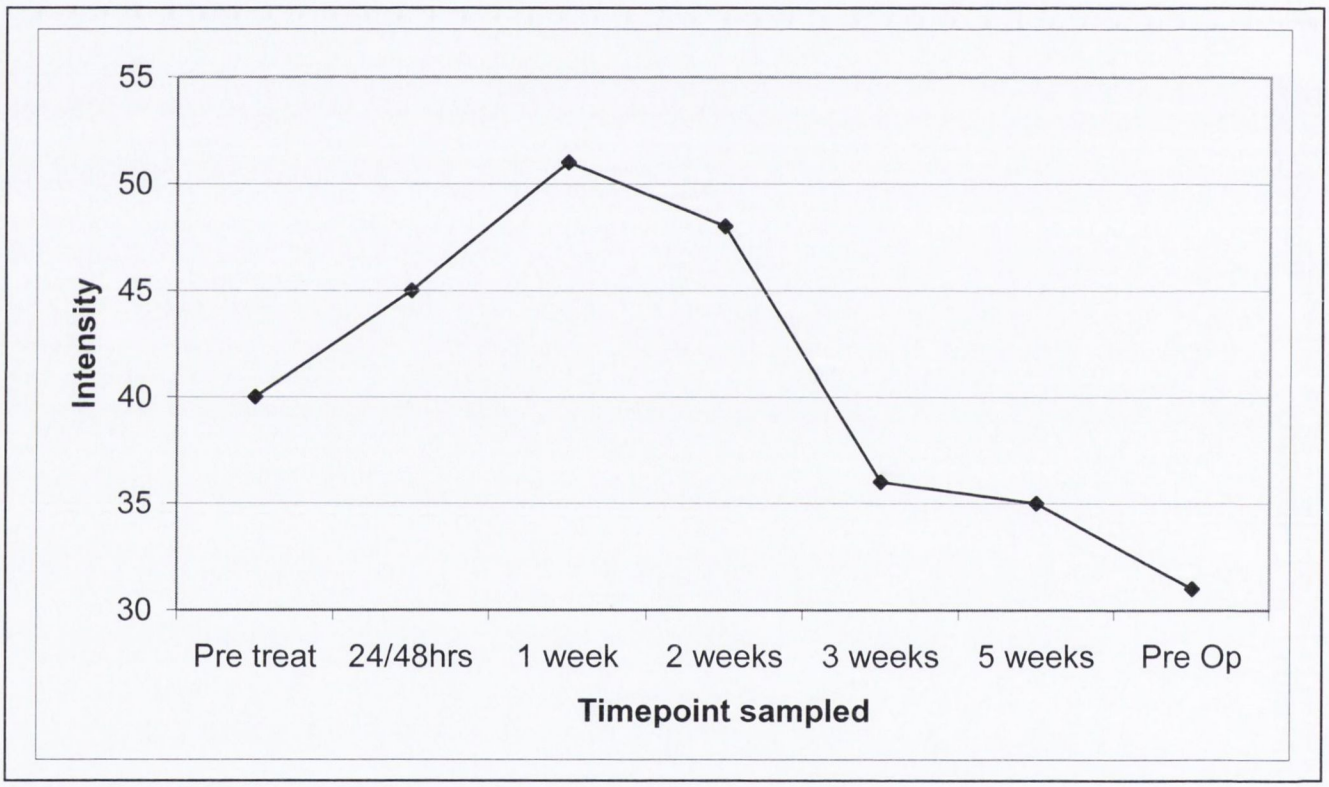
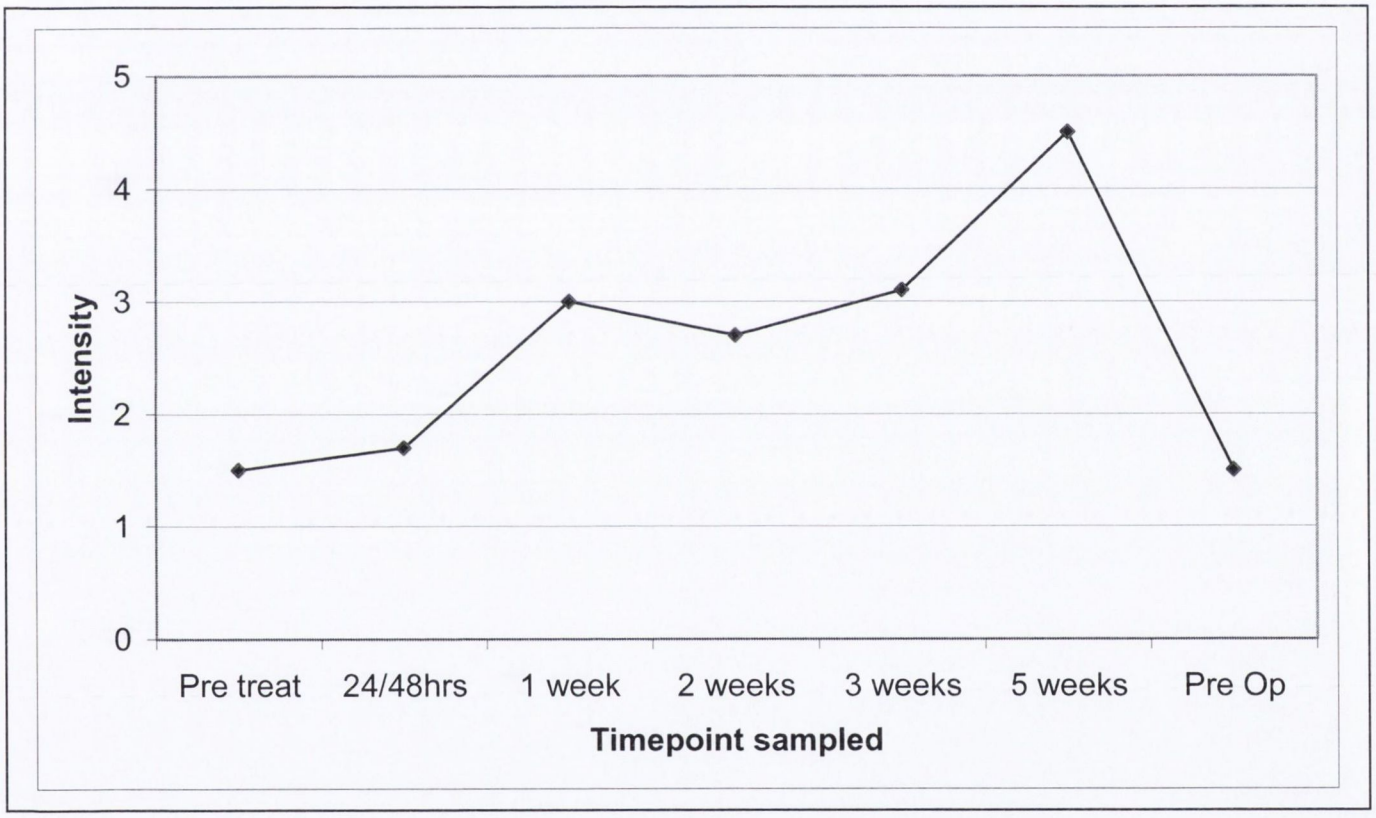


Figure 4.9 Example of differential protein expression over time (Gradual upregulation). Time-course profile of peak at 4160m/z mapped from an individual patient (adapted from a screenshot generated using Ciphergen Biomarker Wizard). Note its gradual >3 fold upregulation during treatment followed by a decrease to pre treatment levels once RCT is stopped.



4.4.3. Classification of response to RCT based on protein spectra

To determine whether a selection of spectral peaks could differentiate good from poor responders at each of the time points sampled, support vector machine (SVM) analysis was performed at each of the timepoints sampled. The performance of each classifier was determined using leave-one-out cross validation. Analyses were performed by F.M.S and Mr Edward Fox. Briefly, the ability of a classifier to predict the response status of an individual, blinded patient sample based on the expression of the remaining patient samples was iteratively assessed, and the optimal sensitivities and specificities determined. These are shown in table 4.2. The greatest classification accuracy was achieved using serum sampled from 18 patients sampled 24 to 48 hours into treatment, where prediction of response could be performed with a sensitivity of 87.5% and a specificity of 80%. This was achieved using a 14-peak classifier comprising 7 positive and 7 negative markers of good response to RCT (Fig 4.10). The majority of these detected peaks were of extremely low molecular weight: 10 of 14 (71%) had *m/z* values of <5000 Da and a further 3 (21%) had m/z values of <10,000Da. While this classifier could in addition be used to predict response at other time points, its accuracy was too low for clinical utility (Table 4.2).

Figure 4.10.

Heatmap of 14-peak classifier at 24/48-hour timepoint. Note clustering of positive markers in good responders and negative markers in poor responders.

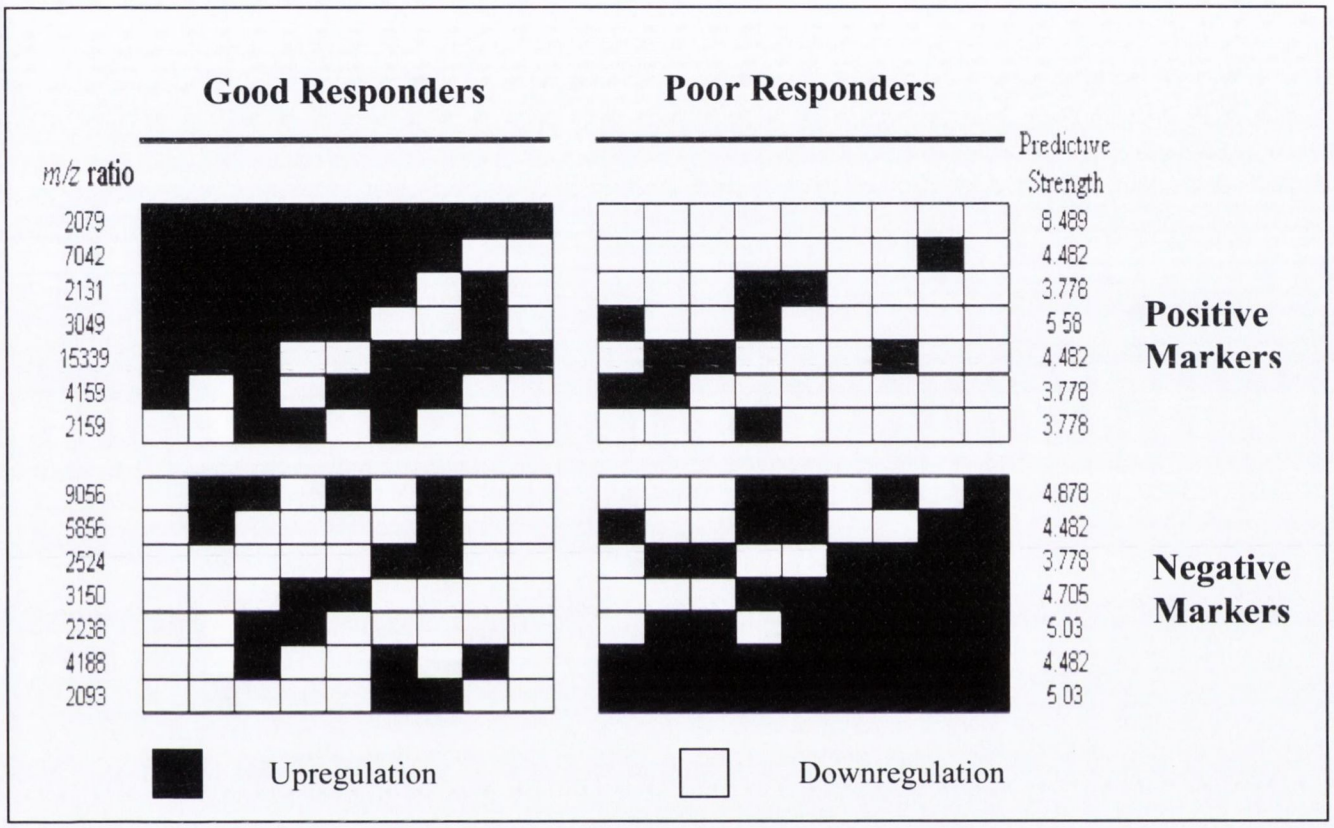


Table 4.2. Maximal sensitivity and specificity of SVM at all timepoints sampled.

SVM	Pre-Tx	24h/48h	1week	2week	3week	LastDay	PreSurgery
Sensitivity	55%	**87.50%**	66%	25%	57%	50%	60%
Specificity	64%	**80%**	80%	66%	75%	80%	80%

4.9 Discussion

In this study we performed serial SELDI-TOF-MS serum profiling in 20 patients with rectal cancer undergoing neoadjuvant RCT. Whilst pre-treatment samples were unable to predict response, serum samples taken 24 or 48 hrs into treatment could predict response with 87.5% sensitivity and 80% specificity based on a pattern of 14 differentially expressed proteins.

The finding may be clinically important as to date the vast majority of studies that have aimed to predict response to RCT in rectal cancer have used endoscopic, pre treatment tissue biopsies(145). This process is based on two assumptions: firstly that the small portion of tumour sampled is representative of the tumour biology as a whole and secondly that molecular expression at this timepoint is best representative of how the tumour will subsequently respond. Increasing evidence from the literature suggests that there is a dynamic response to cytotoxic therapy within tumours and that important changes in gene expression occur in the initial stages of treatment.

In a mouse model, Wang et al have recently demonstrated that total body irradiation induces the expression of the transcription factor nuclear factor κB (NFκB) in intestinal epithelial cells in a time dependent manner(146). They found that an initial rise and fall in NFκB activity two hours after radiation exposure occurred followed by a subsequent rise in levels that persisted for up to 24 hours. This observation is important as NFκB is known to induce the expression of many pro and apoptotic molecules capable of regulating cell survival.

Our laboratory has previously identified that endogenous nuclear NFκB expression in pre treatment biopsies can predict response of oesophageal tumours to neoadjuvant, multimodal therapy(147) and previous studies have shown that inhibition of NFκB activation increases radiation-induced apoptosis and cellular radiosensitivity in colorectal cancer cell lines(148). This evidence therefore suggests that gene transcriptional activation is important both before and in the early stages of treatment.

In a further study by Rodel et al, further evidence for important transcriptional activation in the early stages after irradiation is given(131). In a colorectal cell line model, they demonstrated a marked upregulation of the apoptosis inhibitor survivin 48 hrs after cell irradiation in radioresistant cells, associated with reduced levels of cellular apoptosis. This was in stark contrast to radiosensitive cells where survivin expression remained unchanged.

As a final example, Buchholz et al analysed sequential core biopsies from 5 patients with breast cancer undergoing neoadjuvant chemotherapy(149). Biopsies were taken pre treatment and 24 and/or 48 hrs after treatment and analysed using cDNA microarrays. This study identified significant changes in transcriptional response in patients during chemotherapy. Furthermore, these changes were associated with gene clustering that was distinct in patients with a good or poor response.

This evidence therefore suggests that the early timepoint after initiation of cytotoxic therapy may potentially be even more important than pre treatment sampling because transcriptional profiles of molecules involved in radioresponse have become established.

Serial assessment of molecular tumour profiles, rather than reliance purely on pre treatment tumour biopsies may therefore ultimately prove to be the most informative means of predicting response and understanding endogenous mechanisms of tumour radioresistance. If this hypothesis were correct, it would favour a move away from conventional tissue biopsy, which is invasive and often distressing for patients. Ideally, alternative, non-invasive means of assessing response would be available such as SELDI-TOF-MS analysis, as serum sampling is non-invasive and can easily be repeated at multiple timepoints throughout treatment.

Our study clearly shows the potential utility of SELDI-TOF-MS analysis as a clinical response prediction tool. Not only does our study show that it could predict response after the first or second dose of radiation, but could do so with extremely high sensitivity and specificity based on a combination of 14 key protein differences on an individual patient basis. This is in contrast to previous immunohistochemically-based studies where statistically significant differences in marker have been identified between good and poor response groups but have not been assessed in a prospective manner. Discrepancies between strong statistical differences in marker expression and the true ability of a marker to predict response on an individual basis have previously been discussed in the literature. (150). Analysis of pre-treatment serum in our study illustrates this point. Whilst 12 proteins were differentially expressed between good and poor responders with statistical significance, they were unable to predict response on cross-validation (data not shown). As radiation response is likely to be governed by many interacting molecular pathways, it may be that only techniques such as SELDI-TOF-MS, capable of simultaneously detecting multiple markers, are able to accurately predict response at an individual level.

It remains unclear as to what biological processes the 14 proteins identified in this study are attributed. We hypothesise that they most likely represent pro/anti-apoptosis signalling derived from tumours as part of their initial response to radiation. Increasing evidence suggests that apoptosis is an integral mechanism for tumour radiosensitivity(95-98). Studies in rectal cancer have already shown that high levels of apoptosis in pre treatment biopsies correlate with good response to radiotherapy(36). This hypothesis is supported by temporal assays of circulating nucleosomes, elementary units of chromatin released into the circulation in situations of enhanced apoptosis, in the sera of patients during radiation and chemotherapy. Holdenreider et al have demonstrated that nucleosome levels reach maximum values at 48-72 hrs in the serum of patients receiving radiotherapy for pancreatic cancer(151) and chemotherapy for colorectal cancer (152). This group has not, however, assessed the ability of nucleosome levels to predict histological response to treatment.

SELDI-TOF-MS profiling may offer other clinical possibilities. One of these is the ability to identify and sequence the proteins or peptides responsible for predictive peaks. This in turn may aid the discovery of specific mediators and pathways that are driving radiation response, facilitating the development of novel pharmacological therapies. A recent publication by the Human Proteome Organisation Plasma Proteome Project (HUPO PPP) emphasises this capability and its consistency across multiple geographical sites(108). Here, aliquots of reference serum and plasma samples were analysed by eight different laboratories worldwide. Data from five sites passed preliminary quality assurance tests and amongst these, a high correlation was found between spectra generated. Furthermore, a peak at 9200 m/z was consistently purified and identified in samples originating from these five sites using mass fingerprinting and MS-MS analysis.

4.10 Summary

In this chapter, proteomic profiling of sequential blood samples from 20 patients undergoing neoadjuvant RCT for rectal cancer was performed. Differential expression of proteins was apparent at each timepoint between good and poor responders. Using support vector machine analysis to identify proteins maximally differentially expressed between groups, a response classifier consisting of 14 key proteins was identified. These could be used to predict response with 87.5% sensitivity and 80% specificity 24 or 48 hours into treatment.

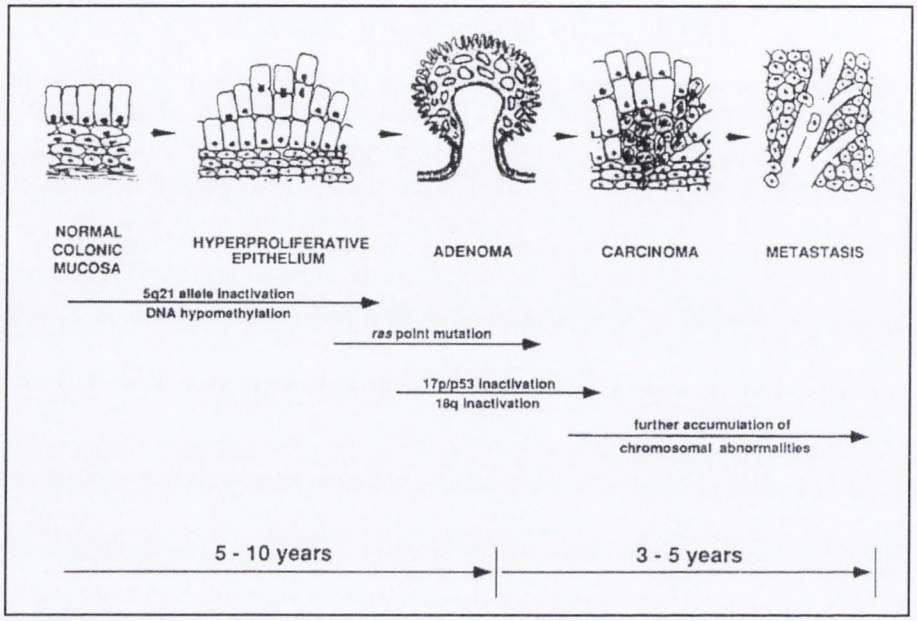
This study is the first to our knowledge to utilise serum proteomic profiling to predict outcome after RCT for rectal cancer. The results of this study suggest that this technique may provide an early indication of response. Furthermore as the ability to sequence and identify proteins exists, a more specific understanding of the molecular basis of resistance to radiation and cytotoxic therapy may be gained allowing for the creation of tailor-made cancer therapy.

Chapter 5: Using SELDI-TOF-MS proteomic profiling of serum as a screening tool for colorectal cancer.

5.1 Introduction

The risk of death from CRC is directly related to the stage of disease at presentation. Patients with early stage tumours have over 90% 5-year survival whereas patients with distant metastatic disease have < 5% 5-year survival(153). Recent figures show that 57% of patients have regional or distant metastases at the time of presentation(154) and it is believed that more than 50% of CRC-related deaths could be prevented through the use of screening tests, identifying the disease at an earlier stage(155, 156). It is widely thought that CRC evolves over time as a result of accumulated molecular alterations in pre-cancerous adenomatous polyps, known as the adenoma-carcinoma sequence (Fig 5.1)(157). The American National Polyp Study showed that endoscopic screening with the removal of adenomatous polyps decreased the incidence of CRC by 76-90% compared to an unscreened population(158). In 1997, the American Gastroenterological Association published guidelines for CRC screening stating that for average risk individuals, screening should start at 50 years of age and consist of annual faecal occult blood testing (FOB) with either flexible sigmoidoscopy every 5 years or double contrast barium enema every 5-10 years; or colonoscopy every 10 years(159). In contrast, the European Union's Advisory Committee on Cancer Prevention recommends that average risk individuals should be screened only using FOB testing with endoscopic assessment only if FOB +ve(160).

Figure 5.1. The colorectal adenoma-carcinoma sequence.



The adenoma-carcinoma sequence, first proposed by Vogelstein and Fearon proposes that colorectal tumorigenesis occurs in a stepwise fashion from normal to adenoma to malignant. This process is underpinned by the accumulation of genetic alterations.

Currently, the optimal screening strategy for CRC is controversial and is limited by the drawbacks of each of the screening techniques. A recent evidence-based review of the current status of colorectal cancer screening cited a large body of evidence stating that persons over 50 years of age should be screened, but concluded that 'the available evidence does not currently support choosing one test over another.'(155)

Although recommendations for CRC screening are currently in place, general uptake has been poor and the optimal means of screening has been hotly debated. Figures from the US show that in 2002, only 40% of adults over 50 had had either a sigmoidoscopy or colonoscopy in the last 5 years and only 22% had received a faecal occult blood test in the last 12 months(161).

It is suggested that the reason for such poor rates of participation are due to several factors such as patient discomfort, cost, lack of awareness and poor acceptability of current screening methods(161). Ideally, the means of screening for CRC should be non invasive, acceptable to patients and cost effective.

Endoscopic assessment, one of the mainstays of current screening, is an invasive, skilled, time-consuming and unpleasant procedure and carries a small but significant risk including colonic perforation(162). For these reasons, colonoscopy is not universally recognised as the screening procedure of choice. Although better tolerated and quicker to perform, flexible sigmoidoscopy increases the risk of missing proximal cancers. This fact was highlighted by Imperiale et al in the New England Journal of Medicine in 2000 showing that almost half of patients with proximal cancers had no distal polyps(163).

In another study by Lieberman et al, again published in the New England Journal of Medicine, it was found that advanced proximal neoplasms would not have been detected 52% of patients if the test was limited to the distal colon(164).

The main alternative to endoscopic screening is FOB testing. This modality has the immediate advantage of being non-invasive and large randomized trials have shown that serial FOB screening is an effective means to reduce mortality from CRC (165-167). An additional advantage of FOB screening is that it is cheap and does not require skilled personnel to perform it. The largest study assessing FOB screening was performed in Minnesota, USA. Here, 46,551 patients aged 50-80 yrs were randomly assigned to one of 3 groups; Group 1 – Annual FOB screening, Group 2 – Biennial FOB screening, Group 3 – Control group.

Patients who tested FOB positive underwent a diagnostic evaluation that included endoscopy and overall vitality was assessed after 13 yrs. The 13-year cumulative mortality per 1000 from colorectal cancer was 5.88 in the annually screened group, 8.33 in the biennially screened group and 8.83 in the control group. The rate in the annually screened group, but not in the biennially screened group, was significantly lower than that in the control group. The reduced mortality seen in the annually screened group was accompanied by an improved survival in patients with colorectal cancer and a shift to detection at an earlier stage of cancer. The use of FOB testing, does, however, have several inherent flaws. The main ones are that cancers may bleed intermittently and the vast majority of gastrointestinal bleeding is from causes unrelated to cancer such as haemorrhoids(161). The result of this is that non-bleeding tumours may be missed and many false positive results are generated by non-malignant pathologies. Current estimates state that only 1 in 10 patients with FOB positivity will truly have CRC(161). An additional factor resulting in false positives is due to the FOB test kits themselves. The most commonly used kits are Guiac-impregnated Haemoccult kits (Smith Kline Diagnostics) where stool is wiped onto a cardboard test plate and a developer solution is added to it causing a peroxidase-based blue colour change to occur. The problem with this method, however, is that dietary haeme found in red meat and plant peroxidases from certain fruits and vegetables can cause false positives and ingestion of excess vitamin C can cause false negatives.

Faced with the limitations of current screening modalities, alternative methods are being actively sought. In particular, non-invasive means are particularly attractive as they are far more acceptable for patients and are generally quickly and easily performed.

At the time of starting this thesis serum proteomic screening using SELDI-TOF mass spectrometry had not been described for CRC but had recently been described in ovarian cancer by Petricoin et al (our NCI collaborators)(110). Their study, using SELDI-TOF-MS showed that the technique was able to distinguish patients with ovarian cancer from those with benign or no disease with 100% sensitvity and 95% specificity. We therefore aimed to replicate this experiment in the setting of CRC.

5.2 Aims

1. To collect and store serum from a large cohort of patients with no colonic pathology, adenomatous polyps or colorectal cancer.

2. To generate proteomic spectra from all serum samples collected.

3. To generate a predictive algorithm for CRC using biostatistical software.

5.3 Materials and Methods

5.3.1. Study design and patients

Before any aspect of this study began, written ethical approval was obtained from the local ethics committee. In addition, our study was conducted in accordance with current ICH-GCP guidelines. All patients recruited gave fully informed consent prior to serum sampling (Appendix 3).

Patients were recruited before undergoing full, endoscopic colonoscopies or prior to undergoing elective surgical resection for colorectal cancer. As the pathological status of each patient's colon was not known prior to colonoscopy, pathology and colonoscopy reports were obtained after their procedures had been performed allowing them to be grouped accordingly. As this study aimed only to differentiate between the different stages of the adenoma-carcinoma sequence, patients found to have infective processes such as active diverticulitis, Crohn's Disease and ulcerative colitis were excluded from the analysis.

5.3.2 Serum Sampling

All serum samples were drawn from patients before they underwent colonoscopy or surgery. The method of serum acquisition, processing and storage was identical to that previously described in chapter 4.

5.3.3 Proteomic analysis

All serum samples were run in duplicate in random order with serum controls. The method of sample and chip preparation and mass-spectrometric analysis was identical to that described in chapter 4.

5.3.4 Data analysis

Spectra were analysed using two different software packages. The first software package to be used was Clementine 8.0 (SPSS Industries, Cary, NJ) and the second was Ciphergen Biomarker Wizard (Ciphergen Biosystems).

5.4 Results

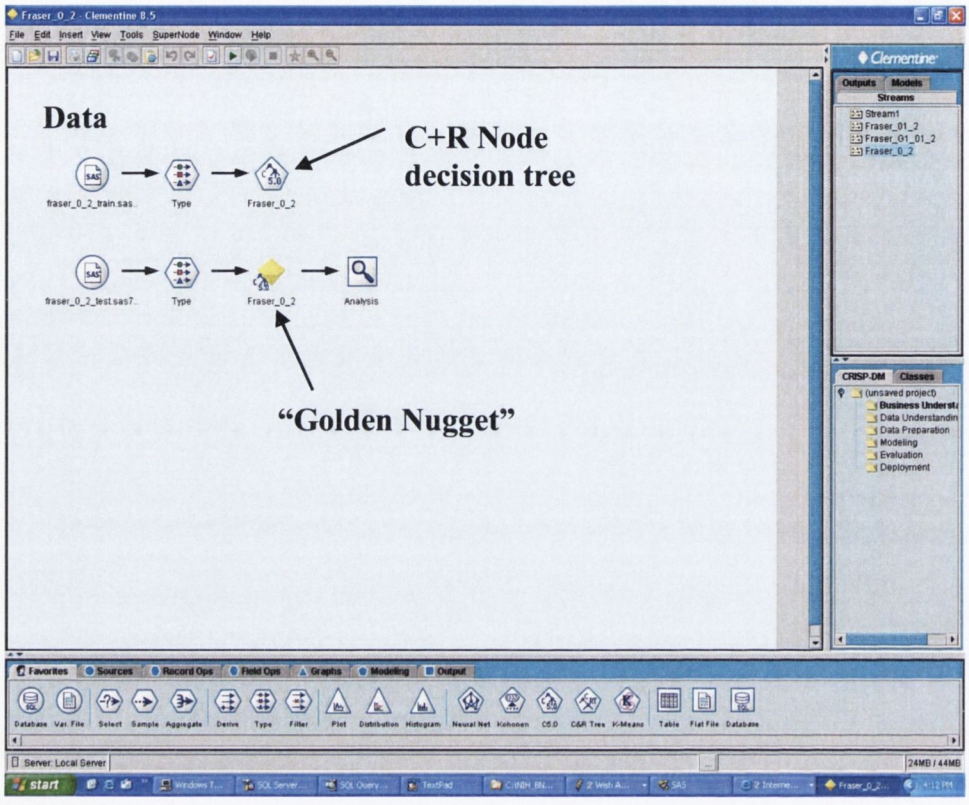
In total, serum from 46 patients found to have normal colons, 23 with adenomatous polyps (single or multiple) and 57 with colorectal cancer were included. This generated a total of 252 spectra representing all samples run in duplicate.

5.4.1 Generation of a predictive algorithm using SPSS

Initially, data was analysed using a classification and regression (C+R) decision tree model using Clementine 8.5 (SPSS, Illinois, USA). Spectra were separated into 2 separate groups prior to analysis: Group 1 (Adenoma or no colonic pathology) and Group 2 (Carcinoma). Each group was then randomly divided by the software package into 2 sub groups: one consisting of 80% of patients (training set) and the other consisting of 20% of patients (test set).

C+R analysis was then performed only on the training set in order to identify peaks that were maximally differentially expressed between benign and malignant disease. This process generated a decision tree algorithm or 'golden nugget' through which the test set was run (Fig5.3). In the initial training set, the golden nugget was capable of discriminating between malignant and non-malignant disease with 80% sensitivity and 85% specificity. When applying it to the test set, however, the sensitivity and specificity fell to 65% and 70% respectively.

Figure 5.2. Screenshot from Clementine/SPSS 8.5 statistical program.



This image shows the generation of a predictive algorithm in a training set of 80% of patients (upper row). This algorithm is then tested in a test set of 20% of patients. As one of the main selling features of Clementine is that it can predict retail consumer purchasing trends, the predictive algorithm is quirkily called the 'golden nugget'.

In order to assess whether there was a more stark difference in spectra only between patients with normal colons and those with malignant disease, the experiment was re-run excluding all patients with adenomas. Here, the sensitivity and specificity in the training set fell to 68% and 69% respectively and in the test set fell even lower to 53 and 56% respectively.

5.4.2 Generation of a predictive algorithm using Ciphergen Biomarker Wizard™.

Subsequent to the poor results gained by analysing spectra using Clementine, we aimed to reassess our results using Ciphergen Biomarker Wizard™. This is the tandem package for the PBS II mass spectrometer and has been used in other published reports assessing SELDI as a screening tool for CRC(141).

Again spectra were divided into 2 groups: Group 1 – Adenoma or no colonic pathology and Group 2 – CRC. This package can be used to build a decision tree (Fig 5.4) that best separates samples into different groups based on differential peak expression. The decision tree can then be tested using leave-one-out cross validation.

A total of 342 peak clusters were identified in our samples, 198 of which were significantly ($P<0.05$) differently expressed between serum from cancer or non-cancer (Fig 5.5). Despite this large number of significant peaks, only 78% of serum from healthy patients and 70% of serum from patients with cancer could be correctly predicted. This result therefore closely mirrors that obtained when using SPSS.

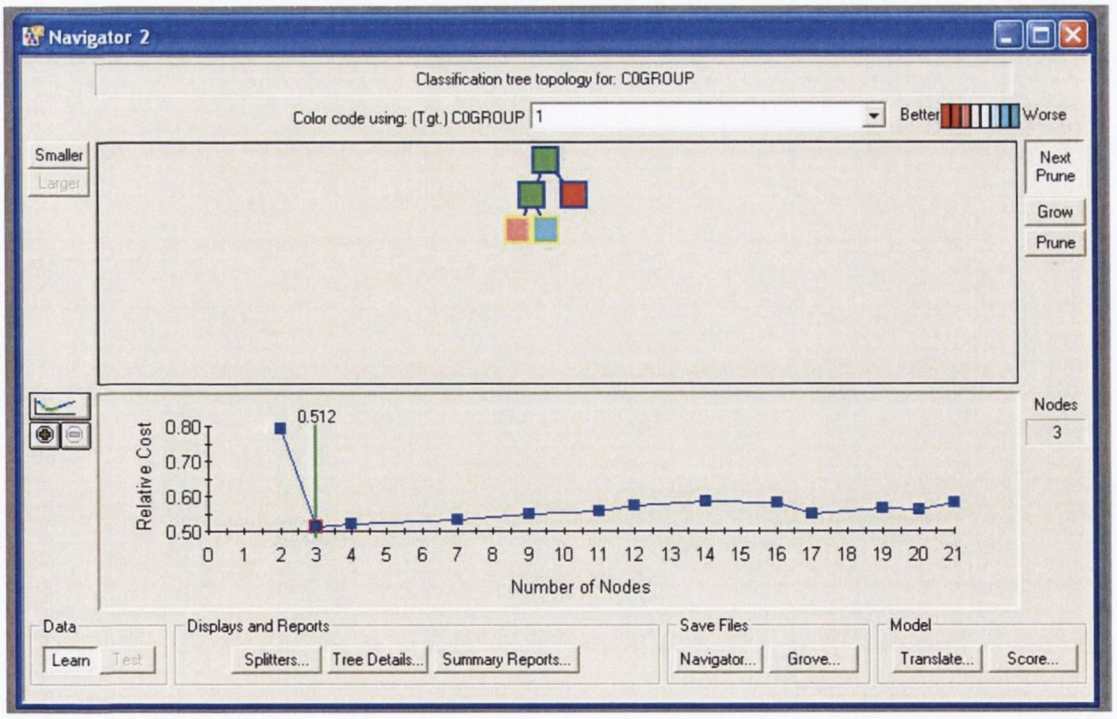
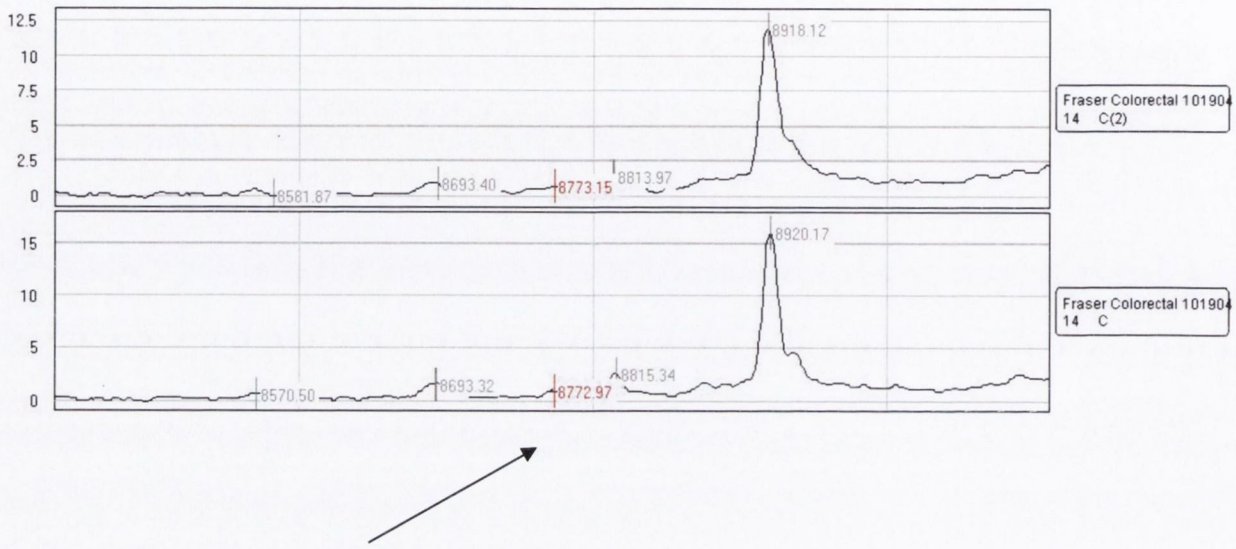
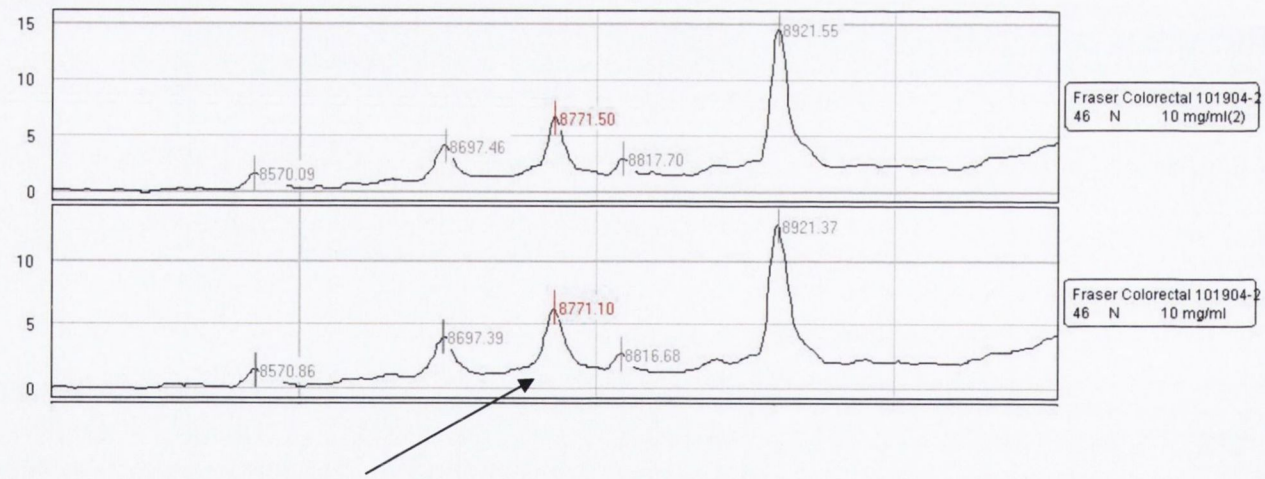


Figure 5.3. Screenshot from Ciphergen Biomarker Patterns software. This image shows the decision tree used to separate cancer from non-cancer that was generated from our data.

Figure 5.4 Close-up view of spectral area showing differential peak intensities between patients with or without cancer.



Absence of peak at 8772.97 Da in a patient with CRC.



Presence of peak at 8772.97 Da in patient with healthy colon.

5.5 Discussion

The prognosis and treatment options for patients with colorectal cancer (CRC) are directly related to the stage of disease at presentation. As CRC often runs an insidious course, many patients remain asymptomatic until the disease is suitably advanced to cause constitutional symptoms. The introduction of screening programs using either fibre optic endoscopy or faecal occult blood testing (FOB) have already shown that it is possible to decrease mortality by detecting tumours at an earlier stage, however, both of these modalities have inherent problems and poor levels of patient acceptability. As such, alternative means of disease screening are currently being sought.

At the time of starting this study, proteomic analysis of serum as a cancer screening modality had only just been described in ovarian cancer where it could differentiate between patients with or without cancer with 100% sensitivity and 95% specificity. The fact that it had not yet been assessed in the setting of CRC in tandem with its non-invasive nature prompted our study. The results are therefore disappointing in that we were unable to differentiate between benign or malignant disease with clinical significance. The explanation for this poor predictive ability is not clear as since our study started, 3 reports using near-identical methodologies to ours have appeared in the literature all of which found that serum proteomics was effective in screening for CRC.

The first of these studies, by Zhao et al analysed serum derived from 73 patients with CRC, 16 with benign colonic disease and 31 with normal colons (141). In this study CRC could be correctly predicted with 97% sensitivity and 100% specificity.

This was promptly followed by 2 further reports. The first of these, by Yu et al analysed serum from 55 patients with CRC, 35 from benign disease and 92 with normal colons. This group found that they were able to correctly classify CRC from both benign disease and from normal colons with sensitivities and specificities approaching 90% (144). The second of these, by Chen et al analysed serum from 55 patients with CRC and 92 age-sex-matched controls. They found four discriminatory peaks able to correctly classify CRC with 91% sensitivity and 93% specificity(113). In addition to its success in screening for ovarian and colorectal cancer, since the instigation of our study pilot studies assessing the screening potential of serum proteomics in multiple other cancer sub-types including breast, lung, laryngeal, prostate have all been successful.

It is therefore unclear as to why our study failed to replicate the good results of others. It was performed in an experienced and established facility, included large numbers of patients per group and all samples analysed were collected and stored in an optimal manner. In addition, two different different statistical packages were assessed when analysing the data. The only possibility to explain our result is that the chip surface used in our study was different to that used in other studies in CRC. Unfortunately this theory is difficult to prove as both Yu et al and Chen et al do not state what chip surface they used in their papers. The paper by Zhao et al, whose results showed 97% sensitivity and 100% specificity, does state that they used IMAC 3 chips. As different chip surfaces bind different groups of proteins, it is possible that the Q10 surface used in our study did not bind the optimal subset of proteins for CRC screening. The benefits of repeating our experiment using a different chip surface at this stage, however, are not clear as to do this would be both expensive and time-consuming in light of the fact that 3 other similar publications are now in existence.

5.6 Summary

In this chapter, the potential for SELDI-TOF-MS analysis of serum to be used as a non-invasive screening tool for colorectal cancer was assessed. Serum from 46 patients with normal colons, 23 with adenomatous polyps (single or multiple) and 57 with colorectal cancer was included. Bioststistical analysis of generated spectra using two different software packages was performed. Neither of these was able to prove that the technique was clinically useful as the best sensitivity and specificity obtained by both techniques was in the order of 75%.

Chapter 6: Global gene expression analysis using microarray profiling of pre-treatment rectal cancer biopsies as a response predictor for neoadjuvant radiochemotherapy.

6.1 Introduction

The aim of this chapter was to generate and compare gene expression microarray profiles between pre treatment biopsies from responsive and non-responsive patients to RCT in rectal cancer. At the time of starting this project there were no published reports where microarrays had been utilised to this end. Furthermore, when our project started, there were no optimised methods in place at St James's Hospital for the collection and storage of fresh tissue or for subsequent RNA extraction.

In order to obtain the most informative results from microarrays, it is imperative that the quality of RNA used is optimal. Central to this process is the avoidance of RNA degradation. This occurs as a result of as ubiquitous endogenous RNAse activity and rapidly degrades tissue RNA after it is removed from a living organism. Inhibition of this process as soon as possible after procuring tissue is therefore important to ensure the most accurate reflection of *in vivo* RNA expression is obtained(168). This ensures the validity of the end results. The way in which tissue is stored and the methods used to extract RNA are therefore all important in determining the ultimate RNA quality and yield. To this end, one of the primary objectives in this study was firstly to optimise our methodology paying particular attention to the inhibition of endogenous RNAses, tissue storage methods and finally the determination of the best means of extracting RNA.

During the preparatory phases of this experiment, a new expression array system from Applied Biosystems was released: The Applied Biosystens Version 2.0 Human Genome Survey Microarray system(169). This was chosen for our study for several reasons. Its design is based upon a complete, accurate gene and transcript library of the human genome.

It also incorporates the sensitive new detection method of chemoluminescence. This is superior to detection using fluorescence as used in other commercially available microarray systems as light used to excite the fluorescent platforms can overlap with their emission spectra. Chemiluminescent reactions, however, produce light when a label binds to a substrate rather than relying on light to excite the bound label, giving the system a more informative and sensitive overall package. Furthermore, each probe in the Applied Biosystems microarray chip is derivative of a relational database including Celera annotations (the company behind the recent sequencing of the human genome) and those in the public domain(170) (Fig 6.1). Probes therefore represent gene sequences that have been fully curated and critically analysed. This enables the rapid inference of biological meaning from the expression events revealed by the microarray data. An additional advantage of the Applied Biosystems microarray platform is that a full set of kits is available to conduct the microarray process from start to finish. Furthermore, corresponding Taq Man targets are commercially available for each probe on the microarray. This enables easy validation of results obtained.

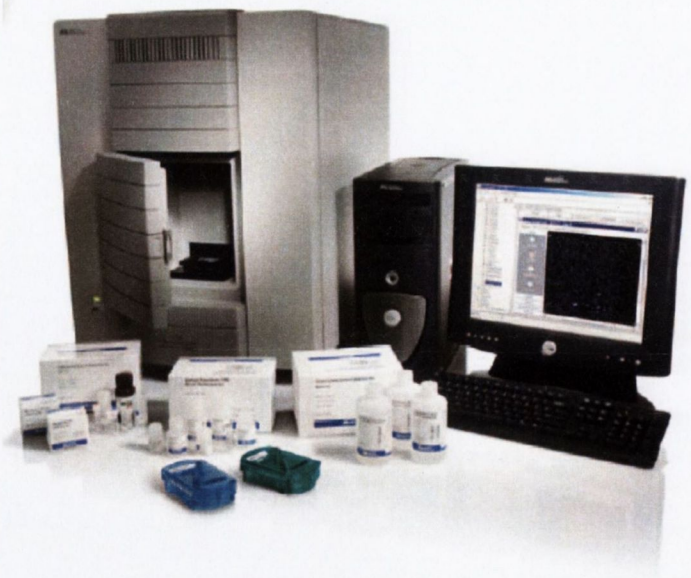


Figure 6.1. Applied Biosystems V2 Expression Array System

6.2 Aims:

1. To optimise collection, storage and extraction techniques for pre treatment rectal cancer biopsies that yield optimal quality RNA suitable for undergoing cDNA microarray analysis.

2. To conduct a comparative cDNA microarray analysis between patients demonstrating good and poor response to RCT.

3. To interpret transcriptional differences between patients demonstrating good and poor response to RCT.

6.3 Materials and methods:

6.3.1. Patients, tumour sampling and ethics

Before any aspect of this study began, written ethical approval was obtained from the local ethics committee. In addition, our study was conducted in accordance with current ICH-GCP guidelines. From July 2003-October 2004, all patients with rectal cancer who were to undergo RCT were prospectively recruited. There were no exclusion criteria. All patients gave fully informed consent for their participation (Appendix 2). In certain circumstances it was not possible to obtain tissue from patients such as patient preference, staff shortage or not being notified of samples by endoscopists. All samples were procured at the time of rigid sigmoidoscopy and were taken with large, rigid biopsy forceps.

6.3.2. Clinical (pre-treatment) and pathological staging

Pre treatment staging consisted of 1) thoracic and abdominal CT scanning and 2) pelvic MRI scanning in all patients. A subset of patients underwent transrectal ultrasound (TRUS) evaluation in addition to MRI to further define local disease. Any discrepancies between MRI and TRUS were discussed and a final pre treatment stage was assigned to each patient according to the TNM classification.

In order to grade pathological response to treatment, diagnostic slides from resected specimens of all patients were retrieved. These included no less than 3 sections of the irradiated area per patient. A single pathologist blinded to patient identifying characteristics reassessed all sections.

Response to RCT was assessed using the scoring system previously described by Mandard et al(19). This system grades response on a 5-point scale according to the relative amounts of residual tumour and fibrosis (Fig 1.2). For analysis purposes in this study, patients with a TRG of 1 or 2 were deemed to have demonstrated a good response and those with a TRG of 3-5 a poor response.

6.3.3. Neoadjuvant regimen

All patients were deemed suitable for RCT after discussion at a weekly multi-disciplinary meeting. Each received 45-50 Gy of radiotherapy, given in 25 fractions delivered on 5 days for 5 weeks. Continuous IV 5-Fluorouracil (225mg/m^2/day) was given concurrently with radiotherapy via a syringe driver. Surgery was performed approximately 6-8 weeks after the last fraction of radiotherapy had been given.

6.3.4. Inhibition of endogenous RNAse activity

There are several reported ways in which to inhibit endogenous RNAse activity, each of which has its own advantages and disadvantages (Table 6.1). It was our aim to assess the 3 main methods (Fig 6.2).

1. Immediate snap freezing of tissue in liquid nitrogen.
2. Placing tissue into a cryomould containing the cryopreservative compound Optimal Cutting Temperature (O.C.T) (Tissue-Tek, USA) followed by immediate snap freezing.
3. Immediate immersion of tissues into the RNA stabilising agent RNAlater (Ambion, Texas, USA).

Figure 6.2: Methodologies used in tissue collection and storage

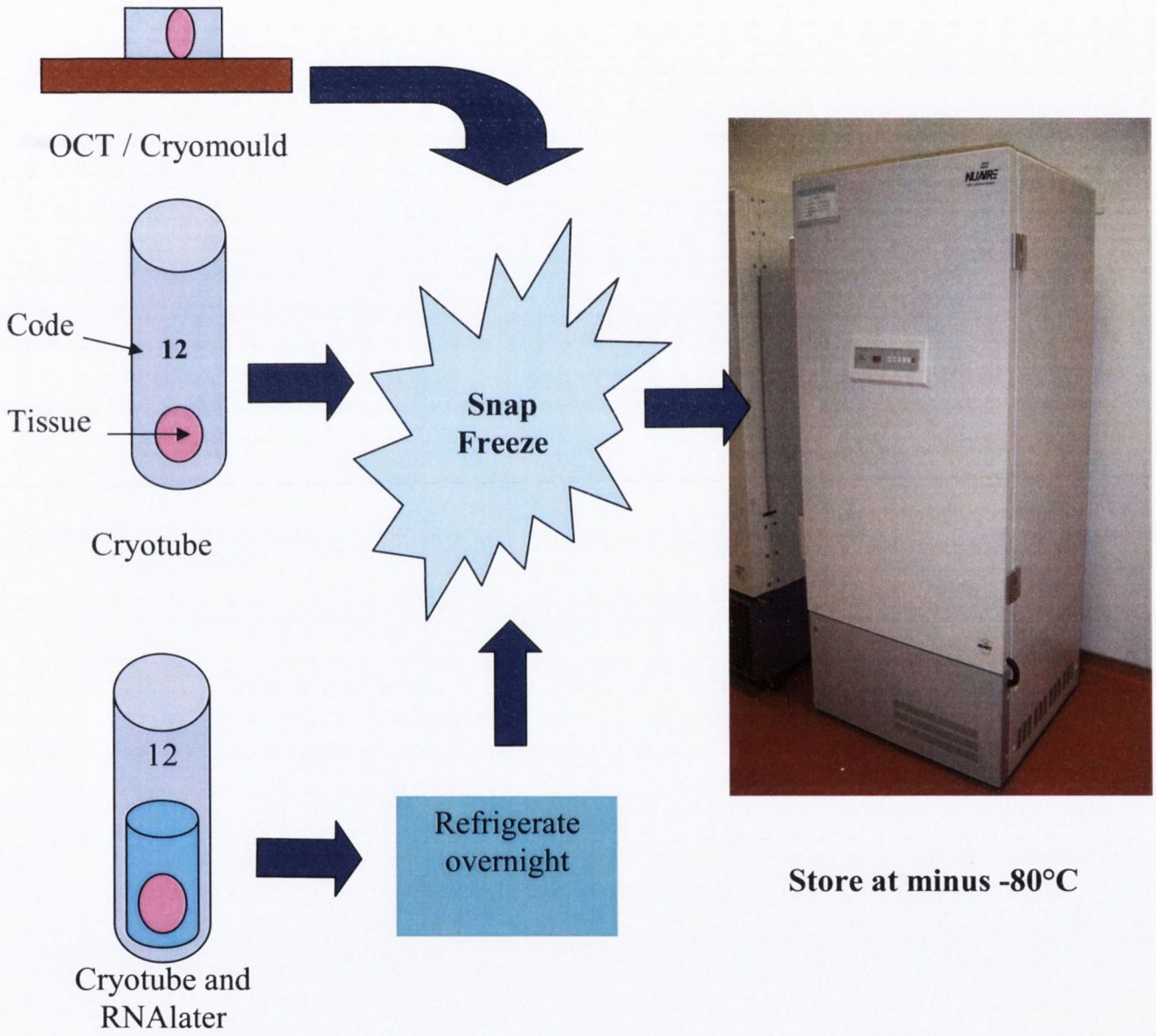


Table 6.1: Advantages and disadvantages of RNAse inhibition/tissue storage techniques

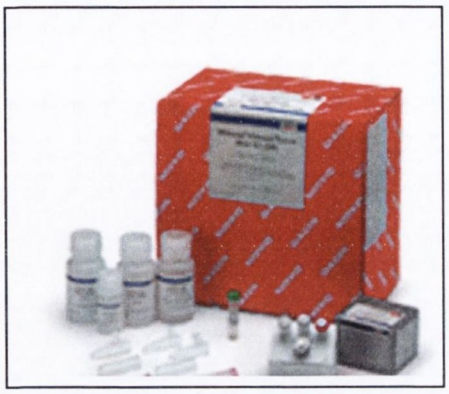
Method	Advantages	Disadvantages
Immediate snap freezing	Tissue may be used for any downstream application such as proteomic analysis, IHC or RNA extraction	Must carry flask of liquid nitrogen. This is dangerous if spilt, time-consuming and requires a local supply of liquid nitrogen
Embedding tissue in OCT-filled cryomoulds and snap freezing.	Tissue may be used for any downstream application such as proteomic analysis, IHC or RNA extraction. Tissue ready mounted for frozen section allowing easy assessment of nature of biopsy and laser capture microdissection.	Time consuming. Requires liquid nitrogen.
Immediate immersion of tissue into RNAlater followed by subsequent snap-freezing	Extremely easy. No need for liquid nitrogen. Tissue stable for up to 24hrs at room temperature and 1 month in the fridge.	Only allows assessment of RNA. Unable to frozen section tissue.

6.3.5. Extraction techniques

In order to ensure that we optimised the quality and quantity of available RNA from the tissue biopsies that we procured, we assessed 3 different extraction methods:

1. Qiagen RNeasy kit (Qiagen, Crawley, UK), (Fig 6.3a)
2. Tissue homogenisation in in QIAzol lysis solution (Qiagen) followed by RNA extraction using the Qiagen RNeasy kit (Qiagen).
3. Versagene^{TM} RNA purification kit (Gentra Systems Inc, USA), (Fig 6.3b).

Figure 6.3 a+b. RNA extraction kits



a) Qiagen RNeasy kit

b) Versagene™ RNA purification kit

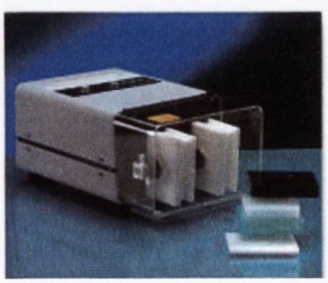
6.3.6. Tissue Homogenisation

We assessed two different methods of tissue homogenisation:

1. Sample agitation with a ball bearing using the Mixer Mill MM300 (Retsch, Leeds, UK), (Fig 6.4a).
2. Sample disruption using a hand-held tissue homogeniser (PRO 200, PRO Scientific, USA), (Fig 6.4b).

Fig 6.4 a+b. Different homogenisation methods

a)



Mixer-Mill 300

b)

Hand-Held Homogeniser

6.3.7. Comparison of 3 different RNAse inhibition and extraction techniques.

In order to standardise all comparisons, 9 equally sized tissue samples were taken from from the same area of the same colorectal tumour. Three samples were stored by immersion in RNAlater, snap freezing or in cryomould/OCT. All samples were then stored at -80°C for 48 hours. Samples were then defrosted and weighed, ready for RNA extraction to be performed. In the case of the Versagene and RNeasy tissue kits, extractions were performed in strict adherence to the manufacturer-supplied standard operating procedures (Appendix 4 and 5 respectively). Samples processed using QIAzol reagent were homogenised and separated into protein/RNA and DNA phases with chloroform. Centrifugation subsequently created an RNA pellet which was then dissolved in RNAse-free water at which point all further steps were performed using the RNeasy tissue Kit.

Quantity and quality of extracted RNA for each kit type were assessed using both the NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Cheshire, UK). High quality RNA was determined both by using 260/280 ratio>2 in the case of the NanoDrop and 28:18S peak ratio>1.4 in the case of the Agilent Bioanalyzer.

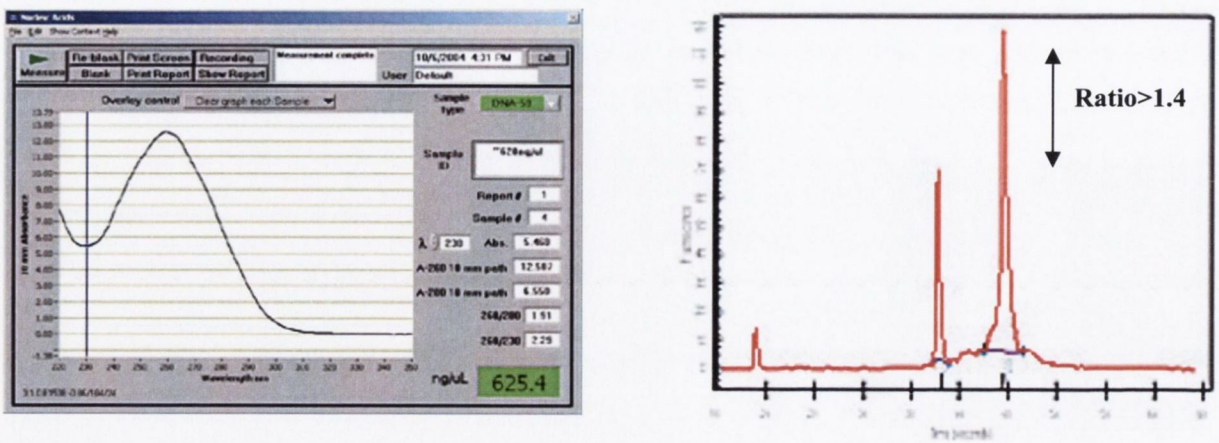


Figure 6.5. Examples of high quality RNA as assessed by NanoDrop (left) and Agilent Bioanalyzer (right)

6.3.8. Evaluation of hand-held rotor tissue homogeniser.

In order to standardise all comparisons, 6 equally sized tissue samples were taken from from the same area of the same colorectal tumour. Three samples were stored by immersion in RNAlater and by snap freezing. All samples were then stored at -80°C for 48 hours. Samples were then defrosted and weighed, ready for RNA extraction to be performed. Tissue homogenisation was performed using a hand-held rotor homogeniser and RNA extraction was performed in an identical manner to section 6.3.7. The quantity and quality of RNA was again assessed using the NanoDrop Spectrophotometer and the Agilent 2100 Bioanalyzer.

6.3.9. Extraction of RNA from archived tumour samples.

Comparison of RNA quantity and quality was performed between all tissue storage and extraction methods performed. The relative merits of each method were discussed and an optimal method was decided upon. From this juncture, all further tissue collection and storage was performed using only this method. When a sufficient number of tissue samples had been collected, RNA extraction was performed in runs using the optimised method. Again, quantity and quality of all extracted RNA was assessed using the NanoDrop Spectrophotometer and the Agilent 2100 Bioanalyzer.

6.3.10. Generation of DIG-labelled cRNA.

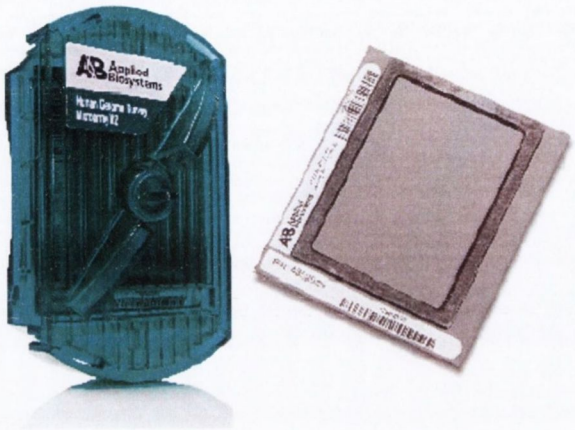
High quality tumour RNA was converted into digoxigenin (DIG)-labelled cRNA prior to hybridisation on to the microarrays. Briefly, RNA was reverse-transcribed and second strand synthesis was performed to generate cDNA. This was then purified with DNA wash buffer in nano-purification columns and eluted through a proprietary fibre matrix.

In vitro transcription (IVT) labelling to generate DIG-labelled cRNA was then performed overnight at 37°C in a thermal cycler. The following morning, this was purified using a series of on-column washes before being eluted into a proprietary storage tube. The quality of the DIG-labelled cRNA was assessed using the Agilent 2100 Bioanalyser and poor quality samples were either repeated or discounted from further study. This whole process was done using the standard operating procedures of the Applied Biosystems Chemiluminescent RT-IVT labelling Kit (V2.0) (Appendix 6).

6.3.11. Hybridisation of DIG-labelled cRNA onto Microarrays

Applied Biosystems V2 Microarrays (Fig 6.6) were pre-hybridised in an oven set at 55°C for 30 minutes. During this time, cRNA was fragmented with cRNA fragmentation buffer for 30 minutes in a thermal cycler then briefly centrifuged. Then 150 µl cRNA was added to a pre-prepared hybridisation mixture, vortexed and quickly transferred into its designated microarray cartridge and placed on a rocking shelf in the oven at 55°C. This process was repeated for all samples to be microarrayed. No more than 4 microarrays were run at one time to reduce the potential for error. All microarrays were then left at 55°C for a period of exactly 16 hours. The experiment was timed to allow this to be of overnight duration.

Figure 6.6. - Photograph of an Applied Biosystems Version 2 Human Genome Microarray.



The following morning, approximately 45 minutes before the microarrays were due to be removed from the oven, the hybridisation and chemiluminescence wash/rinse buffers, enhancing solution and blocking reagent were made. All microarrays were removed from the oven in turn, and placed into a wash tray on a rocking platform whereafter 2 hybridisation washes and 2 chemiluminescent rinses were performed. A further 3 wash steps were performed before the chemoluminescent enhancing solution was applied to the microarrays. At this point, each microarray was loaded in turn into the Applied Biosystems 1700 Chemiluminescent Bioanalyzer for image analysis to be conducted. This procedure was performed using a standard operating procedure supplied with the kit (Appendix 7).

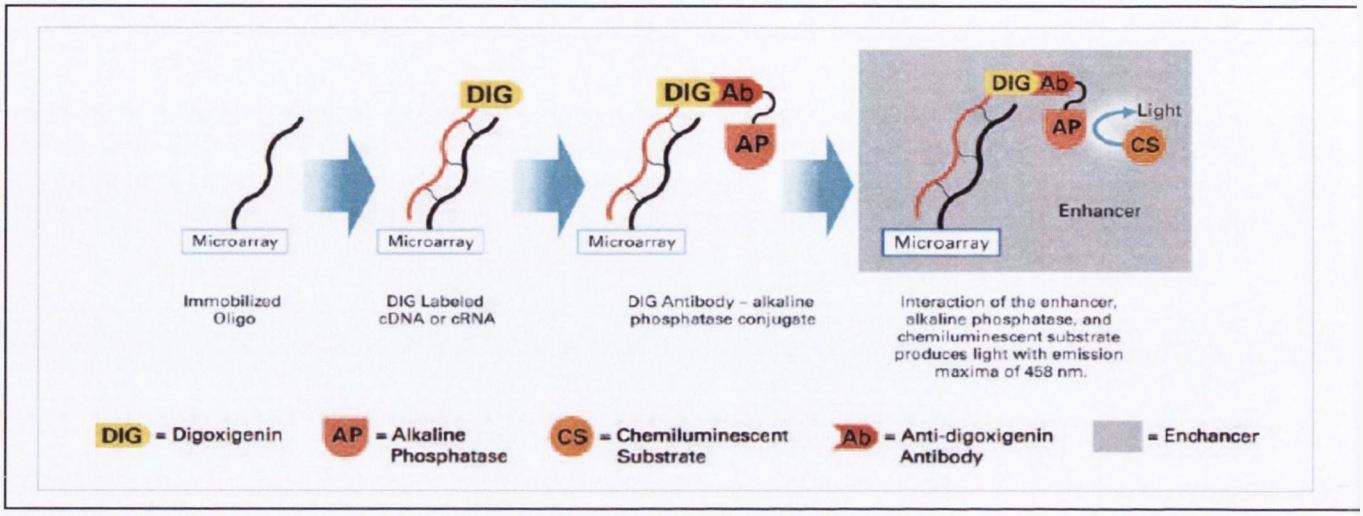


Figure 6.7. Schematic showing the binding and activation of the chemoluminescent detection substrate.

6.3.12. Statistical analysis

Microarray data was analysed using BioConductor R version 2.0 (Analysis by F.M.S and Dr Charles Gillham). This is a free, open source software project designed for the analysis and comprehension of genomic data (171) (R Core Development Team, 2004). Quality assurance was performed on all raw data which included boxplots for signal distribution range, MA plots for signal distribution and signal variability, CV plots for variation amongst hybridisation replicates, scatter plots for correlation between hybridisation arrays, correlation heatmaps for visualisation and signal to noise concordance mapping. A signal to noise ratio threshold of 3 was used. Quantile normalisation was then performed on all data and all quality assurance steps were then repeated. Differential gene expression between good and poor responders was then identified using both the t-test/ANOVA tool and fold change.

All of steps were using the standard operating procedures outlined in appendix 7. Finally, all data was uploaded onto a Microsoft Excel Datasheet (Microsoft Coropration, USA), which allowed easy manipulation of the data to select differentially expressed genes.

6.3.13. Discovery of gene identity, cellular function and pathway of action.

Lists of the genes differentially expressed between good and poor responders were Generated gene lists were uploaded into the PANTHER Classification System (**P**rotein **AN**alysis **TH**rough **E**volutionary **R**elationships). This is an on-line database that is designed to classify proteins (and their genes) in order to facilitate high-throughput analysis. It can classify gene lists by i) family and subfamily (families are groups of evolutionarily related proteins; subfamilies are related proteins that also have the same function), ii) molecular function (The function of the protein by itself or with directly interacting proteins at a biochemical level, e.g. a protein kinase) iii) Biological process (The function of the protein in the context of a larger network of proteins that interact to accomplish a process at the level of the cell or organism, e.g. mitosis) and iv) Pathway (similar to biological process, but a pathway also explicitly specifies the relationships between the interacting molecules). All PANTHER classifications are derivative of the results of human curation as well as sophisticated bioinformatics algorithms.

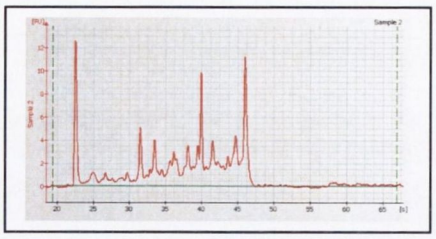
6.4. Results

6.4.1. Optimisation of RNA stabilisation, tissue storage and extraction methods.

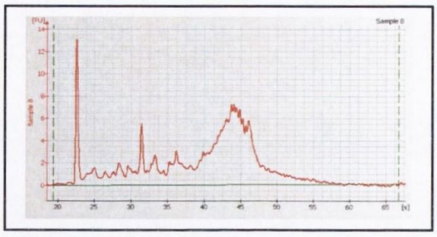
RNA could be extracted using all methods assessed. Initial analysis on the NanoDrop spectrophotomer showed extremely good results for all storage and extraction techniques and found that high concentrations of extracted RNA were present in each sample (Table 6.2). Analysis of the same samples using the Agilent Bioanalyzer, however, found the situation to be somewhat different showing that extracted RNA was of extremely variable quality (Fig 6.7). In particular, RNA generated from all samples stored in OCT compound was found to have undergone significant amounts of degradation (Fig 6.7 a-c) and by far the best quality RNA was that from samples stored in either RNAlater or those that had been snap frozen (Fig 6.7 d-i).

In the first optimisation experiment (6.3.7), theVersagene RNA Purification Kit yielded the best RNA, especially from samples stored in RNAlater (Fig 6.7 a,d and g). The second best kit was the Rneasy Tissue Kit (Fig 6.7 b,e and h). In this experiment samples homogenised in Qiazol yielded poor quality RNA (Fig 6.7 c,f and i). A further point of note was that whilst homogenising several samples in the Mixer Mill MM300, we noted that large chunks of residual tissue remained which could not be disrupted even on further homogenisation. This experiment told us that OCT compound should not be used to store our RNA as it substantially degraded its quality. It also demonstrated that the NanoDrop unreliably demonstrated RNA quality. Lastly, it showed that the Mixer Mill MM300 was incapable of fully homogenising several of our samples. This compelled us to try an alternative means of tissue homogenisation.

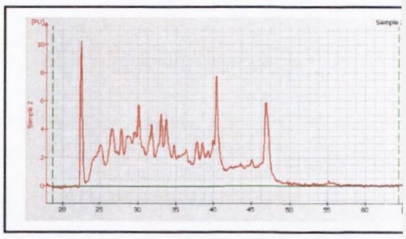
Figure 6.8. Comparison of Agilent Spectra from 1st optimisation experiment.



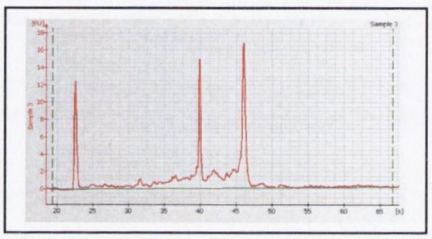
a) Versagene - OCT



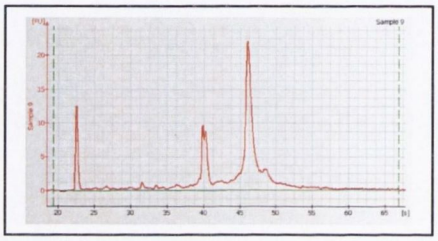
b) RNeasy - OCT



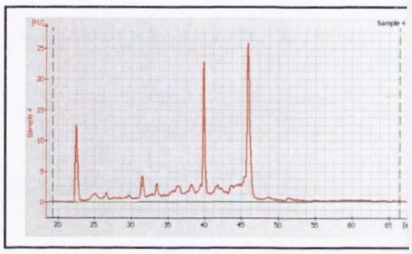
c) RNeasy (Qiazol) - OCT



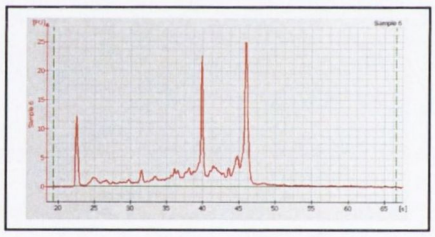
d) Versagene - RNAlater



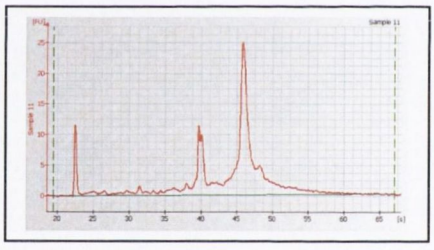
e) RNeasy - RNAlater



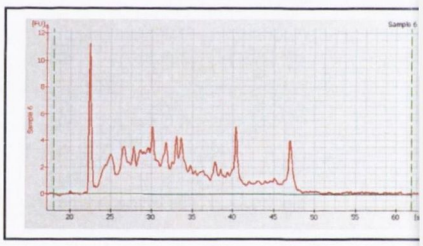
f) RNeasy (Qiazol) –RNAlater



g) Versagene – Snap frozen



h) Rneasy – Snap frozen



i) RNeasy (Qiazol) – Snap froz

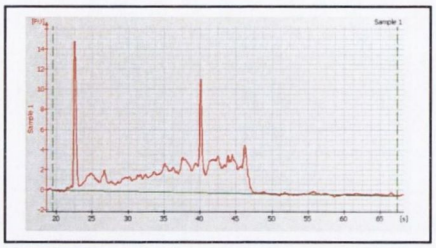
Table 6.2. NanoDrop derived values of extracted RNA quality (1st)

Extraction Type	Nano 260/280	Conc (ng/uL)
RNeasy (Qiazol) OCT	2.1	2070.2
RNeasy (Qiazol) RNAlater	2.13	1285.8
RNeasy (Qiazol) Snap	2.07	2619.7
Versagene OCT	2.11	1398
Versagene RNAlater	2.11	372.3
Versagene Snap	1.91	405.9
RNeasy OCT	2.11	1174.3
RNeasy RNAlater	2.12	1357.7
RNeasy Snap	2.07	2334.2

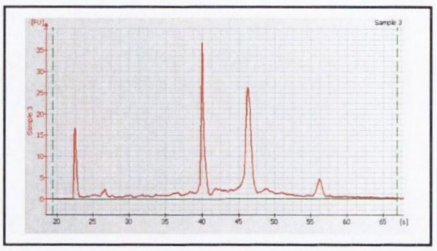
The second optimisation study (6.3.8) followed on from the first study. It re-assessed sample storage **only** in RNAlater and snap-freezing. In addition, all samples were homogenised using a hand-held rotor homogeniser. This second study revealed that tissue stored in RNAlater was very robust and gave consistently good quality RNA (Fig 6.8 a-f). It also showed that snap frozen tissue was capable of giving good quality RNA but not as consistently as tissue stored in RNAlater. The Versagene Kit did not perform well in this experiment (Fig6.8 a+d) on either snap frozen or RNAlater-stored tissue. The best spectra were those where the RNeasy Kit was used with prior homogenisation in Qiazol lysis solution (Fig 6.8 c+f). In addition to the excellent spectral patterns generated, the NanoDrop readings were also of high quality (Table 6.3). This study also revealed that the hand-held homogeniser fully disrupted specimens in contrast to the MixerMill 300.

From the cumulative results of these two experiments, it was decided that all biopsy tissue would be stored in RNAlater solution and would undergo hand-held homogenisation in Qiazol lysis solution followed by on-column ectraction using the RNeasy Kit.

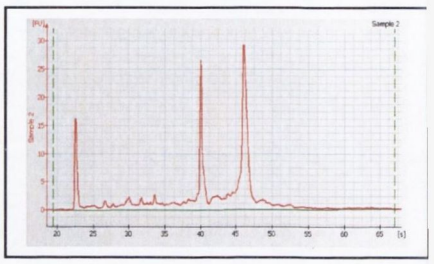
Figure 6.9 a-f. Comparison of Agilent Spectra from 2nd optimisation experiment.



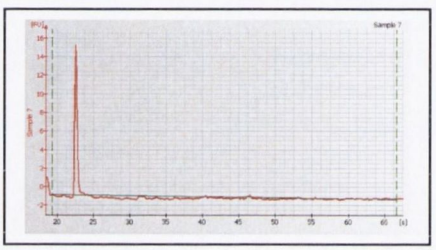
a) Versagene - RNAlater



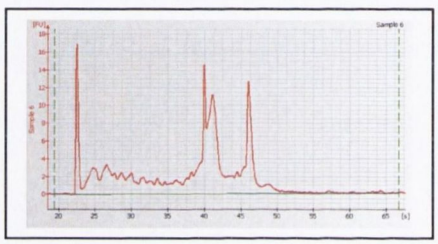
b) RNeasy - RNAlater



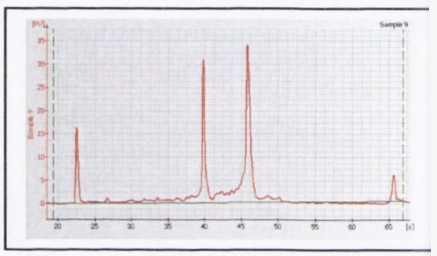
c) Rneasy (Qiazol) - RNAlater



d) Versagene – Snap frozen



e) Rneasy – Snap frozen



f) Rneasy (Qiazol) – Snap frozen

Table 6.3. NanoDrop derived values of extracted RNA quality (2nd)

Extraction Type	Nano 260/280	Conc (ng/uL)
Qiazol RNAlat	2.12	740
Qiazol Snap	2.03	495.4
Versa RNAlat	2.01	502.3
Versa Snap	1.98	465.2
RNeasy RNAlat	2.01	441.5
RNeasy Snap	2.11	988.5

6.4.2. RNA extractions from pre treatment tumour biopsies

Pre treatment tumour biopsies were collected from 19 patients who underwent RCT followed by surgery. The final TRG of these patients along with the means of biopsy storage and final tissue useability are shown in table 6.4. This table shows that RNA of sufficient quality for suitable for microarray analysis was only successfully extracted from 10 patients.

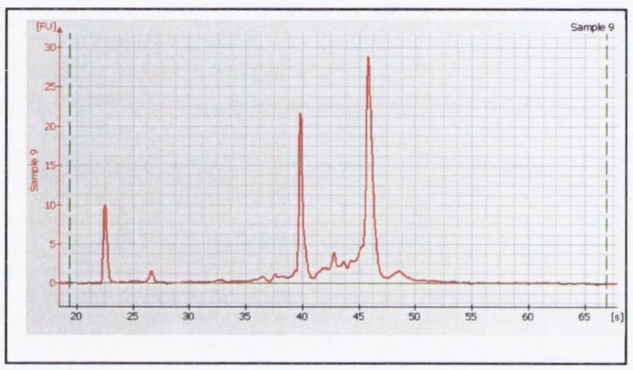
Table 6.4. All patients with pre treatment biopsy tissue showing the method of storage and subsequent tissue useability.

Patient No	TRG	Storage method	Microarrayable?
1	2	RNAlat	Yes
2	2	RNAlat	Yes
3	1	RNAlat	Yes
4	4	RNAlat	Yes
5	2	RNAlat	Yes
6	3	RNAlat	Yes
7	3	RNAlat	Yes
8	3	RNAlat	Yes
9	4	RNAlat	No
10	4	RNAlat	Yes
11	3	RNAlat	Yes
12	4	OCT	No
13	4	OCT	No
14	3	OCT	No
15	2	OCT	No
16	3	OCT	No
17	4	OCT	No
18	4	OCT	No
19	2	OCT	No

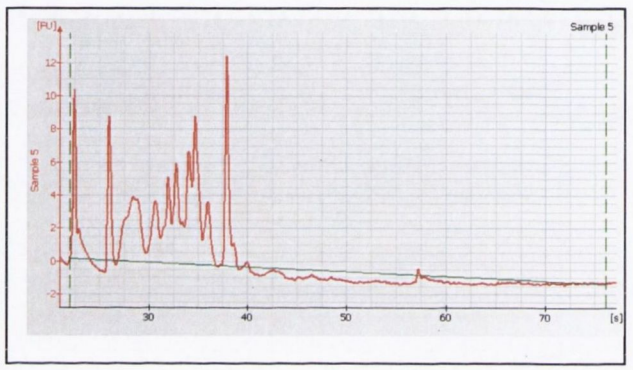
Of note, without exception each patient with good quality RNA had his or her tissue stored in RNAlater. At the start of our project, however, we did not have the facilities necessary to optimise our collection and storage process. As s a result, some of the earlier tumour biopsies had been stored in OCT compound (Tissue-Tek, USA) as it was generally felt at the time that

this technique would yield equivalent tissue useability but ultimately more versatility than other storage techniques. When we came to perform RNA extractions, however, we found that OCT significantly degraded RNA quality rendering it un-useable for microarray analyses.

Figure 6.10. Good and poor quality RNA from pre treatment biopsies (Agilent 2100 Bioanalyser)



Example of excellent quality RNA. The 28/18S ratio for this sample was 1.4. This sample had been immediately immersed in RNAlater and was used to perform microarray.

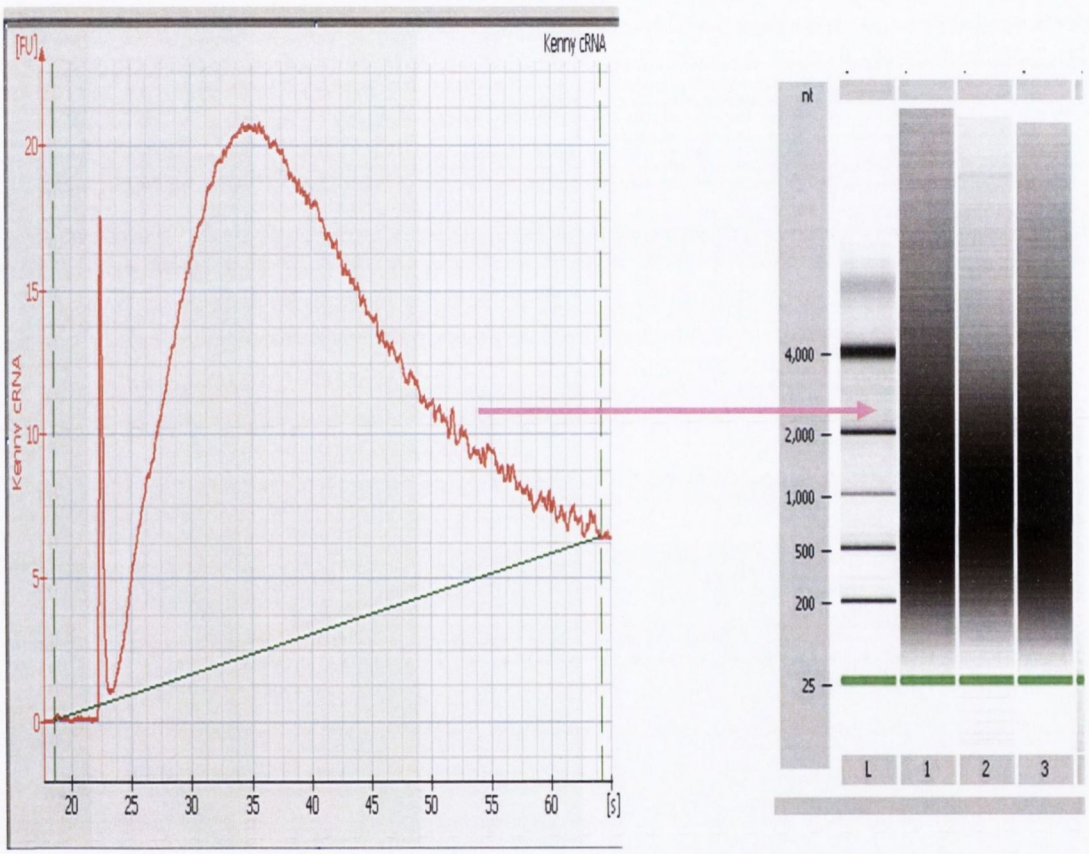


Example of a poor quality RNA sample. This sample had been taken near the beginning of our study before optimisation of collection process and had been stored in OCT. This sample was of too poor quality to microarray.

6.4.3. Generation of cDNA

High quality labelled cRNA suitable for microarray analysis was able to be generated from 9 of the 10 (90%) patients who had good quality RNA. cRNA of this quality was determined by the characteristic bell-shaped curve (spectral view) or 'smudge' (Gel view) on the Agilent Bioprocessor. In one patient, despite several attempts, generated cRNA did not give this appearance and so was not used in further analyses.

Figure 6.11. High quality cDNA suitable for microarray analysis.

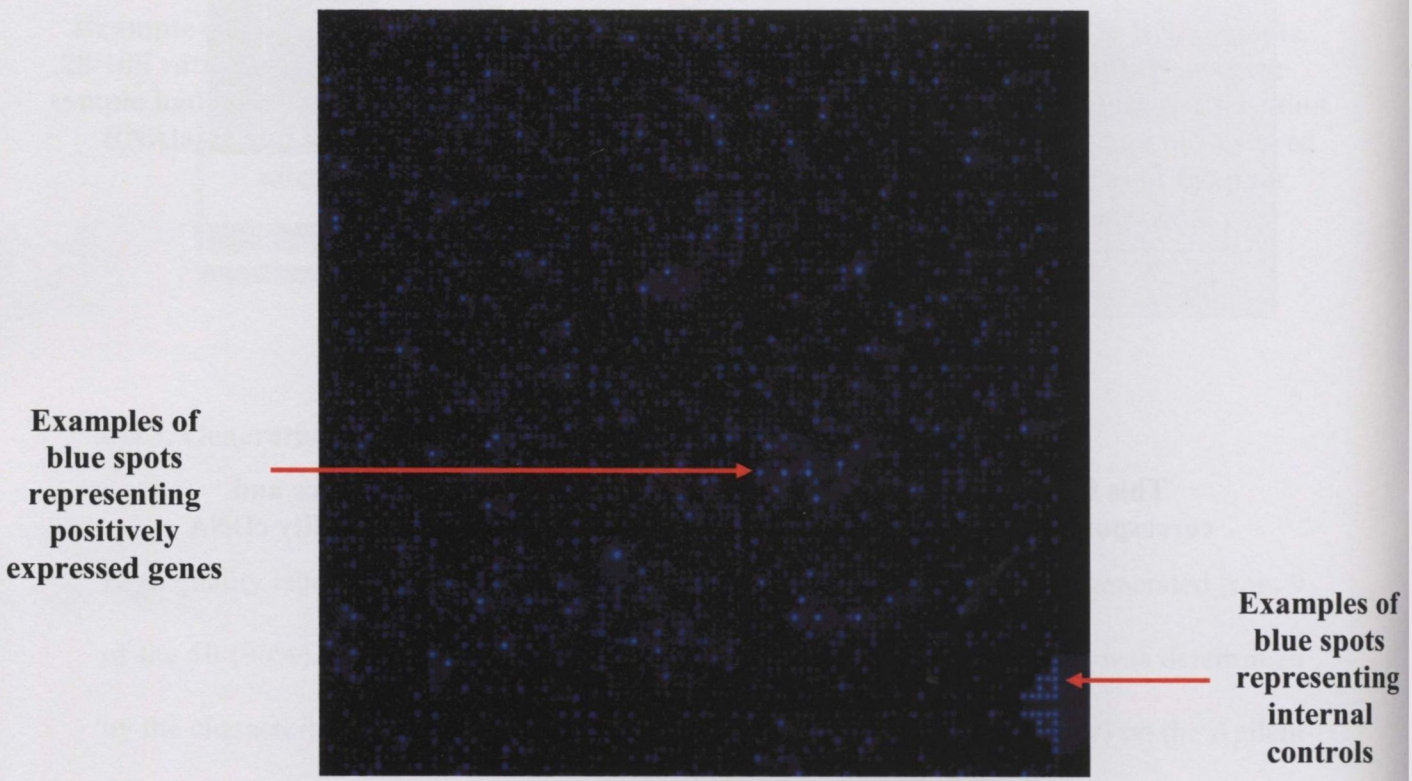


This figure shows the characteristic smooth spectral appearance and corresponding 'smudge' seen on gel view of a sample of high quality cDNA generated from a pre treatment biopsy in our study.

6.4.4. Detection of expressed genes using Applied Biosystems 1700 Chemiluminescent Microarray Bioanalyser.

After the analysis of each V2 microarray on the bioanalyser, a photographic image of each of the test spots on the chip was generated (Fig 6.12). Each blue spot represents an individual expressed gene. Some blue spots, however, represent internal controls, an example of which can be seen in the accompanying figure (Fig 6.12). All images were then read by an integral image analysis system where the relative degree of expression of each gene was determined based on the intensity of generated chemoluminescence.

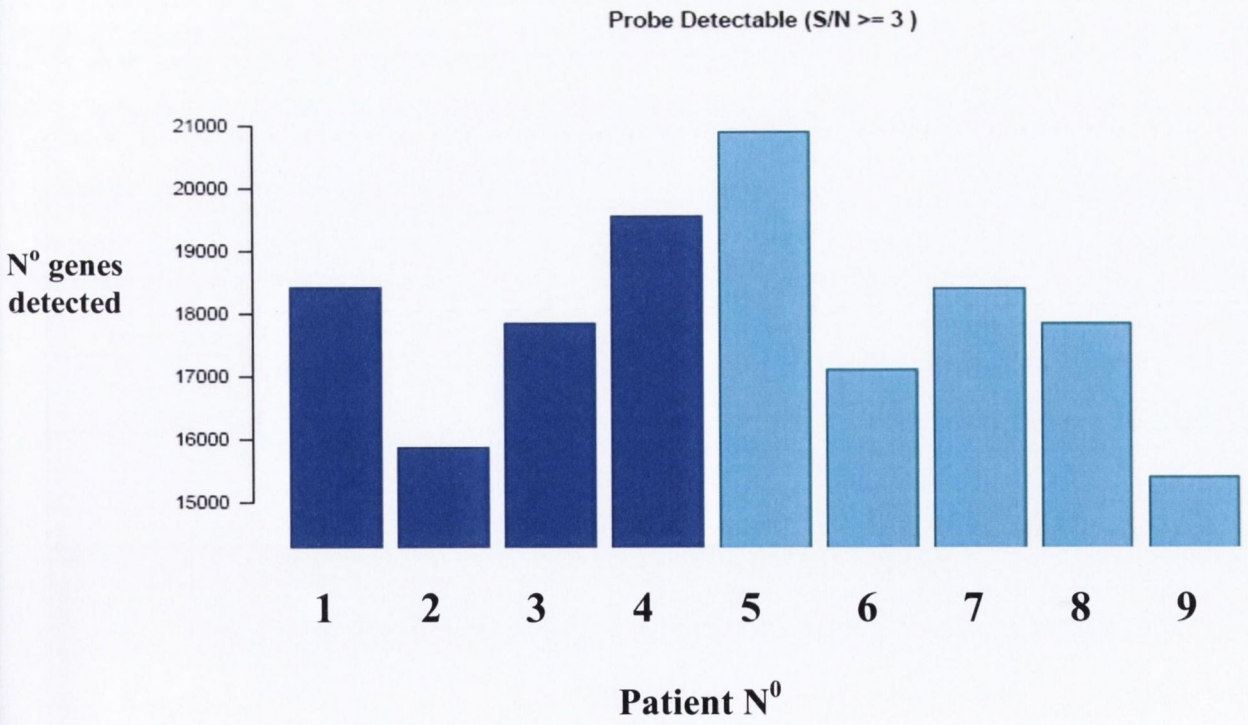
Figure 6.12 Microarray image capture



6.4.5. Paralell comparison of differentially expressed genes between good and poor responders.

Between15,500 and 20,800 genes were detected in each pre treatment tumour biopsy sample analysed (Fig 6.13). Paralell expression analysis was performed using the Bioconductor R statistical package.

Figure 6.13. Bar chart showing number of expressed genes in each sample microarrayed.



An Excel table listing all genes detected with their associated statistical differences and expression fold differences between good and poor responders. This table was then manipulated to display only genes with significant expression differences (i.e. $P<0.05$, $p<0.01$), large fold-differences in expression between good and poor responders (i.e. 2-Fold or 4-Fold) or a combination of these parameters (i.e. All genes with $P<0.01$ and 4-Fold expression differences) (Table 6.5). By adjusting these variables the number of genes differentially expressed could be determined at different levels of significance and fold-change. These were then used to generate gene clustering heat maps and for gene identification using PANTHER.

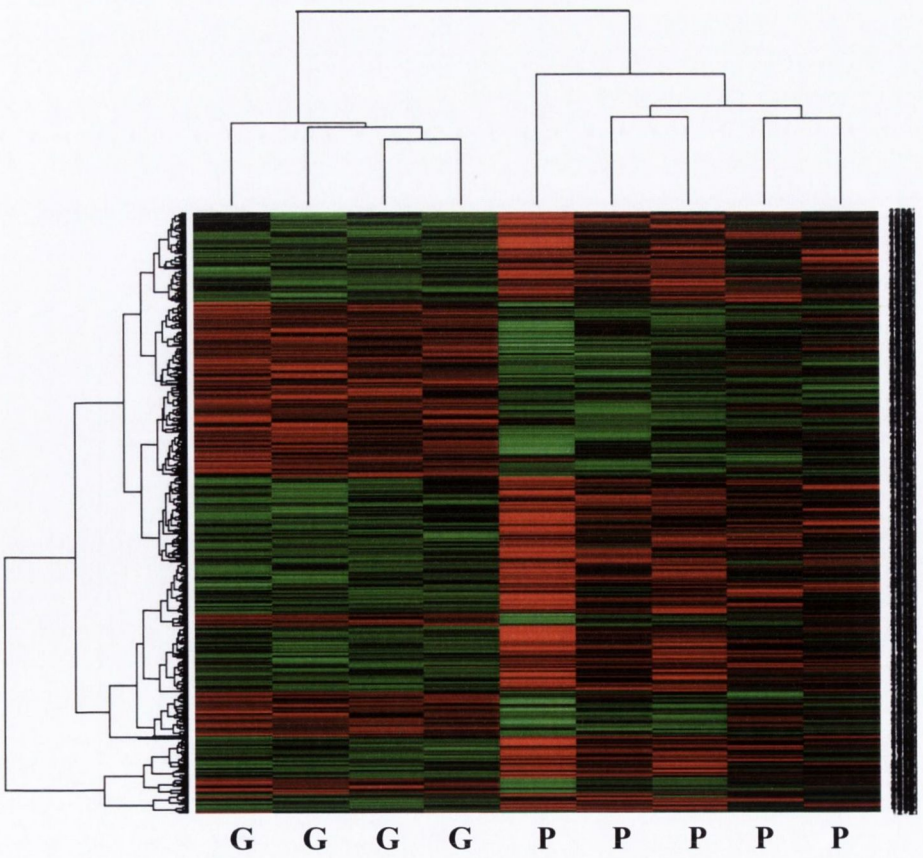
Table 6.5. Summary of number of differentially expressed genes under different classification strategies.

P-value	Fold Change	No Genes Differentially Expressed
<0.05	N/A	516
<0.01	N/A	62
<0.05	>2	237
<0.05	>4	16
<0.01	>2	49
<0.01	>4	3
<0.005	>2	20
<0.005	>4	0

6.4.6. Hierarchical clustering

Hiererchical clustering was performed for each of the 8 sets of differentially expressed genes listed in Table 6.5. This technique provides a visual correlate of clustering where the level of gene expression is represented by different colours and provides a rapid means of identifying cluster pattern differences between good and poor responders. The most visually compelling clustering diagram was obtained using the 516 genes differentially expressed with $p<0.05$ (Fig 6.14)

Figure 6.14. Heatmap (Euclidean) generated for all genes differentially expressed between good and poor responders (P<0.05).



G=Good responder P=Poor responder

Note the strong areas of clustering evidenced between good and poor responders as highlighted by green and red shaded boxes. Each green box represents a gene that is upregulated and each red box a gene that is downregulated.

6.4.7. Gene identification and function analysis using PANTHER Classification Database

Differentially expressed genes identified using the microarrays were uploaded into PANTHER where their molecular and biological functions could be ascertained. For illustrative purposes, these are detailed only for the 49 genes differentially expressed with $p<0.01$ and 2-fold expression change.

For this series, gene expression differences were apparent in genes representing multiple aspects of molecular function such as cell adhesion, transcription factors and cellular transportation (Fig 6.15). The corresponding biological function of these genes included regulation of apoptosis, signal transduction, cell adhesion and intracellular protein trafficking (Fig 6.16). Of note, a large percentage of identified genes have not yet had their molecular and biological functions identified.

Figure 6.15. Pie chart showing the molecular functions of differentially expressed genes.

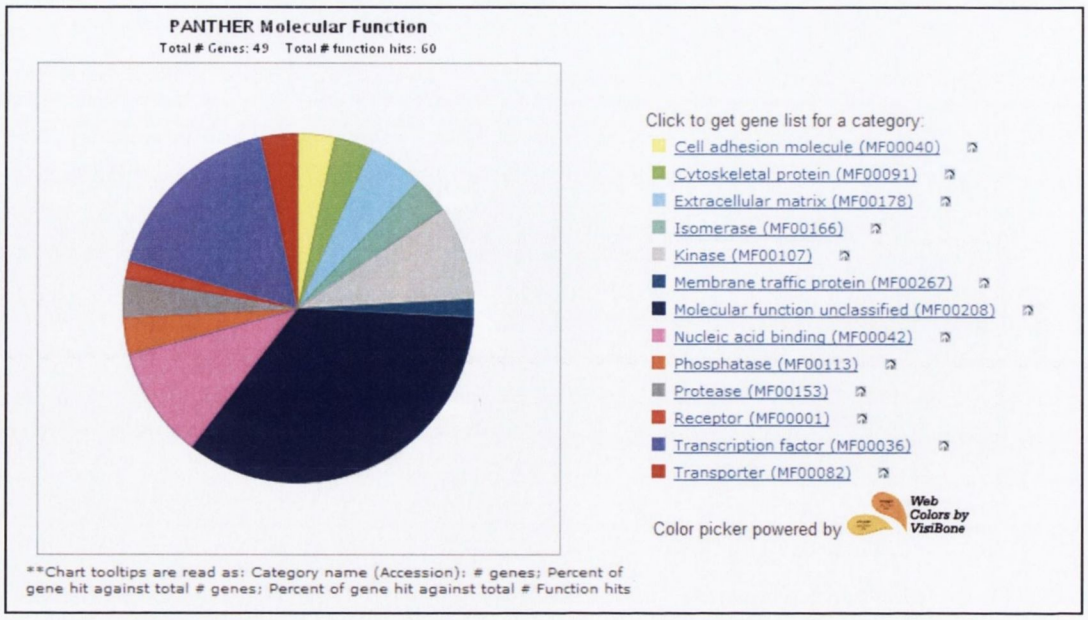


Figure 6.16. Pie chart showing the biological functions of differentially expressed genes.

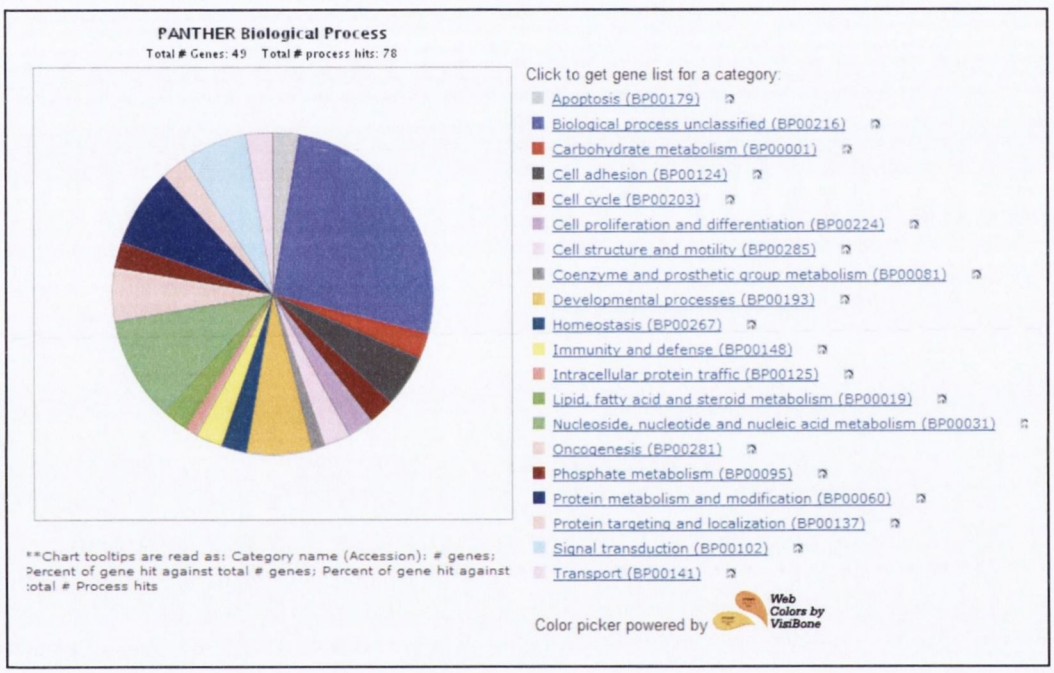


Figure 6.17. List of differentially expressed genes from PANTHER

Species Filter: All | NCBI: H. sapiens | NCBI: M. musculus | FlyBase: D. melanogaster | NCBI: R. norvegicus | Celera: H. sapiens | Celera: M. musculus

clr	all		Gene ID	Gene Name Gene Symbol	PANTHER Best Hit	PANTHER Score	PANTHER Molecular Function	PANTHER Biological Process
☐		1.	GeneID:59336	PR domain containing 13 PRDM13	PR-DOMAIN ZINC FINGER PROTEIN 13 (PTHR23228:SF18)	7E-69●●●	Zinc finger transcription factor	mRNA transcription
☐		2.	GeneID:7145	tensin 1 TNS1	TENS (PTHR12305:SF12)	0E+00●●●	Protein phosphatase Other phosphatase	Phospholipid metabolism Protein phosphorylation Signal transduction Cell adhesion Immunity and defense Induction of apoptosis Cell cycle control Cell proliferation and differentiation Tumor suppressor
☐		3.	GeneID:6840	supervillin SVIL	SUPERVILLIN (PTHR11977:SF4)	0E+00●●●	Non-motor actin binding protein	Cell structure
☐		4.	GeneID:51303	FK506 binding protein 11, 19 kDa FKBP11	FK506-BINDING PROTEIN 11 (PTHR10516:SF13)	7E-97●●●	Other isomerase	Protein folding
☐		5.	GeneID:22852	ankyrin repeat domain 26 ANKRD26	ANK REPEAT-CONTAINING (PTHR18958:SF121)	0E+00●●●	Molecular function unclassified	Biological process unclassified
☐		6.	GeneID:9807	inositol hexaphosphate kinase 1 IHPK1	INOSITOL HEXAPHOSPHATE KINASE (PTHR12400:SF15)	0E+00●●●	Kinase	Biological process unclassified
☐		7.	GeneID:4897	neuronal cell adhesion molecule NRCAM	NEURAL CELL ADHESION MOLECULE (NR-CAM) (PTHR10489:SF42)	0E+00●●●	CAM family adhesion molecule	Cell adhesion-mediated signaling Cell adhesion Neurogenesis
☐		8.	GeneID:4326	matrix metallopeptidase 17 (membrane-inserted) MMP17	MATRIX METALLOPROTEASE 17, MMP-17 (PTHR10201:SF21)	0E+00●●●	Metalloprotease Other extracellular matrix	Proteolysis
☐		9.	GeneID:161835	fibrous sheath interacting protein 1 FSIP1	SUBFAMILY NOT NAMED (PTHR22012:SF3)	0E+00●●●	Molecular function unclassified	Biological process unclassified
☐		10.	GeneID:7627	zinc finger protein 75a ZNF75A	KRAB-RELATED (C2H2-TYPE ZINC-FINGER PROTEIN 500) (PTHR23226:SF11)	0E+00●●●	KRAB box transcription factor Nucleic acid binding	mRNA transcription regulation
☐		11.	GeneID:51450	paired related homeobox 2 PRRX2	PAIRED MESODERM HOMEOBOX PROTEIN (PTHR19418:SF86)	3E-123●●●	Homeobox transcription factor Other DNA-binding protein	mRNA transcription regulation
☐		12.	GeneID:51281	ankyrin repeat and MYND domain containing 1 ANKMY1	SUBFAMILY NOT NAMED (PTHR15897:SF2)	0E+00●●●	Molecular function unclassified	Biological process unclassified
☐		13.	GeneID:23503	zinc finger, FYVE domain containing 26 ZFYVE26	ZINC FINGER FYVE DOMAIN CONTAINING PROTEIN 26 (PTHR22835:SF20)	0E+00●●●	Molecular function unclassified	Biological process unclassified
☐		14.	GeneID:5313	pyruvate kinase, liver and RBC PKLR	PYRUVATE KINASE (PTHR11817)	0E+00●●●	Carbohydrate kinase	Glycolysis
☐		15.	GeneID:5087	pre-B-cell leukemia transcription factor 1 PBX1	PRE-B-CELL LEUKEMIA TRANSCRIPTION FACTOR 1, 2, 3, 4 (PBX) (PTHR11850:SF2)	0E+00●●●	Homeobox transcription factor	Hematopoiesis Oncogene
☐		16.	GeneID:6569	solute carrier family 34 (sodium phosphate),	SLC34A1 RENAL SODIUM-DEPENDENT PHOSPHATE TRANSPORT PROTEIN IIA) (PTHR10010:SF9)	0E+00●●●	Other transporter	Phosphate transport Cation transport Other homeostasis activities
☐		17.	GeneID:7586	zinc finger with KRAB and SCAN domains 1 ZKSCAN1	KRAB-RELATED (C2H2-TYPE ZINC-FINGER PROTEIN 36) (PTHR23226:SF13)	0E+00●●●	KRAB box transcription factor Nucleic acid binding	mRNA transcription regulation
☐		18.	GeneID:8581	lymphocyte antigen 6 complex, locus D LY6D	No Panther Hit	1E+00	Molecular function unclassified	Biological process unclassified
☐		19.	GeneID:23230	vacuolar protein sorting 13A (yeast) VPS13A	VACUOLAR PROTEIN SORTING-ASSOCIATED PROTEIN (VPS13A) (PTHR16166:SF22)	0E+00●●●	Molecular function unclassified	Protein targeting and localization
☐		20.	GeneID:401056	hypothetical LOC401056 LOC401056	No Panther Hit	1E+00	Molecular function unclassified	Biological process unclassified
☐		21.	hCG16134	pyruvate kinase, liver and RBC PKLR	PYRUVATE KINASE (PTHR11817)	0E+00●●●	Carbohydrate kinase	Glycolysis
☐		22.	hCG96024	unassigned unassigned	HUMAN UNCHARACTERIZED PROTEIN (PTHR12138)	1E-10 ●	Molecular function unclassified	Biological process unclassified
☐		23.	hCG1984861	vacuolar protein sorting 13A (yeast) VPS13A	VACUOLAR PROTEIN SORTING-ASSOCIATED PROTEIN (VPS13A) (PTHR16166:SF22)	0E+00●●●	Molecular function unclassified	Protein targeting and localization
☐		24.	hCG38404	unassigned unassigned	PANTOTHENATE KINASE (PTHR12280:SF6)	1E-51●●●	Other kinase	Coenzyme metabolism
☐		25.	hCG1811573	ankyrin repeat and MYND domain containing 1 ANKMY1	SUBFAMILY NOT NAMED (PTHR15897:SF2)	0E+00●●●	Molecular function unclassified	Biological process unclassified

			ANKMY1				
26.	hCG1811886	tensin 1 TNS1	TENS (PTHR12305:SF12)	0E+00●●●	Protein phosphatase Other phosphatase	Phospholipid metabolism Protein phosphorylation Signal transduction Cell adhesion Immunity and defense Induction of apoptosis Cell cycle control Cell proliferation and differentiation Tumor suppressor	
27.	hCG1777537	unassigned unassigned	CDC50-RELATED (PTHR10926:SF5)	0E+00●●●	Molecular function unclassified	Biological process unclassified	
28.	hCG2040196	unassigned unassigned	NUCLEAR PORE MEMBRANE GLYCOPROTEIN GP210-RELATED (PTHR23019)	1E-23 ●●	Membrane traffic protein	Nuclear transport	
29.	hCG21913	zinc finger, FYVE domain containing 26 ZFYVE26	ZINC FINGER FYVE DOMAIN CONTAINING PROTEIN (PTHR22835:SF26)	0E+00●●●	Molecular function unclassified	Biological process unclassified	
30.	hCG30596	paired related homeobox 2 PRRX2	PAIRED MESODERM HOMEOBOX PROTEIN (PTHR19418:SF86)	3E-123●●●	Homeobox transcription factor Other DNA-binding protein	mRNA transcription regulation	
31.	hCG23011	pre-B-cell leukemia transcription factor 1 PBX1	PRE-B-CELL LEUKEMIA TRANSCRIPTION FACTOR 1, 2, 3, 4 (PBX) (PTHR11850:SF2)	0E+00●●●	Homeobox transcription factor	Hematopoiesis Oncogene	
32.	hCG31601	matrix metallopeptidase 17 (membrane-inserted) MMP17	MATRIX METALLOPROTEASE 17, MMP-17 (PTHR10201:SF21)	0E+00●●●	Metalloprotease Other extracellular matrix	Proteolysis	
33.	hCG2040619	unassigned unassigned	No Panther Hit	1E+00	Molecular function unclassified	Biological process unclassified	-
34.	hCG2041697	unassigned unassigned	No Panther Hit	1E+00	Molecular function unclassified	Biological process unclassified	-
35.	hCG1642966	FK506 binding protein 11, 19 kDa FKBP11	FK506-BINDING PROTEIN 11 (PTHR10516:SF13)	7E-97●●●	Other isomerase	Protein folding	-
36.	hCG20127	adhesion molecule with Ig-like domain 3 AMIGO3	ALIVIN 1 (PTHR23154:SF42)	0E+00●●●	Receptor Extracellular matrix	Other receptor mediated signaling pathway Developmental processes	-
37.	hCG20135	inositol hexaphosphate kinase 1 IHPK1	INOSITOL HEXAPHOSPHATE KINASE (PTHR12400:SF15)	0E+00●●●	Kinase	Biological process unclassified	-
38.	hCG1731806	unassigned unassigned	No Panther Hit	1E+00	Molecular function unclassified	Biological process unclassified	-
39.	hCG2024096	zinc finger with KRAB and SCAN domains 1 ZKSCAN1	KRAB-RELATED (C2H2-TYPE ZINC-FINGER PROTEIN 36) (PTHR23226:SF13)	0E+00●●●	KRAB box transcription factor Nucleic acid binding	mRNA transcription regulation	-
40.	hCG17111	neuronal cell adhesion molecule NRCAM	NEURAL CELL ADHESION MOLECULE (NR-CAM) (PTHR10489:SF42)	0E+00●●●	CAM family adhesion molecule	Cell adhesion-mediated signaling Cell adhesion Neurogenesis	-
41.	hCG41577	solute carrier family 34 (sodium phosphate), member 1 SLC34A1	SLC34A1 RENAL SODIUM-DEPENDENT PHOSPHATE TRANSPORT PROTEIN 2 (SODIUM/PHOSPHATE COTRANSPORTER IIA) (PTHR10010:SF9)	0E+00●●●	Other transporter	Phosphate transport Cation transport Other homeostasis activities	-
42.	hCG1818572	zinc finger protein 75a ZNF75A	KRAB-RELATED (C2H2-TYPE ZINC-FINGER PROTEIN 500) (PTHR23226:SF11)	0E+00●●●	KRAB box transcription factor Nucleic acid binding	mRNA transcription regulation	
43.	hCG2041852	unassigned unassigned	No Panther Hit	1E+00	Molecular function unclassified	Biological process unclassified	
44.	hCG25239	ankyrin repeat domain 26 ANKRD26	ANK REPEAT-CONTAINING (PTHR18958:SF121)	0E+00●●●	Molecular function unclassified	Biological process unclassified	
45.	hCG40063	supervillin SVIL	SUPERVILLIN (PTHR11977:SF4)	0E+00●●●	Non-motor actin binding protein	Cell structure	
46.	hCG38698	fibrous sheath interacting protein 1 FSIP1	SUBFAMILY NOT NAMED (PTHR22012:SF3)	0E+00●●●	Molecular function unclassified	Biological process unclassified	
47.	hCG16702	PR domain containing 13 PRDM13	PR-DOMAIN ZINC FINGER PROTEIN 13 (PTHR23228:SF18)	7E-69●●●	Zinc finger transcription factor	mRNA transcription	
48.	hCG39309	lymphocyte antigen 6 complex, locus D LY6D	No Panther Hit	1E+00	Molecular function unclassified	Biological process unclassified	
49.	hCG1798407	unassigned unassigned	No Panther Hit	1E+00	Molecular function unclassified	Biological process unclassified	

Hits 1-49 of 49 [page: (1)]

Key: PANTHER relation
●●● closely related
●● related
● distantly related

6.5 Discussion

In this chapter, we optimised the collection and storage of pre treatment rectal cancer biopsies in to facilitate extraction of high quality RNA for scientific analysis. Furthermore, we went on to generate whole genome microarray profiles from 9 pre treatment tumour biopsies. Parallel analysis between good and poor responders to RCT allowed us to identify genes that were differentially expressed. The molecular and biological function of these genes was then identified using the PANTHER gene classification database. In total, 49 genes were differentially expressed with a significance of $p<0.01$ and 2-fold expression difference between good and poor responders.

This result therefore supports the concept that response to RCT is a multifactorial phenomenon, evidenced by the fact that multiple differentially expressed genes were found which had a wide variety of molecular and biological functions. Assuming that this is the case, our results suggest that in order to best detect biomarkers of response, a paradigm shift away from methodologies such as IHC assessing single markers is required. Whilst many of such studies have found statistical relationships between marker expression and radiation response, no single marker as yet has been able to predict response with clinical utility(172). Microarray data therefore suggest that pursuing this mode of study may be futile if radiation response is a multifactorial phenomenon.

An example of this can be found in our own study assessing COX 2 overexpression as a response predictor(173). This molecule was assessed as there has been considerable evidence that it mediates radiation response *in vitro*. This was confirmed in our study where we found

that tumours that overexpressed COX 2 were statistically more likely to demonstrate a poor response to RCT with statistical significance.

Whilst one can therefore correctly derive the conclusion that as a group, patients with COX 2 overexpression will have a worse response to RCT, on an individual patient basis, not all patients with COX 2 overexpression have a poor response and vice versa. Similar to other IHC-assessed markers such as p53 and p21, COX 2 overexpression is therefore an important, but far from exclusive, mechanism in mediating radiation response. Only methods capable of concurrently assessing multiple markers such as microarray are likely to be of optimal clinical utility for prospective response prediction on an individual patient basis.

Of the 49 genes identified in our study, there are none that correspond with individual protein targets previously identified as being of potential predictive importance by IHC such as p53, p21, COX 2 etc. Whilst one could argue that this represents inaccuracies of the microarray technique, more likely these results demonstrate the exact opposite, highlighting the strength of microarray analysis to identify truly important predictive biomarkers using blinded, genome-wide screening of human samples. Our microarray data is therefore exciting, as it has identified 49 genes that are differentially expressed with high statistical significance between good and poor responders that are currently unassessed as response predictors for rectal cancer.

Analysis of the differentially expressed genes in our study shows that 16% are as yet unnamed. Furthermore, 42% have not yet had their molecular or biological functions classified, highlighting the importance of microarrays for novel biomarker discovery.

Although in time, all genes detected and their corresponding biological functions will no doubt be identified, at present microarrays are allowing exploration of 'uncharted molecular territory'.

Despite the relatively large proportion of unclassified genes detected in our study, several interesting known genes have been identified. 6 (12%) genes identified in our study encoded zinc-finger transcription factors (Fig 6.17). This family of transcription factor is characterised by its possession of Cys_2His_2 motifs which consist of a sequence of 30 amino acids containing two histidines, two cysteines and three hydrophobic residues, which are all at conserved positions. Zinc finger transcription factors form a small, independently folded domain stabilized by Zn^{2+}, which can be used repeatedly in a modular tandem fashion to achieve sequence-specific recognition of DNA. Their molecular structure thus offers a large number of combinatorial possibilities for DNA recognition and as such, zinc finger transcription factors are the most selective of the DNA-binding transcription factors(174, 175). Of note, the protein survivin, assessed in the IHC chapter of this thesis, is a member of the zinc finger transcription factor family. Although survivin itself was not one of the genes identified in this study, it is interesting that a significant number of other zinc finger proteins were significantly differentially expressed between tumour response grades. Whilst the specific zinc finger proteins identified in our study are currently unassessed in radiation response, cell line studies have shown that other members of this family are involved in regulating apoptosis and radiation response via p53 dependent and independent pathways(176-178).

Two (4%) of genes that were differentially expressed were in the matrix metalloproteinases family (MMPs) (Fig 6.17). These are a family of proteins with many biological functions,

including extracellular matrix degradation, cell proliferation and angiogenesis and MMPs have been implicated at many levels in carcinogenesis(179). 2 (4%) of these genes were found in our study (Fig 6.17). MMPs have previously been assessed in rectal in association with neoadjuvant short-course radiotherapy. Here, quantitative RT PCR analysis of RNA derived from rectal tumours before and after treatment found that MMPs were significantly upregulated by 6 or 7-fold. Perhaps more interestingly, however, that this same group found that MMP expression was maximal 48hrs after receiving a dose of radiation in a cell line model. This data therefore not only supports our microarray finding that MMP expression may be important in predicting response, but also supports our proteomic data of chapter 4 that it is maximal in the early stages after radiation is initiated(180, 181).

Collective analysis of the genes represented in our results using PANTHER revealed that they had multiple different molecular functions and were involved in many biological processes. Looking at molecular function, apart from those genes that were unclassified, the largest percentage of genes was found in transcription factors and nucleic acid binding factors. This highlights the fact that gene transcription and transcriptional regulation are of primary importance important in mediating radiation response. Analysis of the biological processes represented by differentially expressed genes showed that they were involved in multiple processes pertaining to cellular viability and functioning such as carbohydrate metabolism, cell cycle, cell structure and motility, immunity and defense. Perhaps surprisingly, in light of the evidence from IHC studies, only one gene involved in the initiation of apoptosis was found. This gene, tensin 1, is capable of initiating of apoptosis via the PI3 kinase-signalling pathway(182). We hypothesise that this lack of representation can be explained in 2 ways. The first is that initiation or avoidance of apoptosis is only a small part of many processes involved in radiation response. The second and most likely

explanation, however, is that initiation of apoptosis is the end effector of most of the other processes represented by the remaining differentially expressed genes. As such this single, terminal pathway represents a small proportion of all other upstream pathways represented.

Our data therefore gives a remarkable insight into factors differentially expressed in pre-treatment tissues that may ultimately govern radioresponsiveness. As such, this work therefore represents a great milestone for our laboratory as it shows that we have been able to develop and optimise the techniques of high quality RNA procurement and extraction and then go on to perform trans-genomic microarray analysis of tumours. On the other hand, this data also represents only the beginning of our understanding of factors mediating radioresistance.

Despite the informative results of this study, its clinical relevance is questionable in its current form. One main reason for this is that the overall number of patients in this study is very small with only 4 good responders and 5 poor responders. Whilst this number was unavoidable as it was literally all the tissue that we had, further samples will be needed in order to give a more representative data set from which to base further studies. Another reason is that at this stage, we have not validated our results in any way. We are acutely aware of both of these points and are they are currently being addressed. In order to resolve both of these issues, further patient samples are being actively collected and validation of our original results by real-time quantitative PCR (RtPCR) is already underway. The validation step has been relatively easily performed to date as stated in the introduction to this chapter, one of the advantages of using the Applied Biosystems microarray package is that targets for all of the genes represented on the microarray platform can be immediately purchased from the company.

During the process of performing this study, another very similar study was published by Ghadimi et al(120). This study found 54 genes were differentially expressed between good and poor responders to RCT. The ability of these genes to predict response was evaluated using leave-one-out cross validation enabling correct prediction of tumour behaviour with 78% sensitivity and 86% specificity.

Despite sharing an identical end goal to our own study, there are a number of interesting differences between our own study and that of Ghadimi resulting in relative merits and downfalls of each. The first of these is that Ghadimi et al assessed 30 pre treatment biopsies, 16 from good responders and 14 from poor responders, therefore its sample size is over 3 times greater than ours. The second important point about this study is that they generate a predictive algorithm based upon a training set of 23 patients that is then tested on 7 patients not used to generate the predictive algorithm. Due to the small sample size in our study, we are unable to do this. The third interesting and important difference between the study by Ghadimi et al and our own is that we used TRG as a downstaging endpoint. In contrast, Ghadimi et al used a decrease in T stage as whilst they also assessed TRG, they found that only 5 genes were differentially expressed at $p<0.01$ with the latter. They concluded that concluded that this could be down to chance and so excluded TRG from subsequent analyses. Our study, on the other hand, did not replicate these results, in fact we found that 62 genes were differentially expressed between patients with good or poor TRG. Furthermore, we were able to narrow down these genes to 49 that had a 2-fold level of differential expression. The reason for the discrepancy in these results is not readily apparent. One possible explanation is that Ghadimi et al used a National Cancer Institute microarray chip incorporating 9, 984 cDNA features and 22,321 oligonucleotides whereas we used the

Applied Biosystems chips consisting of transcript sequences representing a set of 31,700 probes, 26% of which have been curated by Celera only and are not available in the public domain.

The difference in microarray systems and the gene probes contained on them may have given rise to these differences. A further explanation is that Ghadimi et al verified the quality of their RNA using the NanoDrop System. We found that whilst the NanoDrop could accurately detect and quantify the presence of RNA, it did not accurately predict the quality of RNA. To this end we used the Agilent 2100 to assess the quality of all of our RNA. The profiles that we obtained therefore are all of optimal quality and again may be a source of the discrepancy between the two studies.

Summary

Inability to predict response to RCT in patients with rectal cancer results in the majority of patients deriving sub-optimal benefit from treatment. The ability to predict response has therefore become a focus of recent research. Our study is one of the first to assess response prediction using trans-genomic microarray analysis and found that 49 genes were differentially expressed between pre treatment biopsies of good and poor responders. This approach, in contrast to IHC studies is capable of assessing the multi-factorial nature of radiation response and offers a powerful means to discover novel predictive biomarkers. Ultimately, this technique may hold the key to finding a predictive 'signature' of multiple genes that may better guide therapy.

Chapter 7: Pre-and post treatment MRI scanning as a response predictor for rectal cancer undergoing neoadjuvant radiochemotherapy

7.1 Introduction

Currently only 10-30% of patients obtain a complete pathological response (pCR) after RCT, characterised by the complete absence of tumour cells in their resected surgical specimens. As previously outlined, it is not possible to identify these patients until their specimens have undergone formal histopathological analysis. The result of this, patients must undergo empirical rather than individualised surgical procedures. This means that despite no longer having viable tumour cells, patients with pCR must still undergo radical surgery. Furthermore, these patients are at risk of many other surgical complications including anastomotic leaks, pelvic nerve damage and even post-operative death(21). The ability to accurately determine patients who had undergone pCR after undergoing RCT for rectal cancer would be of huge clinical advantage allowing more confident implementation of either observational(21) or local treatments(183) in good responders in place of radical surgery.

At the time of starting this thesis there was only one published report whereby MRI scanning was used as a post RCT staging modality. Although this report, by Hoffman et al found that it was unable to provide accurate staging information, we wished to confirm these results in our own centre(39).

As well as simply correlating tumour and nodal stage correlation between post RCT scans and pathological reports, we aimed to investigate whether MRI staging could be correlated with biological response of tumours measured by TRG. It was felt that this could be an important area as favourable TRG has recently been shown to be an independent prognostic factor in rectal cancer after RCT.

7.2 Aims:

1. **To audit the re-staging capabilities of MRI scanning after RCT for rectal cancer.**

2. **To correlate MRI T and N stage with the degree of tumour regression.**

7.3 Materials and methods

7.3.1. Consent and inclusion criteria

As pre and post RCT MRI scanning was already standard of care in St James's Hospital, consent was not obtained from all patients. In total, pre and post RCT MRI scans from 20 patients with rectal cancer undergoing RCT were included in this study.

7.3.2 Clinical (pre-treatment) and pathological staging

Pre treatment staging consisted of 1) thoracic and abdominal CT scanning and 2) pelvic MRI scanning in all patients. A subset of patients underwent transrectal ultrasound (TRUS) evaluation in addition to MRI to further define local disease. Any discrepancies between MRI and TRUS were discussed and a final pre treatment stage was assigned to each patient according to the TNM classification.

In order to grade pathological response to treatment, diagnostic slides from resected specimens of all patients were retrieved. These included no less than 3 sections of the irradiated area per patient. A single pathologist blinded to patient identifying characteristics reassessed all sections. Response to RCT was assessed using the scoring system previously described by Mandard et al(19). This system grades response on a 5-point scale according to the relative amounts of residual tumour and fibrosis (Fig 1.2). For analysis purposes in this study, patients with a TRG of 1 or 2 were deemed to have demonstrated a good response and those with a TRG of 3-5 a poor response.

7.3.3. Neoadjuvant regimen

All patients were deemed suitable for RCT after discussion at a weekly multi-disciplinary meeting. Each received 45-50 Gy of radiotherapy, given in 25 fractions delivered on 5 days for 5 weeks. Continuous IV 5-Fluorouracil (225mg/m^2/day) was given concurrently with radiotherapy via a syringe driver. Surgery was performed approximately 6-8 weeks after the last fraction of radiotherapy had been given.

7.3.4. Interpretation of MRI staging

Two senior radiologists independently reviewed all scans (Dr Mary Keoghan, Consultant and Dr David Tuite, SpR). Staging was then discussed then a final stage was given. T stage was determined by the degree of tumour penetration into or through the rectal wall. Nodes were identified as characteristic hypodense entities in the mesorectal fat (Fig 7.1). Nodal positivity was determined by size criteria where all nodes >5mm were deemed positive, based on a previous study by Brown et al (184), (Fig 7.2).

7.3.5. Statistical analysis

Statistical analyses were performed using SPSS Version 6 (SPSS, Chicago, IL). Only p values of <0.05 were considered significant.

Figure 7.1. T2-weighted MRI depicting transverse view of rectum. Nodes can be clearly seen as hypodense areas marked with arrows.

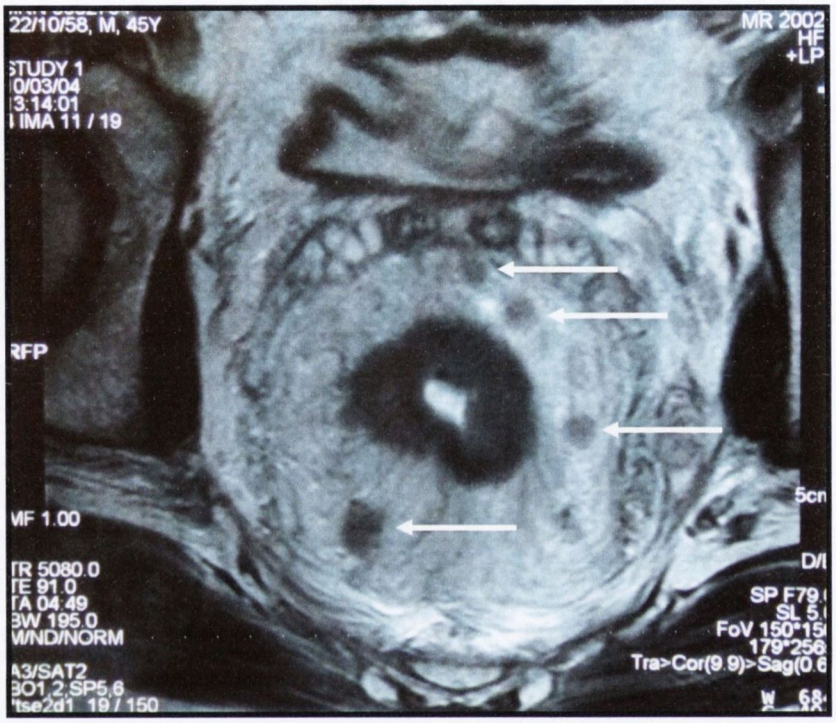


Figure 7.2 – Histogram showing the number of normal (white bars) and malignant (grey bars) nodes seen on MRI images, according to diameter (mm) taken from Brown et al (184).

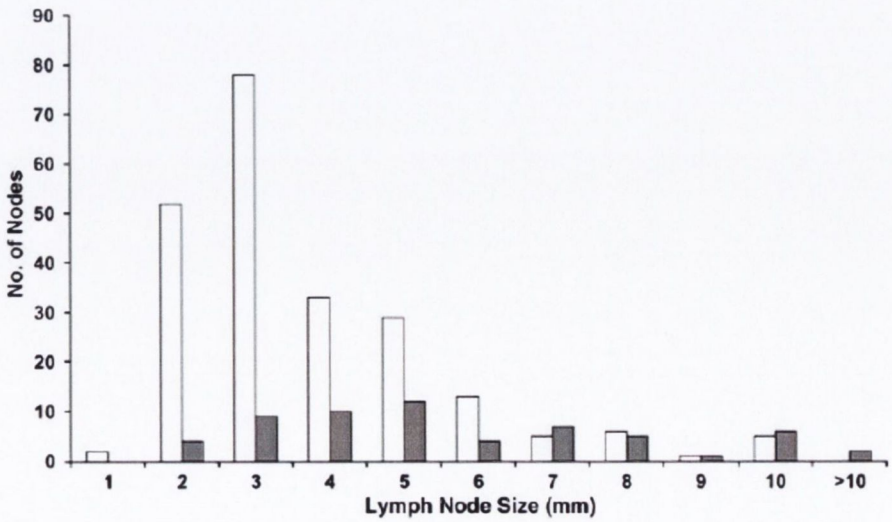


Figure 1. Histogram shows the number of normal (white bars) and malignant (gray bars) nodes seen on MR images, according to diameter (which is measured in millimeters).

7.4. Results

Pre and post treatment MRI scans from 20 patients who underwent RCT followed by TME were available for study. All patients were staged T3/T4 + node positive with the exception of one patient. This patient's pre treatment MRI scan showed T2 disease. At the time of surgery, however, the tumour was tethered and so she was referred for RCT. Both T and N stage were compared between histological reports and post RCT MRI scans (Table 7.1).

Table 7.1. Pathological versus MRI T and N stage between individuals

Patient No	Post Rx T Stage (MRI)	Post Rx N Stage (MRI)	Pathological T Stage	Pathological N Stage
1	3	1	4	1
2	3	0	0	0
3	2	0	1	0
4	3	1	3	1
5	3	0	3	1
6	3	0	3	0
7	3	1	3	2
8	3	0	1	0
9	3	0	3	2
10	3	1	3	0
11	3	0	2	0
12	3	1	3	2
13	3	1	3	1
14	3	1	3	1
15	3	1	3	0
16	3	1	3	1
17	3	1	0	0
18	3	1	3	2
19	3	0	0	0
20	3	2	3	2

7.4.1. Accuracy of T and N stage prediction in post RCT MRI scans.

MRI correctly predicted T stage of only 13 of 20 (65%) patients. Of note, all patients whose T stage was correctly predicted were T3 both by MRI and pathological analysis. MRI was unable to correctly predict patients whose T stage increased or decreased post RCT (100% inaccurate). N stage prediction by MRI *per se* was also poorly accurate at assessing nodal stage with the exception of N1 patients. (Table 7.2).

Table 7.2. Comparison between pathological versus MRI nodal stage

Path	MRI N0	MRI N1	MRI N2	Accuracy Path vs MRI (%)
N0=9	6	3	0	66
N1=6	1	5	0	83
N2=5	1	3	1	20

7.4.2. Accuracy of Post RCT MRI scanning in predicting node positive vs node negative.

The accuracy of post RCT MRI was then assessed in determining simply whether a tumour was node positive or negative on histopathological staging. In comparison to predicting individual nodal stage, this approach was only slightly more accurate, being 67% sensitive and 75% specific (Table 7.3).

Table 7.3. Comparison between pathological and MRI node positive and negative

MRI Node status	pN-ve	pN+ve
Neg = 8	6	2
Pos =12	3	9

MRI N0 vs pN0

Sensitivity = 67%
Specificity = 75%

7.4.3. Relationship between nodal stage by MRI and TRG

After RCT, a total of 8 patients (40%) demonstrated a good response to treatment (TRG 1+2). The remaining 12 patients (60%) demonstrated a poor response. The relations between TRG and MRI nodal status can be seen on an individual patient basis in table 7.4.

Of the 8 patients who underwent a good response to RCT, 6 were node negative on post RCT MRI. Furthermore, of the 12 patients who underwent a poor response, 10 were node positive on post RCT MRI scanning. This relationship between nodal positivity on MRI and TRG had a sensitivity of 75%, a specificity of 83% and met statistical significance (table 7.6) ($p<0.05$, Chi-square).

In order to determine if patients who were node negative on pathological examination had an associated good response to RCT, comparison was made between pN0 stage and good response by both TRG (table 7.5). It was found that pN0 predicted good response with 78% sensitivity and 91% specificity. This was statistically significant ($P<0.05$, Fisher's Exact Test) (Table 7.7).

Table 7.4. Individual comparison between MRI nodal stage and TRG

Patient No	Post Rx N Stage (MRI)	TRG	Response
1	1	4	Poor
2	0	1	Good
3	0	2	Good
4	1	3	Poor
5	0	4	Poor
6	0	2	Good
7	1	3	Poor
8	0	2	Good
9	0	4	Poor
10	1	3	Poor
11	0	2	Good
12	1	2	Good
13	1	3	Poor
14	1	4	Poor
15	1	4	Poor
16	1	4	Poor
17	1	1	Good
18	1	4	Poor
19	0	1	Good
20	2	4	Poor

Table 7.5. Statistical comparison between MRI nodal stage and TRG.

TRG	MRI Nodal Status		Total
	MRI Node Neg	MRI Node Pos	
1=Good Response (TRG 1+2)	6	2	8
2=Poor Response (TRG 3-5)	2	10	12
Total	8	12	20

P<0.05 Fisher's Exact Test

Table 7.6. Individual comparison between pathological nodal negativity versus histological downstaging and TRG.

Patient No	pT	pN	TRG	Response
1	4	1	4	Poor
2	0	0	1	Good
3	1	0	2	Good
4	3	1	3	Poor
5	3	1	4	Poor
6	3	0	2	Good
7	3	2	3	Poor
8	1	0	2	Good
9	3	2	4	Poor
10	3	0	3	Poor
11	2	0	2	Good
12	3	2	2	Good
13	3	1	3	Poor
14	3	1	4	Poor
15	3	0	4	Poor
16	3	1	4	Poor
17	0	0	1	Good
18	3	2	4	Poor
19	0	0	1	Good
20	3	2	4	Poor

Table 7.7. Statistical comparison between pathological nodal negativity versus histological downstaging and TRG.

TRG	Pathological N stage 1=Node Negative 2=Node Positive		Total
	1	2	
1=Good Response (TRG 1+2)	7	1	8
2=Poor Response (TRG 3-5)	2	10	12
Total	9	11	20

P<0.05 Fisher's Exact Test

7.5 Discussion

MRI scanning is a readily available facility in most hospitals; therefore it would be of huge importance if it were found to be capable of predicting response to RCT. In this study we have demonstrated that MRI has 100% inaccuracy in detecting change in T stage after RCT. We have also found that the accuracy of MRI scanning was between 20 and 83% in correctly determing N stage after RCT but was 66% sensitive and 75% specific in predicting whether patients were node positive or negative. In contrast, we found that N0 staging by MRI predicted high biological response to RCT (TRG 1 +2) with 75% sensitivity and 83% specificity. In addition, comparison of pN0 status by histopathological reporting and TRG showed that absence of nodal metastases predicted a favourable TRG with 78% sensitivity and 91% specificity. Whilst MRI may not yield sufficient clinical accuracy in the prediction of nodal status *per se*, N0 status on post RCT MRI may identify patients who have undergone a large biological response.

This finding may be clinically important for several reasons. Firstly, several recently published studies have shown that there is a strong association between post RCT T stage and rates of nodal positivity. Specifically, patients who obtain a decrease in T stage after RCT are less likely to harbour lymphatic metastases. The first of such reports was by Read et al in 2004(24). This group analysed data from 644 patients treated with RCT folled by TME and found a strongly significant correlation between T and N status. Specifically, metastatic nodes were found in 2%, 4%, 23%, 47 and 48% of T0, T1, T2, T3 and T4 patients respectively. A further 2 studies have supported this original finding. Bujko et al studied 316

patients undergoing RCT(22). They found metastatic nodal involvement in 5%, 8%, 26% and 55% in T0, T1, T2, T3-4 patients respectively.

Finally, Puciarelli et al assessed a cohort of 235 patients undergoing RCT(23). They found metastatic nodal involvement in 2%, 15%, 17%, 38% and 33% in T0, T1, T2, T3 and T4 patients respectively. All of these authors concluded that, as the rate of nodal metastasis is so low in patients with a low T stage after RCT, local excision could be considered in these cases. These data are confirmed in our own study where a reciprocal relationship was found between pN0 and both TRG and tumours staged T2 or less ($P<0.05$, Fisher's Exact Test).

The current limitation with offering local excision to patients who obtain a low T stage after RCT is that there are no accurate means of predicting a decrease in T stage either clinically or radiologically. This is primarily because imaging modalities cannot differentiate between the intermingled areas of residual tumour and radiation-induced fibrosis(172). The importance of our study, therefore, is that it has demonstrated that by focussing purely on post RCT nodal status, an overall picture of tumour response may be gained. This is because nodes are generally placed more peripherally around tumours, and so are far less likely to be obscured by radiation-induced fibrosis. They may therefore be more reliably identified. Our study has found a significant relationship between MRI N0 and both tumour regression to TRG 1+2 and downstaging to T2 or less. Furthermore, we are the first group to our knowledge to show that tumours staged as pN0 are more likely to have a favourable TRG.

In our study, only 6 of 8 (75%) patients who were node negative on post RCT MRI scanning had a favourable TRG. Similarly, only 10 of 12 (83%) patients who were node positive on post RCT MRI scanning had an unfavourable TRG.

Whilst these figures alone are insufficient to wholly advocate making clinical decisions based on MRI N0 status, they highlight the potential for nodal MRI status as an adjunctive staging method when selecting patients for conservative or local therapy post RCT. Furthermore, they highlight the importance that complete nodal downstaging is a harbinger of good overall tumour response. This relationship may be further developed with evolving radiological techniques aimed at improving the accuracy of lymphatic staging by MRI. Several reports now exist where injection of ultra-small particles of iron oxide (USPIOs) into patients prior to performing MRI scanning has greatly improved lymphatic staging accuracy.

USPIOs are lymphographic contrast agents, which are administered intravenously. They are then transferred by a process known as transcytosis from capillaries into the interstitial space and then, via the lymphatic vessels into the lymph nodes(185). Iron particles are then phagocytosed by nodal macrophages and thus result in signal loss in normal nodes on T2 weighted sequencing. In nodes with metastases, however, the areas infiltrated by tumour are relatively unaffected by USPIOs and remain of high signal intensity, thus allowing their characterisation. The ability to improve the accuracy of nodal staging in malignancy has been recently demonstrated. In 44 patients, 29 with cervical and 15 with endometrial cancer, Rockall et al found that sensitivity and specificity increased from as low as 27% and 94% using conventional T2 weighted scans to 100% and 91% respectively after the injection of the USPIO agent Ferumoxtran-10(186). In a smaller study assessing 10 patients with penile cancer, Tabatabaei et al found that USPIOs could correctly differentiate metastatic from normal nodes with a sensitivity and specificity of 100% and 97% respectively(187).

Only one study by Koh et al has assessed the ability of USPIOs to enhance nodal staging in rectal cancer(185). This study included 12 patients who were candidates for primary TME

without RCT. Here, the appearance and signal intensity of lymph nodes at T2 weighted imaging were recorded before and after USPIO administration. These radiological appearances were then correlated with surgical specimens where node-by-node comparison was made. A total of 74 lymph nodes were found in this study of which 6 were malignant on histopathological examination. Because of this small number of malignant nodes, the authors were unable to fully characterise their of MRI appearances but concluded that high signal intensity and eccentric high signal intensity patterns may be useful in the identification of malignant nodes. Non-malignant nodes, on the other hand, frequently demonstrated either uniform or central low signal intensity patterns. They state that they are following up this study with a larger prospective study.

Chapter 8

Overview and Discussion of Thesis

Overview and discussion

The aim of this thesis was to identify predictors of response of rectal cancer to neoadjuvant radiochemotherapy (RCT). From the outset, one of our primary objectives was not only to use traditional research techniques, but also to develop translational research methods that were not yet established in our laboratory. To this end we performed traditional immunohistochemical analysis on archival pre-treatment biopsies from a large cohort of patients who had previously undergone RCT followed by surgery. In addition, we collected fresh tissue and blood from a prospective cohort of patients undergoing RCT on which we used SELDI-TOF serum proteomics and microarray analysis for novel biomarker discovery. The ability of MRI scanning to predict the degree of response after RCT was also assessed. During the course of these various projects, the opportunity arose to perform an additional study, assessing of the capabilities of SELDI-TOF serum proteomics as a minimally invasive disease-screening tool for colorectal cancer.

In chapter 3, archival pre-treatment biopsies from a historical cohort of patients who had received RCT followed by surgery were analysed using IHC. Here, along with measuring intrinsic levels of tumour apoptosis and proliferation, the degree of COX 2 and survivin staining was determined and compared to tumour response. Our study found that patients who over-expressed COX 2 in pre treatment biopsies were statistically more likely to have a poor response to RCT (P=0.026, Chi Square). There was also a strong association between high levels of spontaneous apoptosis and a good tumour response to RCT. This was even evident across individual tumour regression grades (P=0.007). The expression of both survivin and proliferation, on the other hand, were not found to be capable of predicting

response (P>0.05). We were unable to identify any inter-relations or co-dependence between any of the markers assessed (P>0.05).

There are currently no markers that can be used clinically in order to predict response and hence guide therapy for patients with rectal cancer undergoing RCT. Given that RCT is expensive, time-consuming and increases the risks of both peri-operative morbidity and developing secondary radiation-related tumours(123), such markers would be of unquestionable clinical utility. In contrast to patients with rectal cancer, staining for IHC markers is now an established part of treatment planning in other disease types. In breast cancer, for example, hormonal/HER 2 status are now routinely assessed in order determine the type of adjuvant therapy that will be most efficacious(188-190). Our finding that COX 2 is overexpressed in radioresistant tumours may therefore represent an important clinical finding in rectal cancer as COX 2 overexpression not only predicts a poor response, but COX 2 inhibition also exists as a therapeutic modality with comparatively cheap and widely available COX-2 inhibitors already in the marketplace. Although there have recently been concerns over their long term safety, there are no data to suggest that short term useage is harmful(137). Accordingly, there may be therapeutic rationale in using COX 2 inhibitors either before or during treatment in patients found to over express COX 2 in an attempt to improve overall rates of response, a concept which has already been successfully applied in animal models(124, 125).

High levels of spontaneous apoptosis were also found to be a strong predictor of good response to RCT in our study, a finding mirrored in several other studies in rectal cancer(123). This suggests that by identifying intrinsic molecular regulators of apoptosis within tumours, they may be targeted in order to improve overall response to cytotoxic

therapies. In light of this evidence, it was therefore surprising to find that expression of survivin; a relatively recently discovered member of the inhibitor of apoptosis protein (IAP) family(130) was unrelated to either response to RCT or levels of spontaneous apoptosis in pre treatment biopsies. Previous research found that a reciprocal association between survivin expression and rates of spontaneous apoptosis existed(64), however, these results were not replicated in our study. The reason for this was not clear, as we were confident that both of our antibodies were well optimised and gave reliable staining.

It is becoming increasingly clear that the concept of biomarker identification using single IHC markers is unlikely to reflect the multiplicity of pathways controlling radiation response(139) This is exemplified by the fact that other markers such as p53 gene mutations(30, 31) and p21 expression(54) have also been shown to predict radioresistance in rectal cancer with statistical significance. The strength and advantage of IHC studies lies in the fact that they are cheap, repeatable and easily performed in any hospital setting, often using automated staining machines. As such, they may represent an ideal means to routinely assess panels of markers identified using translational methods.

In chapters 4, 5 and 6, the emerging translational methods of SELDI-TOF-MS serum proteomics and gene expression microarray were assessed as a biomarker detection tools for response to RCT in patients with rectal cancer (Ch4 and 6) and as a general screening tool for colorectal cancer (Ch 5). In chapter 4, SELDI-TOF-MS was performed on sequential serum samples taken from patients with rectal cancer throughout the course of RCT. Our study found that differences in spectral profiles were apparent between good and poor responders at individual timepoints. In addition, the technique could identify up and down regulation of protein/peptide species in all patients throughout the course of treatment. More importantly,

we also generated an algorithm capable of predicting response using a data-mining software package. Here, we found that response could be predicted with 87% sensitivity and 80% specificity based on the differential expression of 14 peaks after 24/48hrs of treatment. This finding may have several important clinical implications.

The first of these is that it demonstrates that analysis of the low molecular weight region of the serum proteome may be capable of predicting response to neoadjuvant therapy at an extremely early stage into treatment. Using this technique, therefore, patients predicted to be non-responders could be excluded from undergoing the full 5 weeks of treatment after only one or two fractions of radiotherapy. They would then be candidates for alternative treatment regimens or recruitment into Phase 2 clinical trials assessing novel biological agents.

A second important clinical implication of this study is that it highlights that biomarker analysis in the early stages of starting treatment rather than at the pre-treatment timepoint was optimal for predicting response. This is important as to date, the vast majority of studies have focussed on the collection and analysis of purely pre-treatment biopsy tissues. Although performed in serum and not in tissue, our study found that protein patterns in serum induced in the early stages of radiation onset provided a better delineation of response when compared to the pre-treatment timepoint. If this same principle were manifest in tumour tissue itself, studies limited to analysing only pre-treatment biopsies would miss this potentially important biological timepoint. As the collection of serum is minimally invasive, it would be better suited to intra-treatment sampling as it is acceptable to patients and can easily be repeated on multiple occasions. This is especially applicable to patients with rectal cancer for whom procurement of tissue biopsies is often an unpleasant and embarrassing process.

In chapter 5, we assessed the ability of serum proteomics to screen for colorectal cancer. We found that the technique could not adequately discriminate between patients with benign or malignant disease, yielding at best a sensitivity and specificity in the region of 75%. These results were disappointing, as since the inception of this study, 3 further studies have all found that the technique does have good potential as a screening tool with sensitivity and specificity in the order of 90%. The reasons for the failure of our study to predict response are unclear. We included large patient cohorts in each group, used standardised collection and storage techniques and performed analyses in a laboratory where the technique was well optimised. The only explanation for the discrepancy in our results that we can think of is that the chip type used (Q10) was different to that used in the other published reports. As a result, different spectral profiles, yielding less informative peaks may have been generated. Because several other reports now exist in the literature that confirm the efficacy of the technique, however, we do not plan to take our study any further by re-analysing our samples.

At present, proteomic analysis of serum is a relatively new and evolving field. The first published study relating it to cancer was by Petricoin et al in 2002(110). Since then there has been an exponential interest in the technique especially as a tool for cancer screening where it has now been successfully assessed in multiple different cancer sub-types. Surprisingly, there is a paucity of literature assessing serum proteomics as a tool to predict response to cytotoxic therapy. We could only source one report, by Pustazi et al, which assessed proteomic differences in plasma in patients with breast cancer(191). Here, plasma taken on day zero before chemotherapy and on day-3 post treatment from 69 patients undergoing paclitaxel or FAC chemotherapy and 15 healthy volounteers was analysed using SELDI-TOF-MS. In this study only one peak of interest, induced by chemotherapy, was identified at

m/z 2790. This was detectable in 80% of patients treated preoperatively and 40% treated post operatively. Unlike our findings, however, there was no clear correlation in the induction of m/z 2790 and histological response. The discrepancy between the results of this study and our own is most readily explained by the fact that Pustazi et al only looked at pre- and post-treatment plasma and not the 24/48 hour timepoint timepoint which we found so informative. Furthermore, although the cohort of patients examined in their study group was larger than ours, there was variability in both the timing and type of chemotherapy, which may lead to discrepant results.

Whilst our results were disappointing with respect to using serum proteomics as a screening tool for colorectal cancer, our initial results assessing the technique as a response predictor are extremely promising. If our study is repeatable, it will have important implications in how response to treatment is assessed. Furthermore, the capabilities of serum proteomics will allow our results to be taken further to provide a better understanding of mechanisms of response and means of enhancing it. A recent advance in serum protemics is the ability to both sequence and identify mass peaks of interest. Although no data relating peak identification to response to cytotoxic therapies exists, this information is starting to be published in studies assessing SELDI-TOF as a cancer-screening tool. One such publication is by Li et al. (192). This study is elegant in that in a previous experiment, the group had already identified the m/z values of the two sequenced markers using SELDI-TOF-MS. In this publication, however, they independently validated the results of their previous study using a second cohort of serum from 61 women with locally invasive breast cancer, 32 with DCIS, 37 with various benign breast diseases and 46 age-matched healthy women. The expression patterns of the two previously identified biomarkers were found to be consistent with their initial study set. Furthermore, they went on to identify these biomarkers using

seriel chromatography, 1-dimensional gel electrophoresis, in-gel digestion, peptide mass fingerprinting and tandem mass sequencing. The two candidate biomarkers were found to be complement component C3a (desArg) and a C-terminal-truncated form of C3a (desArg).

If serum proteomics was to be introduced as a clinical tool, it would have to be proven that generated spectra and data derived from them were robust and reproducible across different laboratories and geographical sites. Only recently has data been published by The Human Proteome Organisation Plasma Proteome Project (HUPO PPP) demonstrating that this is possible(193). Here, aliquots of reference serum and plasma samples were analysed by eight different laboratories worldwide. Data from five sites passed preliminary quality assurance tests and amongst these, a high correlation was found between spectra generated. Furthermore, a peak at m/z 9200 was consistently purified and identified in samples originating from these five sites using mass fingerprinting and MS-MS analysis.

In a similar experiment, Semmes et al recently published the results of the first stage of a National Cancer Institute/Early Detection Research Network-sponsored multi-institutional evaluation and validation for using SELDI to detect prostate cancer(194). This study assessed two different parameters. Firstly they determined whether the output from the mass spectrometer demonstrated acceptable inter-laboratory reproducibility. This was done by measuring mass accuracy, resolution, signal-to-noise ratio and normalised intensity of three peaks that were present in a standardised pooled serum sample. Secondly, they evaluated the ability of each 'standardised' instrument, to differentiate between cancer or control serum using an algorithm that had previously been established. They found that the inter-laboratory spectra obtained were equivalent to intra laboratory measurements of the same peaks. In

addition, they found that all six sites obtained perfect-blinded classification of all samples when boosted alignment of raw intensities were used.

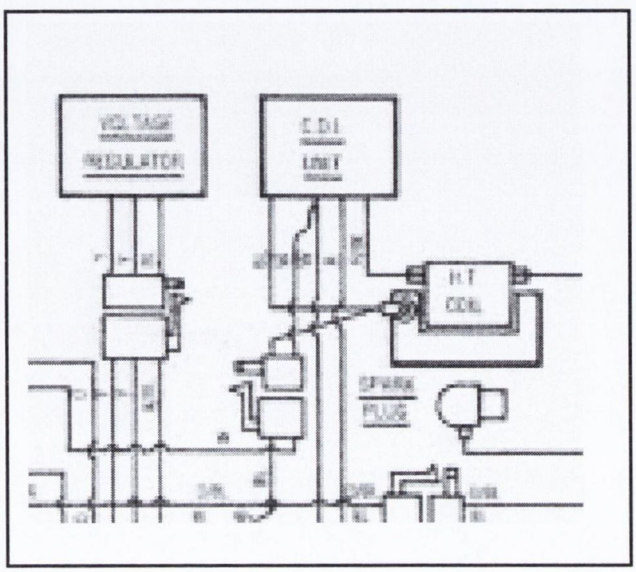
There therefore seems to be no doubt that the technique of serum proteomics holds great promise in the field of early cancer detection. Its ability to predict response to cytotoxic therapies is equally exciting but as yet remains a largely unassessed field of research. Further studies validating our own are therefore clearly needed.

In chapter 6, gene expression microarray profiling was performed on RNA extracted from the pre treatment tumour biopsies of 9 patients who underwent RCT. Paralell analysis using biostatistical software of samples from good and poor responders identified 49 genes that were differentially expressed with $p<0.01$ and 2-fold expression difference. Using PANTHER, an on-line database, the molecular and biological functions of these genes was determined(195). This process revealed that inherent transcriptional differences were apparent between multiple different cellular processes in good and poor responders. Furthermore, a large proportion of identified genes had molecular and biological functions that were yet to be discovered.

This experiment demonstrates the vast capabilities of the microarray platform as a means of biomarker discovery, active over the entire, known human genome. In contrast to traditional laboratory methods, microarrays can identify multiple expression differences between patients and in turn these represent multiple intrinsic molecular pathways. This platform is therefore ideally suited to profiling tumours undergoing cytotoxic therapies as it is capable of concurrently monitoring gene expression in all of the multi-factorial pathways involved in mediating response. The ultimate clinical endpoint of microarrays, therefore, would be to

identify all of the active pathways of resistance that were manifest in a patient's tumour before or during cytotoxic therapy offering revolutionary clinical possibilities. The first of these is that instead of conventional histological descriptions of tumours, the pathology report of the future may resemble something more like a "wiring-diagram" highlighting the molecular characteristics of a tumour rather than simply describing its microscopic cellular appearance, as is currently the case (Fig 8.1). This type of report would therefore highlight the active cellular pathways in a tumour and provide insight to pathways amenable to cytotoxic therapy. As a direct result of this, a second possibility would be that instead of having either single or small numbers of chemotherapeutic agents, each of which may have unpleasant toxic side effects, chemotherapy regimes based on microarray profiles could take the form of multiple agents, given in smaller less toxic doses, that were specifically aimed at molecular pathways highlighted by microarrays. This may therefore decrease the horrendous side effects of current chemotherapeutic agents such as hair loss and intractable nausea whilst at the same time increase cytotoxic efficiency. In addition, dynamic tumour profiling over several timeoints would allow these cytotoxic regimens to be modified in accordance to changes in gene expression during treatment.

Figure 8.1. Wiring diagram.



Limitations of current biomarker research

Despite the clinical potential of accurate predictive biomarkers, there are several intrinsic sources of error in studies performed to date that will have to be addressed. One of these is the general lack of standardisation of both methods and statistical analyses that currently precludes meaningful comparison of results. Looking only at studies assessing IHC staining, for example, even studies assessing the same marker can often not be compared due to inter-observer variation, variation in scoring systems used and batch-to-batch variability(196). These issues may be resolved in part using computerised image-analysis software to score sections but this technique is expensive and has an associated steep learning curve. In addition, implementation of more sophisticated IHC techniques such as tissue microarray, which allows representative samples from multiple tumours to be compared concurrently, may minimise batch-to-batch variability and make experimental results more robust(197).

Similar problems exist in more advanced translational research methods such as microarray and proteomic analysis. This is a particular problem at the moment as due to the relative youth of these techniques, different companies vieing for their share of the market are producing research platforms designed to outstrip their competitors. A good example of this is that the Applied Biosystems V2 microarray kit (as used in our study) has 8336 unique, copyrighted probes based on the Celera Human Genome Project. Whilst this arguably makes these chips the most informative on the market, it also makes meaningful comparison between this kit and others without these probes extremely difficult. This may in part explain why our microarray results were so different from those of Ghadimi et al who used an alternative microarray platform.

Other important potential source of error lies in using pre treatment tissue samples for biomarker discovery. First, there can be no guarantee that the superficial aspect of tumours, which is amenable to endoscopic biopsy, truly reflects the biology of the tumour as a whole and means that comparison between responsive and non-responsive tumours may be inaccurate due to sampling error. Secondly, even if biopsies are taken from equally representative areas of tumours, biopsies often consist of varying amounts of stroma, blood vessels and lymphocytes(172, 198). In microarray-based research, these subpopulations themselves contribute to contribute to gene expression profiles introducing a potential source of error. Whilst it is possible to use laser capture microdissection to specifically study tumour cells alone, there is emerging evidence that the stroma itself may be involved in tumour behaviour.

A final yet important source of error lies in the analysis of the vast quantity of data that translational studies generate. There are currently multiple different software packages on the market designed for user-friendly data analysis. Somewhat worryingly, a recent publication by Millenaar et al found that analysis of the same microarray data using 6 commercially available softeware packages gave highly discrepant results with overlap between informative gene signatures as low as 27-36% between analysed groups(199). This finding has also been highlighted in several other studies(200, 201) and could represent a major limiting step in the adoption of microarrays and other translational methods as clinical decision-making tools where high precision and accuracy are imperative. Similar to the technical aspects of studies, therefore, it will be important to standardise methods of data analysis across studies if meaningful comparisons are to be made.

Validation of results

In addition to identifying and correcting all sources of experimental error, another important area in ensuring meaningful results are derived from translational research studies is reproducing and verifying the results of single-experiments. Both of these factors will be of particular importance in determining the true clinical significance of our own results. In order to do this it is now important to increase our experimental sample sizes. In our proteomics study, for example, whilst we included a relatively large number of patients, our sample size did not allow us to validate our predictive algorithm on an independent patient dataset, as has been performed in other proteomic studies including more patient samples(110, 113). To this end, further clinical samples from all patients undergoing RCT are being actively collected. Furthermore, samples from our original experiment are being re-analysed in a proteomics facility in Dublin.

The results gained from our microarray study are also in the process of being validated. Here, real-time PCR is being used to perform gene expression measurements for 15 genes selected from the 49 that were differently expressed ($p<0.01$ and 2-fold expression change) between good and poor responders. This method of verification is well recognised in the literature and the validity of our results will therefore be confirmed if genes found to be significantly up or down regulated by microarray remain so with PCR(202). In addition to validating the results of our initial microarray data set, we are also collecting further fresh pre treatment biopsies on which additional microarrays will be performed. This will not only serve to reduce the risk of type 2 error but the addition of further samples will allow us to generate and test predictive algorithms(198).

A vision for the future

As a result of recent technological and computational advances, the last few years have seen an exponential rise in the use of translational research. Whilst this whole field is still in its relatively infancy, it has become clear that it represents an incredibly powerful biomedical research tool. The collection of high quality, fresh, biological samples that are collected and stored in an optimised and standardised fashion is required for translational research. More than ever there is now a need for such tissue, yet there is no formal system in place in hospitals to ensure that it is collected. Instead individual researchers are responsible for collecting what samples they need for their own research projects. Looking at the broader picture, however, this system is less than ideal, as it makes no allowance for studying the disease entity as a whole and means that scientists themselves spend a large amount of their time collecting samples. In the last 3 years at St James's Hospital, we have identified and tried to address this issue. Firstly, collaboration with the Department of Histopathology and The Department of Surgery has seen the introduction of a biobank for fresh biological samples. Here, after firstly obtaining fully informed consented, samples are taken from patients included in clinical research projects in sufficient quantity for multiple uses. In addition, for each patient being studied, a broad range of samples is taken (snap frozen tissue, tissue stored in RNAlater, serum and whole blood). All samples are then logged into a fully annotated computerised database and given a unique storage location in a fully alarmed and backed-up –80°C freezer. Secondly, there is a full-time laboratory technician now employed in the lab who is responsible for collecting biological specimens, transporting them to the laboratory and logging them into the database. This therefore frees up researchers to pursue their research rather than collecting samples.

We hope that our model becomes generally adapted amongst the surgical community for several reasons. Recent publications have confirmed that a provider volume phenomenon exists in oncological surgery meaning that centres that specialise in surgery for cancer have the best oncological results(203). As a result of this, the whole field of surgery is moving towards increasing sub-speciality. This means that patients with cancer are increasingly being operated on by a small number of surgeons in specialist centres. Under these circumstances, the concentration and volume of patients provides an ideal opportunity for study. There is therefore arguably an ethical obligation to ensure that biological samples are procured from these patients for research purposes and that they are done so in a manner that maximises their possible future uses and inter-laboratory comparability.

Final conclusion

The ultimate goal of biomarker research such as that conducted in this thesis is to identify a means of predicting and understanding mechanisms underlying response to cytotoxic therapies. Recent advances in translational research are bringing us ever closer to this goal by providing trans-genomic tumour profiling and the ability to monitor response during treatment. The ultimate goal is that all patients will have tailor-made therapy allowing them to obtain maximal benefit from cytotoxic therapies with minimal morbidity and that non-response will be a thing of the past.

Figure 8.2. Medicine in the molecular era

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Appendix 1

Preparation of sinapic acid

1. Pipette 2.5ml deionised water into a labelled 15ml plastic, conical tube.

2. Add 25µl Trifluroacetic acid (TFA) (Sigma-Aldrich) to the deionised water. Mix gently by shaking.

3. Pipette 2.5ml Acetonitrile to the deionised water/TFA solution. Mix gently by shaking.

4. Weigh out approximately 25mg sinapic acid (SPA) (Sigma-Aldrich) using a covered balance.

5. Transfer SPA powder to a second labelled 15ml plastic conical tube. Screw on the tube cap. Vigorously shake the tube to move all the SPA powder to the bottom of the tube.

6. Add 2.5ml of matrix diluent to yield a final 10mg/ml solution of SPA.

7. Vortex the SPA matrix solution for a minimum of 2 minutes and longer if all SPA is not yet dissolved.

8. Wrap aluminium foil around SPA matrix solution and store in the dark until ready for use (as it is light sensitive).

Appendix 2

Consent form used to for studies assessing predictors of response in rectal cancer.

Patient Information and Consent

1. Title of Study RECTAL CANCER: PREDICTORS OF RESPONSE TO NEOADJUVANT CHEMORADIOTHERAPY.

2. Introduction

We would like to invite you to participate in a research project studying the effects that the combination of chemotherapy and radiotherapy have on rectal cancer. You are under no obligation to participate and if, when you have read and heard about the study you would prefer not to do so, we will accept your decision without question.

Background

Each year colorectal cancer affects over 1500 individuals in Ireland alone. In cancers that are deemed operable, radiotherapy and chemotherapy both have accepted roles in pre operative treatment. Radiotherapy acts to both shrink the tumour and help stop it recurring in the pelvis. Chemotherapy makes the tumour cells more easily damaged by radiotherapy and may also kill cancer cells elsewhere in the body.

Although some patients receive good benefit from radiotherapy and chemotherapy, many patients will have only a partial response to both of these therapies and some may have no response at all.

The purpose of this study is to identify features of the cancer, which will predict the best response for patients.

3 Procedures that you will undergo.

All patients who are undergoing radiotherapy and chemotherapy for rectal cancer will be invited to participate.

It is planned that all the tests that are needed for the trial will be done whilst you are attending the hospital for **routine** treatment anyway.

If you participate, we will monitor your progress through treatment in **3 different ways**.

i) Tissue samples

By participating, you will give us your consent to study the initial biopsies that were taken to make your diagnosis and the surgical specimen after it is removed at operation. You will also be asked to have another sample taken 1 week into treatment. This will be whilst you are attending for **routine** follow up. It will be done in much the same way as your initial biopsy was taken and will allow us to see how the cancer is changing during treatment.

We plan to stain these samples with dyes that stain specific molecules. By looking at the pattern of staining in the tissue before during and after treatment, we will be able to identify any changes that have taken place.

In the future we may perform further ethically approved tests on these samples in other work on rectal cancer.

ii) Blood tests

During the course of your treatment we will take a number of blood tests. These will be taken when you are having **routine** blood tests taken anyway. This will amount to 3 extra 5ml samples taken one day a week for 5 weeks from the same needle as your other **routine** tests. We will also take 3 extra 5ml samples of blood 24 and 48 hours after you get your first dose of radiotherapy.

In the future we may perform further ethically approved studies on these samples in other work on rectal cancer.

iii) Scans

Before your surgery, you will have a **routine** scan called a MRI scan which takes around 15 – 20 minutes to perform. This gives us pictures of the rectum and lets us see the cancer in great detail. If you participare in this study, you will stay in the scanner for a further 15 – 20 minutes to allow us to perform a second scan called MRS. This uses a special computer program to look at the molecules in your body. After our chemoradiotherapy is completed, another **routine** MRI scan will then be performed to help plan your surgery and to see how the cancer is responding to the treatment. Again we will perform an additional MRS scan taking 10 – 15 extra minutes.

4. Benefits

Although this study may have no direct benefit to you, the results may benefit subsequent patients like you in the future.

5. Risks

All of the tests except the MRS scan that you will be getting are **routine**. The MRS scan is not routine but only involves lying flat for 10 – 15 minutes after your **routine** MRI scan.

If you are a woman of childbearing age, you may participate in this study only if you are surgically sterilised or are using the contraceptive pill. You must not be pregnant or lactating and you must have a negative pregnancy test before the study begins. The effects of radiochemotherapy on the fetus or child are known to be harmful. If you should become pregnant, in spite of all the precautions, please notify your doctor immediately.

6. Exclusion from participation:

Nothing should exclude you from participating in this study if you are having routine pre operative radiochemotherapy.

7. Alternative treatment

If you should choose not to participate then there would be no change in the treatment which you will receive and no change in the length of your of hospital stay.

8. Confidentiality

Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone outside the hospital.

*9. Compensation

Your doctors are covered by standard medical malpractice insurance. The medical practitioners involved in this study have current medical malpractice insurance cover. The medical practitioners will comply with the ABPI guidelines and Irish Law (statutory and otherwise) in the unlikely event of your becoming ill or injured as a result of participation in this clinical study. Nothing in this document restricts or curtails your rights.

10. Voluntary Participation

You have volunteered to participate in this study. You may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits which you had before entering the study.

11. Stopping the study

You understand that your doctor may stop your participation in the study at any time without your consent.

12. Permission

This study has been approved by the St James' Hospital and Federated Dublin Voluntary Hospitals Joint Research Ethics Committee.

13. Further information

You can get more information or answers to your questions about the study, your participation in the study, and your rights, from Mr Fraser Smith who can be telephoned at 416 3750. If your doctor learns of important new information that might affect your desire to remain in the study, he or she will tell you.

ST JAMES'S HOSPITAL AND FEDERATED DUBLIN VOLUNTARY HOSPITALS
JOINT RESEARCH ETHICS COMMITTEE

Consent Form

Title of research study: RECTAL CANCER : PREDICTORS OF RESPONSE TO NEOADJUVANT CHEMORADIOTHERAPY.

This study and this consent form have been explained to me. My doctor has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.

I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor.

I understand that in the future additional ethically approved testing may be performed on the samples of my tissue in other work on colorectal cancer.

PARTICIPANT'S NAME:

PARTICIPANT'S SIGNATURE:

Date:

Date on which the participant was first furnished with this form:

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the subject is a minor (under 18 years old) the signature of parent or guardian must be obtained:-

NAME OF CONSENTOR, PARENT or GUARDIAN:

SIGNATURE:

RELATION TO PARTICIPANT:

Where the participant is capable of comprehending the nature, significance and scope of the consent required, but is physically unable to sign written consent, signatures of two witnesses present when consent was given by the participant to a registered medical practitioner treating him or her for the illness.

NAME OF FIRST WITNESS: **SIGNATURE:**

NAME OF SECOND WITNESS: **SIGNATURE:**

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Physician's signature:

Date:
(Keep the original of this form in the participant's medical record, give one copy to the participant, keep one copy in the investigator's records, and send one copy to the sponsor (if there is a sponsor).

Appendix 3

Consent form used to gather serum to assess serum proteomics as a screening tool for colorectal cancer.

ST JAMES'S HOSPITAL AND FEDERATED DUBLIN VOLUNTARY HOSPITALS

PATIENT INFORMATION

1.Title of Study: Apoptosis (Cell Death) in Colonic Polyps

2. Introduction: We are doing a research study to learn more about how cells die (apoptosis) in colonic polyps, which are known to grow into cancer. If we can determine what stimulates apoptosis in polyps, this may help us devise new treatment options for treating colonic tumours.

For the purpose of this study, we need to examine a blood sample, normal colonic tissue and a small fraction of any growth that is detected at colonoscopy/operation. Biopsy samples of tissue for diagnosis and research and a blood sample will be taken during the same procedure. This study will not require you to attend for any additional visits or procedures.

3. Procedures: You will be selected based on your symptoms as to whether you need colonoscopy/surgical operation. No additional procedures are involved in this study. All polyp / tumour biopsies will be examined by a pathologist and part of the biopsy tissue will be used for research. Your blood sample will be analysed whether you have a polyp / tumour or not i.e. if your colonoscopy is clear. In that case your blood sample will act as a "control", to compare with the blood of patients with polyps and tumours.

In addition, tissue that is retained within the laboratory may be used for further ethically approved research studies on colorectal disease.

4. Benefits: No direct benefit of this study may be applicable to you. This study will give a better understanding of colonic polyp and tumour development and hopefully will help in devising better therapies for the future

5. Risks: No additional risks of this study to your health other than the procdure/operation itself which will be explained to you.

6. Exclusion from participation: *No exlusion criteria applies for this study,if you need the procedure of colonoscopy because of your symptoms or referral for the procedure,*

7. Alternative treatment: NA

8. Confidentiality: Your identity will remain confidential. Your name will not be published and will not be disclosed to anyone outside the hospital. Your research sample will be assigned a unique number and anonymised i.e. it will no longer be linked to you. The results obtained from your sample will be incorporated with results of other patients' biopsies but will not be traceable to you.

***9. Compensation:**

Your doctors are covered by standard medical malpractice insurance. Nothing in this document restrict or curtails your rights.

10. Voluntary Participation: *You have volunteered to participate in this study. You may quit at any time. If you decide not to participate, or if you quit, you will not be penalised and will not give up any benefits which you had before entering the study.*

11. Stopping the study: *You understand that your doctor may stop your participation in the study at any time without your consent.*

12. Permission: Permission sought.

13. Further information: *You can get more information or answers to your questions about the study, your participation in the study, and your rights, from Dr Fraser Smith who can be telephoned at 01 406 3750. If your doctor learns of important new information that might affect your desire to remain in the study, he or she will tell you.*

ST JAMES'S HOSPITAL AND FEDERATED DUBLIN VOLUNTARY HOSPITALS
JOINT RESEARCH ETHICS COMMITTEE
Consent Form

Title of research study: Apoptosis (Cell Death) in Colonic Polyps

This study and this consent form have been explained to me. My doctor has answered all my questions to my satisfaction. I believe I understand what will happen if I agree to be part of this study.
I have read, or had read to me, this consent form. I have had the opportunity to ask questions and all my questions have been answered to my satisfaction. I freely and voluntarily agree to be part of this research study, though without prejudice to my legal and ethical rights. I have received a copy of this agreement and I understand that, if there is a sponsoring company, a signed copy will be sent to that sponsor. **In understand that in addition, tissue that is retained within the laboratory may be used for further ethically approved research studies on colorectal disease.**

Name of sponsor:

PARTICIPANT'S NAME:

PARTICIPANT'S SIGNATURE:

Date::
Date on which the participant was first furnished with this form:

Where the participant is incapable of comprehending the nature, significance and scope of the consent required, the form must be signed by a person competent to give consent to his or her participation in the research study (other than a person who applied to undertake or conduct the study). If the subject is a minor (under 18 years old) the signature of parent or guardian must be obtained:-

NAME OF CONSENTOR, PARENT or GUARDIAN:
SIGNATURE:
RELATION TO PARTICIPANT:

Where the participant is capable of comprehending the nature, significance and scope of the consent required, but is physically unable to sign written consent, signatures of two witnesses present when consent was given by the participant to a registered medical practitioner treating him or her for the illness.

NAME OF FIRST WITNESS: SIGNATURE:
NAME OF SECOND WITNESS: SIGNATURE:

Statement of investigator's responsibility: I have explained the nature, purpose, procedures, benefits, risks of, or alternatives to, this research study. I have offered to answer any questions and fully answered such questions. I believe that the participant understands my explanation and has freely given informed consent.

Physician's signature:
Date:
(Keep the original of this form in the participant's medical record, give one copy to the participant, keep one copy in the investigator's records, and send one copy to the sponsor (if there is a sponsor).

Appendix 4

Standard Operating Procedures for Versagene RNA extraction Kit

Protocol

VERSAGENE™ RNA Purification Kit

RNA Purification from up to 40 mg Animal Tissue

Expected Yield Range: 0.5 - 5 µg RNA/mg tissue (actual yields will vary depending on tissue type)

Important Protocol Information

1. Protocol modifications have been developed for RNA isolation from tissues with high levels of RNase activity, such as pancreas. When working with these types of tissue, please see suggestions and protocol modifications in Protocol #400135-000: Helpful Hints for Isolating RNA from High RNase-Containing Tissues.
2. All steps, including centrifugations, should be performed at room temperature.

Before starting

1. Add TCEP to the Lysis Solution (4 µl TCEP per every 400 µl Lysis Solution) for all samples that will be processed. TCEP is stable in the Lysis Solution for 30 days at room temperature.
2. Mix Lysis Solution by shaking prior to each use.
3. If performing the optional DNase treatment, reconstitute the DNase according to the instructions provided with the DNase Kit (Cat. #VGR-0050D, 50 samples)

Tissue Homogenization and Cell Lysis

Weight of Tissue	Volume of Lysis Solution	Volume of TCEP (0.5 M)	Number of Purification Columns	Number of PRECLEAR Columns
0.5 – 40 mg	400 µl	4 µl	1	1

1. Add 4 µl TCEP reducing agent to 400 µl Lysis Solution.
2. Add 0.5-40 mg of tissue to the Lysis Solution/TCEP containing and homogenize thoroughly.
3. If excessive foam is present in the lysate after homogenization, centrifuge at 400 x g for 1 minute to eliminate the foam. Gently rock solution to resuspend any pellet formed during centrifugation. For detailed information on homogenization, please contact Gentra Systems' Technical Services.

PRECLEAR™ Column

1. Pipet 400 µl of lysate onto PRECLEAR Column (in a green tube). Centrifuge at 400 x g for 1 minute.
   NOTE: The PRECLEAR Column will remove large particulates from the lysate. The amount of particulates trapped in the PRECLEAR Column will vary depending on the tissue type and extent of the homogenization.
2. Discard basket containing PRECLEAR Column.
   NOTE: Insufficient homogenization will result in sample loss, which may be visibly trapped in the PRECLEAR Column along with tissue particulates. In some cases, additional volume may be recovered from the column by centrifuging 1-2 minutes longer, however, do not centrifuge at a higher speed.

RNA Binding and Wash 1

1. Pipet lysate up and down to resuspend any tissue particulates.
2. Pipet 400 µl lysate onto the Purification Column in a clear tube.
3. Change the centrifuge setting to maximum speed. Centrifuge at 13,000 – 16,000 x g for 1 minute. The white membrane of the column may become discolored, but this will have no effect on the purified RNA.
   NOTE: If residual lysate is left on the column after each centrifugation, rotate the tube 180 degrees in the centrifuge and centrifuge again at 13,000 -16,000 x g for 1 minute.

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4. Transfer basket containing Purification Column to a new tube.
5. Add 400 µl Wash 1 **Solution** to the Purification Column.
6. Centrifuge at 13,000 – 16,000 x g for 1 minute.
7. Transfer basket containing Purification Column to a new tube.

DNase Treatment (Optional)

1. Apply 50 µl **DNase Solution** to the Purification Column and incubate at room temperature for 15 minutes.
2. Add 200 µl **DNase Wash Solution** to the Purification Column.
   NOTE: Before centrifugation **DNase Wash Solution** must be added to the Purification Column. Failure to do so will result in loss of RNA.
3. Centrifuge at 13,000-16,000 x g for 1 minute.
4. Add 200 µl **DNase Wash Solution** to the **Purification Column.**
5. Centrifuge at 13,000-16,000 x g for 2 minutes.
6. Transfer Purification Column to a new clear tube (provided in the DNase Kit)

Wash 2

1. Add 200 µl Wash 2 **Solution** to the Purification Column.
2. Centrifuge at 13,000 – 16,000 x g for 1 minute.
3. Add an additional 200 µl Wash 2 **Solution** to the Purification Column.
4. Centrifuge at 13,000 – 16,000 x g for 2 minutes.

RNA Elution

1. Carefully transfer basket containing Purification Column to a new tube. Do not allow the Purification Column to come into contact with the waste in the tube prior to transferring.
2. Add 50 – 100 µl **Elution Solution** to the Purification Column to elute the RNA.
3. Centrifuge at 13,000 – 16,000 x g for 1 minute.
4. Discard basket containing the Purification Column.
5. Place the tube containing the purified RNA on ice. Store the purified RNA at –70° to –80°C.

Appendix 5

Standard Operating Procedures for Qiagen RNeasy Tissue RNA Extraction Kit

RNeasy Mini Protocol for Isolation of Total RNA from Animal Tissues

Determining the correct amount of starting material

It is essential to begin with the correct amount of tissue in order to obtain optimal RNA yield and purity with RNeasy columns. A maximum amount of 30 mg tissue can generally be processed with RNeasy mini columns. For most tissues, the binding capacity of the column (100 µg RNA) and the lysing capacity of Buffer RLT will not be exceeded by these amounts. Average RNA yields from various sources are given in Table 2 (page 17).

Some tissues such as spleen, parts of brain, lung, and thymus are more difficult to lyse or tend to form precipitates during the procedure. The volume of lysis buffer may need to be increased to facilitate complete homogenization and to avoid significantly reduced yields, DNA contamination, or clogging of the RNeasy column. See protocol for recommended amounts of lysis buffer to use.

Total RNA isolation from skeletal muscle, heart, and skin tissue can be difficult due to the abundance of contractile proteins, connective tissue, and collagen. The specialized protocol in Appendix C (page 93) includes a proteinase digestion and optimized RNA isolation procedure for these tissues.

If you have no information about the nature of your starting material, we recommend starting with no more than 10 mg of tissue. Depending on the yield and purity obtained, it may be possible to increase the amount of tissue to 30 mg.

Do not overload the column. Overloading will significantly reduce yield and quality.

Important notes before starting

- If using RNeasy or RNeasy Protect Kits for the first time, read "Important Points before Using RNeasy Kits" (page 16).
- If working with RNA for the first time, read Appendix A (page 88).
- **For best results, stabilize animal tissues immediately in RNA*later* RNA Stabilization Reagent following the protocol on page 47. Tissues can be stored in RNA*later* RNA Stabilization Reagent for up to 1 day at 37°C, 7 days at 18–25°C, 4 weeks at 2–8°C, or for archival storage at –20°C or –80°C.**
- Fresh, frozen, or RNA*later* stabilized tissue can be used. To freeze tissue for long-term storage, flash-freeze in liquid nitrogen, and immediately transfer to –70°C. Tissue can be stored for several months at –70°C. To process, do not allow tissue to thaw during weighing or handling prior to disruption in Buffer RLT. Homogenized tissue lysates (in Buffer RLT, step 4) can also be stored at –70°C for several months. To process frozen lysates, thaw samples and incubate for 15–20 min at 37°C in a water bath to dissolve salts. Continue with step 5.
- **β-Mercaptoethanol (β-ME) must be added to Buffer RLT before use. β-ME is toxic; dispense in a fume hood and wear appropriate protective clothing. Add 10 µl β-ME per 1 ml Buffer RLT. Buffer RLT is stable for 1 month after addition of β-ME.**

- **Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.**
- Generally, DNase digestion is not required since the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). In these cases, the small residual amounts of DNA remaining can be removed using the RNase-Free DNase Set (cat. no. 79254) for the optional on-column DNase digestion (see page 99) or by a DNase digestion after RNA isolation. For on-column DNase digestion with the RNase-Free DNase Set, prepare the DNase I stock solution as described on page 99 before beginning the procedure.
- Buffer RLT may form a precipitate upon storage. If necessary, redissolve by warming, and then place at room temperature.
- Buffer RLT and Buffer RW1 contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach. Guanidine is an irritant. Take appropriate safety measures and wear gloves when handling.
- All steps of the RNeasy protocol should be performed at room temperature. During the procedure, work quickly.
- All centrifugation steps are performed at 20–25°C in a standard microcentrifuge. Ensure that the centrifuge does not cool below 20°C.

1. **Excise the tissue sample from the animal or remove it from storage. Remove RNA*later* stabilized tissues from the reagent using forceps.**
2. **Determine the amount of tissue. Do not use more than 30 mg.**

   Weighing tissue is the most accurate way to determine the amount. See page 50 for guidelines to determine the amount of starting material.
3. **For RNA*later* stabilized tissues:**

   If the entire piece of RNA*later* stabilized tissue can be used for RNA isolation, place it directly into a suitably sized vessel for disruption and homogenization, and proceed with step 4.

   If only a portion of the RNA*later* stabilized tissue is to be used, place the tissue on a clean surface for cutting, and cut it. Determine the weight of the piece to be used, and place it into a suitably sized vessel for homogenization. Proceed with step 4.

   RNA in the RNA*later* treated tissue is still protected while the tissue is processed at 18 to 25°C. This allows cutting and weighing of tissues at ambient temperatures. It is not necessary to cut the tissue on ice or dry ice or in a refrigerated room. The remaining tissue can be placed into RNA*later* RNA Stabilization Reagent for further storage. Previously stabilized tissues can be stored at –80°C without the reagent.

For unstabilized fresh or frozen tissues:

If the entire piece of tissue can be used for RNA isolation, place it directly into a suitably sized vessel for disruption and homogenization, and proceed immediately with step 4.

If only a portion of the tissue is to be used, determine the weight of the piece to be used, and place it into a suitably sized vessel for homogenization. Proceed immediately with step 4.

RNA in tissues is not protected after harvesting until the sample is treated with RNA*later* RNA Stabilization Reagent, flash frozen, or disrupted and homogenized in protocol step 4. Frozen animal tissue should not be allowed to thaw during handling. The relevant procedures should be carried out as quickly as possible.

Note: The remaining fresh tissue can be placed into RNA*later* RNA Stabilization Reagent for stabilization (see Protocol for RNA Stabilization in Tissues with RNA*later* RNA Stabilization Reagent, page 47). However, previously frozen tissue samples thaw too slowly in the reagent, thus preventing it from diffusing into the tissue quickly enough before the RNA begins to degrade.

4. **Disrupt tissue and homogenize lysate in Buffer RLT. (Do not use more than 30 mg tissue.)**

Disruption and homogenization of animal tissue can be performed by 4 alternative methods (a, b, c, or d). See pages 20–24 for a more detailed description of disruption and homogenization methods.

After storage in RNA*later* RNA Stabilization Reagent, tissues may become slightly harder than fresh or thawed tissues. Disruption and homogenization of tissue samples using standard methods is usually not a problem. For easier disruption and homogenization it is recommended to increase the volume of lysis Buffer RLT to 600 µl as recommended for tissues that are difficult to lyse.

Note: Incomplete homogenization will lead to significantly reduced yields and can cause clogging of the RNeasy column. Homogenization with rotor–stator homogenizers generally results in higher total RNA yields than with other homogenization methods.

a. **Rotor–stator homogenization:**

Place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in a suitably sized vessel for the homogenizer. Add the appropriate volume of Buffer RLT (see below). Homogenize immediately using a conventional rotor–stator homogenizer until the sample is uniformly homogeneous (usually 20–40 s). Continue the protocol with step 5.

Rotor–stator homogenization simultaneously disrupts and homogenizes the sample.

Note: Ensure that β-ME is added to Buffer RLT before use (see "Important notes before starting").

Amount of starting material	Volume of Buffer RLT
<20 mg	350 µl or 600 µl*
20 to 30 mg	600 µl

** Use 600 µl Buffer RLT if preparing RNA from tissues that have been stabilized in RNAlater RNA Stabilization Reagent or that are difficult to lyse.*

b. **Mortar and pestle with QIAshredder homogenization:**

Immediately place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer RLT (see below). Pipet the lysate directly onto a QIAshredder spin column placed in 2 ml collection tube, and centrifuge for 2 min at maximum speed. Continue the protocol with step 5.

Grinding the sample using a mortar and pestle will disrupt the sample, but it will not homogenize it. Homogenization is carried out by centrifugation through the QIAshredder spin column.

Note: Ensure that β-ME is added to Buffer RLT before use (see "Important notes before starting").

Amount of starting material	Volume of Buffer RLT
<20 mg	350 µl or 600 µl*
20 to 30 mg	600 µl

** Use 600 µl Buffer RLT if preparing RNA from tissues that have been stabilized in RNAlater RNA Stabilization Reagent or that are difficult to lyse.*

c. **Mortar and pestle with needle and syringe homogenization:**

Immediately place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube (not supplied). Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

Add the appropriate volume of Buffer RLT (see below), and homogenize by passing lysate at least 5 times through a 20-gauge needle fitted to an RNase-free syringe. Continue the protocol with step 5.

Grinding the sample using a mortar and pestle will disrupt the sample, but it will not homogenize it. Homogenization is carried out with the needle and syringe.

Note: Ensure that β-ME is added to Buffer RLT before use (see "Important notes before starting").

Amount of starting material	Volume of Buffer RLT
<20 mg	350 µl or 600 µl*
20 to 30 mg	600 µl

** Use 600 µl Buffer RLT if preparing RNA from tissues that have been stabilized in RNAlater RNA Stabilization Reagent or that are difficult to lyse.*

d. **Mixer Mill MM 300:**

See "Appendix E: Disruption and Homogenization of RNA*later* Stabilized Tissues Using the Mixer Mill MM 300" (page 101) for guidelines.

The Mixer Mill MM 300 simultaneously disrupts and homogenizes the sample.

5. **Centrifuge the tissue lysate for 3 min at maximum speed in a microcentrifuge. Carefully transfer the supernatant to a new microcentrifuge tube (not supplied) by pipetting. Use only this supernatant (lysate) in subsequent steps.**

In some preparations, very small amounts of insoluble material will be present, making the pellet invisible.

6. **Add 1 volume (usually 350 µl or 600 µl) of 70% ethanol to the cleared lysate, and mix immediately by pipetting. Do not centrifuge. Continue without delay with step 7.**

If some lysate is lost during steps 4 and 5, adjust volume of ethanol accordingly.

A precipitate may form after the addition of ethanol, but this will not affect the RNeasy procedure.

7. **Apply up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy mini column placed in a 2 ml collection tube (supplied). Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm). Discard the flow-through.**

Reuse the collection tube in step 8.

If the volume exceeds 700 µl, load aliquots successively onto the RNeasy column, and centrifuge as above. Discard the flow-through after each centrifugation step.†

Optional: QIAGEN offers the RNase-Free DNase Set (cat. no. 79254) for convenient on-column DNase digestion during RNA purification. Generally, DNase digestion is not required since the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). If using the RNase-Free DNase Set, follow the steps shown on pages 99–100 after performing this step.

† *Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach.*

8. **Add 700 µl Buffer RW1 to the RNeasy column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through and collection tube.***

   Skip this step if performing the optional on-column DNase digestion (page 99).

9. **Transfer the RNeasy column into a new 2 ml collection tube (supplied). Pipet 500 µl Buffer RPE onto the RNeasy column. Close the tube gently, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.**

   Reuse the collection tube in step 10.

   Note: Buffer RPE is supplied as a concentrate. Ensure that ethanol is added to Buffer RPE before use (see "Important notes before starting").

10. **Add another 500 µl Buffer RPE to the RNeasy column. Close the tube gently, and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to dry the RNeasy silica-gel membrane. Continue directly with step 11, or, to eliminate any chance of possible Buffer RPE carryover, continue first with step 10a.**

    It is important to dry the RNeasy silica-gel membrane since residual ethanol may interfere with downstream reactions. This centrifugation ensures that no ethanol is carried over during elution.

    Note: Following the centrifugation, remove the RNeasy mini column from the collection tube carefully so the column does not contact the flow-through as this will result in carryover of ethanol.

10a. **Optional: Place the RNeasy column in a new 2 ml collection tube (not supplied), and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min.**

11. **To elute, transfer the RNeasy column to a new 1.5 ml collection tube (supplied). Pipet 30–50 µl RNase-free water directly onto the RNeasy silica-gel membrane. Close the tube gently, and centrifuge for 1 min at ≥8000 x *g* (≥10,000 rpm) to elute.**

12. **If the expected RNA yield is >30 µg, repeat the elution step (step 11) as described with a second volume of RNase-free water. Elute into the same collection tube.**

    To obtain a higher total RNA concentration, this second elution step may be performed by using the first eluate (from step 11). The yield will be 15–30% less than the yield obtained using a second volume of RNase-free water, but the final concentration will be higher.

** Flow-through contains Buffer RLT or Buffer RW1 and is therefore not compatible with bleach.*

Appendix D: Optional On-Column DNase Digestion with the RNase-Free DNase Set

The RNase-Free DNase Set (cat. no. 79254) provides efficient on-column digestion of DNA during RNA purification. The DNase is efficiently removed in subsequent wash steps.

Note: Standard DNase buffers are not compatible with on-membrane DNase digestion. Use of other buffers may affect the binding of the RNA to the RNeasy silica-gel membrane, reducing the yield and integrity of the RNA.

Lysis and homogenization of the sample and binding of RNA to the silica-gel membrane are performed according to the standard protocols. After washing with a reduced volume of Buffer RW1, the RNA is treated with DNase I while bound to the silica-gel membrane. The DNase is removed by a second wash with Buffer RW1. Washing with Buffer RPE and elution are then performed according to the standard protocols.

- Generally, DNase digestion is not required since the RNeasy silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be necessary for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). DNA can also be removed by a DNase digestion following RNA isolation.
- **Prepare DNase I stock solution before using the RNase-Free DNase Set for the first time. Dissolve the solid DNase I (1500 Kunitz units) in 550 µl of the RNase-free water provided. Take care that no DNase I is lost when opening the vial. Mix gently by inverting the tube. Do not vortex.**
- **Do not vortex the reconstituted DNase I.** DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube.
- For long-term storage of DNase I, remove the stock solution from the glass vial, divide it into single-use aliquots, and store at –20°C for up to 9 months. Thawed aliquots can be stored at 2–8°C for up to 6 weeks. Do not refreeze the aliquots after thawing.

Procedure

Carry out lysis, homogenization, and loading onto the RNeasy mini column as indicated in the individual protocols. Instead of continuing with the Buffer RW1 step, follow steps D1–D4 below.

D1. Pipet 350 µl Buffer RW1 into the RNeasy mini column, and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash. Discard the flow-through.

Reuse the collection tube in step D3.

D2. Add 10 µl DNase I stock solution (see above) to 70 µl Buffer RDD. Mix by gently inverting the tube.

Buffer RDD is supplied with the RNase-Free DNase Set.

Note: DNase I is especially sensitive to physical denaturation. Mixing should only be carried out by gently inverting the tube. Do not vortex.

D3. Pipet the DNase I incubation mix (80 µl) directly onto the RNeasy silica-gel membrane, and place on the benchtop (20–30°C) for 15 min.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy silica-gel membrane. DNase digestion will be incomplete if part of the mix sticks to the walls or the O-ring of the RNeasy column.

D4. Pipet 350 µl Buffer RW1 into the RNeasy mini column, and centrifuge for 15 s at ≥8000 x *g*. Discard the flow-through.* Continue with the first Buffer RPE wash step in the relevant protocol.

Note: In most of the protocols, the immediately following BufferRW1 wash step is skipped (as indicated in the protocol). Continue with the first Buffer RPE wash step.

Appendix 6

Standard Operating Procedures for Applied Biosystems V2

RT-IVT Labelling Kit

Applied Biosystems

Applied Biosystems Chemiluminescent RT Labeling Kit

For safety and biohazard guidelines, refer to the "Safety" section in the *Applied Biosystems Chemiluminescent RT Labeling Kit Protocol* (PN 4339628). For all chemicals in **bold** type, read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Performing Reverse Transcription Labeling

Materials Needed

- 2 to 5 µg mRNA sample(s)
- Oligo (dT) Primer
- Control RNA
- Nuclease-free water
- **10× RT Buffer Mix**
- DIG-dUTP
- RT Enzyme Mix
- Ice bucket
- 0.2-mL MicroAmp® reaction tube(s)
- Pipettor: 1- to 20-µL range
- Pipette tips
- Vortexer
- Microcentrifuge
- Thermal cycler

Procedure

1. Prepare sample and labeling reagents:
   a. Thaw on ice:
      - 2 to 5 µg mRNA sample
      - Oligo (dT) Primer
      - Control RNA
      - Nuclease-free water
      - **10× RT Buffer Mix**
      - DIG-dUTP

   b. Vortex tubes, then centrifuge briefly.
2. Pipette components into a 0.2-mL MicroAmp reaction tube on ice:

Component	Volume (µL)
Oligo (dT) primer	3.0
Control RNA	2.0
2 to 5 µg mRNA sample and nuclease-free water	17.0
Total	22.0

3. Heat and cool the RNA and primer mixture in a thermal cycler:

Stage	Temperature	Time	Reaction Volume
1	70 °C	5 minutes	22 µL
2	4 °C	Indefinite hold	

4. After the run, place the tube on ice.
5. Check the **10× RT Buffer Mix** for precipitates.

   Note: If precipitates are present, warm the buffer at 37 °C for 2 to 3 minutes, then vortex it briefly before using.
6. Add reverse transcription reagents to the reaction tube and mix thoroughly by pipetting:

Component	Volume (µL)
10× RT Buffer Mix	3.0
DIG-dUTP (approximately 2 nmol)	2.0
RT Enzyme Mix	3.0

7. Perform reverse transcription in a thermal cycler:

Stage	Temperature	Time	Reaction Volume
1	25 °C	10 minutes	30 µL
2	42 °C	3 hours	
3	70 °C	15 minutes	
4	4 °C	Indefinite hold	

8. After the run, remove the tube from the thermal cycler.

Degrading RNA

Materials Needed

- 2.5 M **NaOH**
- 1 M Tris-HCl, pH 7.0
- Pipettor: 1- to 20-µL range
- Pipette tips
- Thermal cycler

Procedure

1. Add 3.0 µL of 2.5 M **NaOH** to the reaction tube and mix thoroughly by pipetting up and down.
2. Incubate the mixture in a thermal cycler:

Temperature	Time	Reaction Volume
37 °C	15 minutes	33 µL

3. *Immediately* after the 15-minute incubation, add 20 µL of 1 M Tris-HCl, pH 7.0 to the reaction tube and mix thoroughly.

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Purifying cDNA

Materials Needed

- **DNA Binding Buffer**
- **100% ethanol**
- **DNA Wash Buffer**
- DNA Elution Buffer
- DNA purification column(s)
- 2-mL receptacle tube(s)
- 1.5-mL elution tube(s)
- Pipettors: 1- to 20-µL range, 20- to 200-µL range, 100- to 1000-µL range
- Pipette tips
- Microcentrifuge

Procedure

1. In a new 1.5-mL nuclease-free microcentrifuge tube, combine:
   - **DNA Binding Buffer**: 100 µL
   - **100% ethanol**: 100 µL
2. Add the entire RT reaction (53 µL) to the DNA Binding Buffer-ethanol mixture and mix thoroughly by pipetting.
3. Begin the purification:
   a. Insert a DNA purification column into a 2-mL receptacle tube.
   b. Transfer the RT reaction-DNA Binding Buffer-ethanol mixture (253 µL) to the column, then close the tube.
   c. Centrifuge the column and tube at 13,000 × *g* for 1 minute.
   d. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.
4. Wash the cDNA (1):
   a. Add 700 µL of **DNA Wash Buffer** to the column, then close the tube.
   b. Centrifuge the column and tube at 13,000 × *g* for 1 minute.
   c. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.
5. Wash the cDNA (2):
   a. Add 700 µL of **DNA Wash Buffer** to the column, then close the tube.
   b. Centrifuge the column and tube at 13,000 × *g* for 1 minute.
   c. Remove the column from the tube, discard the liquid, then reinsert the column into the tube.
   d. Centrifuge the column and tube at 13,000 × *g* for 1 minute.
6. Elute the cDNA (1):
   a. Transfer the column to a new 1.5-mL elution tube.
   b. Pipette 30 µL of DNA Elution Buffer onto the fiber matrix at the bottom of the column, then close the tube.

   IMPORTANT! Do not let the pipette tip touch the fiber matrix.
   c. Incubate the column at room temperature for 1 minute.
   d. Centrifuge the column and tube at 13,000 × *g* for 1 minute for an elution volume of 30 µL.
7. Elute the cDNA (2):
   a. Pipette 30 µL of DNA Elution Buffer onto the fiber matrix at the bottom of the column, then close the tube.

   IMPORTANT! Do not let the pipette tip touch the fiber matrix.
   b. Incubate the column at room temperature for 1 minute.
   c. Centrifuge the column and tube at 13,000 × *g* for 1 minute for an elution volume of 60 µL.
8. Elute the cDNA (3):
   a. Pipette 30 µL of DNA Elution Buffer onto the fiber matrix at the bottom of the column, then close the tube.

   IMPORTANT! Do not let the pipette tip touch the fiber matrix.
   b. Incubate the column at room temperature for 1 minute.
   c. Centrifuge the column and tube at 13,000 × *g* for 1 minute for a final elution volume of 90 µL.
   d. Discard the column, then close the tube.

Storing cDNA Product

- –15 to –25 °C for up to 2 months or
- –80 °C for long-term storage

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Appendix 7

Standard Operating Procedures for Applied Biosystems V2

Microarray Chemoluminescence Detection Kit

Applied Biosystems

Applied Biosystems Chemiluminescence Detection Kit

For safety and biohazard guidelines, refer to the "Safety" section in the *Applied Biosystems Chemiluminescence Detection Kit Protocol* (PN 4339627). For all chemicals in **bold** type, read the MSDS and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Setting Up the Hybridization Oven

Power on the hybridization oven and set the temperature to 55 °C.

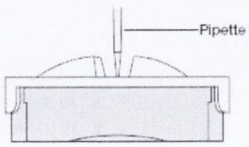
Note: The oven may take up to 3 hours to heat up and equilibrate to 55 °C.

Prehybridizing Microarrays

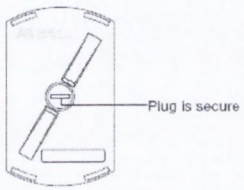
1. Verify that the oven temperature is 55 °C.
2. Warm at 37 °C for 30 minutes, then vortex to dissolve any precipitates:
   - **Hybridization Buffer**
   - **Hybridization Denaturant**
   - **Blocking Reagent**
3. Equilibrate microarrays to room temperature.
4. Prepare prehybridization mixture in a nuclease-free tube and vortex to mix:

Component	Volume (µL)/ Microarray
Nuclease-free water	150
Hybridization Buffer	330
Hybridization Denaturant	100
Blocking Reagent	420
Total/Microarray	1000

5. Transfer prehybridization mixture into each microarray cartridge (repeat for each cartridge):
   a. Remove the foil wrapping from a cartridge.
   b. Remove the plug and pipette prehybridization mixture (1 mL) into the port with the pipette tip at a 90° angle.



   c. Dry the port surface with lint-free tissue and seal the port with the plug. Turn the plug 90 degrees to secure it.



   d. Gently tilt the array several times to wet evenly.

IMPORTANT! Do not shake the cartridge.

   e. Rock all cartridges in a vertical orientation on the rocking platform shaker for 5 minutes.

6. Place all cartridges in the oven, making sure that each cartridge is level and held securely by the clamps, then close the oven door.
7. Incubate the cartridge(s) in the oven:
   - Temperature: 55 °C
   - Agitation: 100 rpm
   - Time: 1 hour

Fragmenting cRNA

During prehybridization, fragment the cRNA if you used the Applied Biosystems Chemiluminescent RT-IVT Labeling Kit and have DIG-labeled cRNA targets.

1. Allow reagents to equilibrate to room temperature 1 hour, then vortex and centrifuge briefly before use:
   - **cRNA Fragmentation Buffer**
   - **cRNA Fragmentation Stop Buffer**
2. Combine components into a 0.2-mL MicroAmp® reaction tube on ice, then mix by pipetting:

Component	Volume (µL)
cRNA Fragmentation Buffer	10.0
10 µg DIG-labeled cRNA and nuclease-free water	90.0
Total	100.0

3. Heat the tube in a thermal cycler:

Temperature	Time	Reaction Volume
60 °C	30 minutes	100 µL

4. Neutralize the reaction:
   a. Remove the tube from the thermal cycler, then centrifuge the tube briefly.

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b. Add 50 µL of **cRNA Fragmentation Stop Buffer**, then mix by pipetting.

c. Place the tube on ice or add immediately to hybridization mixture as below.

Hybridizing Samples to Microarrays

1. Vortex Hybridization Controls, then centrifuge the tube briefly.
2. If you used the Applied Biosystems Chemiluminescent RT Labeling Kit, add nuclease-free water to the cDNA targets to bring the volume to 150 µL.

   Note: If you used up to 2 µg mRNA input or up to 40 µg total RNA input, you can use the entire DIG-labeled cDNA product on one microarray.

3. For each microarray, prepare hybridization mixture in a 1.5 mL nuclease-free microcentrifuge tube:

Component	Volume (µL)/ Microarray
Nuclease-free water	100
Hybridization Buffer	170
Hybridization Controls	30
cDNA targets and nuclease-free water or fragmented cRNA targets	150
Hybridization Denaturant	50
Total/Microarray	500

4. Vortex the hybridization mixture, then centrifuge the tube briefly.
5. Take to the hybridization oven:
   - Tube(s) with hybridization mixture
   - 1000-µL pipettor
   - 1000-µL pipette tips
   - Lint-free tissues
6. *Quickly* transfer hybridization mixture into each microarray cartridge (repeat for each cartridge):

IMPORTANT! Add hybridization mixture quickly.

   a. Remove one cartridge from the oven and close the door.

   b. Remove the plug and pipette hybridization mixture (500 µL) into the port.

   c. Dry the port with lint-free tissue and seal it with the plug. Turn the plug 90 degrees to secure it.

   d. Return the cartridge to the oven. Make sure that the cartridge is level and held securely by the clamps.

7. Incubate cartridge(s) in the oven:
   - Temperature: 55 °C
   - Agitation: 100 rpm
   - Time: 16 hours

IMPORTANT! Do not vary the incubation time. Variations may affect reproducibility.

Preparing AB 1700 Wash Buffers and Reagents

Volumes provided are for one **set of washes** with one to four microarrays in one AB wash tray.

1. Equilibrate to 22 °C before use:
   - **Hybridization Wash Buffer Concentrate**
   - Hybridization Wash Detergent Concentrate
   - **Chemiluminescence Rinse Buffer Concentrate**
   - **Chemiluminescence Enhancing Rinse Concentrate**
   - Nuclease-free deionized water
2. Heat to 37 °C for 30 minutes, then mix well. Allow to equilibrate to room temperature before use:
   - **Chemiluminescence Enhancing Solution**
   - **Blocking Reagent**
3. Prepare hybridization wash buffer 1:

Component	Volume (mL)
Hybridization Wash Buffer Concentrate	30
Hybridization Wash Detergent Concentrate	60
Nuclease-free deionized water	210
Total	300

4. Prepare hybridization wash buffer 2:

Component	Volume (mL)
Hybridization Wash Buffer Concentrate	1.5
Nuclease-free deionized water	298.5
Total	300.0

5. Prepare CL rinse buffer:

Component	Volume (mL)
Chemiluminescence Rinse Buffer Concentrate	75
Nuclease-free deionized water	1425
Total	1500

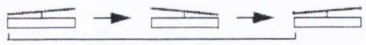
6. Prepare CL enhancing rinse buffer:

Component	Volume (mL)
Chemiluminescence Enhancing Rinse Concentrate	15
Nuclease-free deionized water	585
Total	600

Rocking Platform Shaker Settings

Verify the tilt settings of the recommended VWR rocking platform shaker before performing hybridization washes, antibody binding, antibody washes, and the CL reaction:

- Tilt angle: 10°
- Tilt speed: 30 tilts back and forth per minute



1 tilt back and forth

Performing Hybridization Washes

Perform at room temperature (19 to 30 °C).

IMPORTANT! Do not allow the microarrays to dry out. After completing a step, *immediately* proceed to the next step. Perform all steps in AB wash trays, one tray at a time, with up to 4 arrays per tray. Do not shake loose the secured microarray.Do not touch the microarray surface.

1. Perform hybridization wash 1:
   a. Add 300 mLs hybridization wash buffer 1 to a clean wash tray.

Note: *Quickly* transfer each microarray, one at a time, from the oven to the wash tray (repeat for each microarray) as below:

   b. Open the oven, remove one cartridge, then close the door to restart agitation.
   c. Open the cartridge and take the microarray out. Make sure that some hybridization volume (>0.75 mL) remains.
   d. Decant the liquid, then shake and tap the microarray gently to remove excess liquid.
   e. Place the microarray securely in the wash tray and submerge the microarray in the buffer. Repeat for up to 4 microarrays per tray until all arrays are in trays. Ensure that each microarray is properly inserted.
   f. Place the wash tray on the rocker with the arrays vertically arranged in the wash tray. Agitate on the rocking platform for 5 minutes.
   g. Decant the buffer by tilting the wash tray.
2. Perform hybridization wash 2:
   a. Add 300 mLs hybridization wash buffer 2 to the wash tray, then make sure that all microarrays are submerged in buffer.
   b. Place the wash tray on the rocker with the arrays vertically arranged in the wash tray. Agitate on the rocking platform for 5 minutes.
   c. Decant the buffer by tilting the wash tray.
3. Perform CL rinse 1:
   a. Add 300 mLs CL rinse buffer to the wash tray, then make sure that all microarrays are submerged in buffer.
   b. Place the wash tray on the rocker with the arrays vertically arranged in the wash tray, then agitate on the rocking platform shaker for 5 minutes.
   c. Decant the buffer by tilting the wash tray.
4. Perform CL rinse 2:
   a. Add 300 mLs CL rinse buffer to the wash tray, then make sure that all microarrays are submerged in buffer.
   b. Place the wash tray on the rocker with the arrays vertically arranged in the wash tray. Agitate on the rocking platform for 5 minutes.
   c. Cover the wash tray, remove it from the rocking platform, then place it on the bench top.

Note: You may leave the microarrays in CL rinse buffer at room temperature for up to 1 hour.

Performing Antibody Binding

1. Combine components for the CL blocking buffer/antibody mixture in a nuclease-free tube and mix well by inversion. Do not vortex.

Component	Volume/ Microarray
Nuclease-free water	2.8 mL
Chemiluminescence Rinse Buffer Concentrate	0.2 mL
Blocking Reagent	1.0 mL
Anti-digoxigenin-AP	15 µL
Total/Microarray	4.015 mL

2. Decant the CL Rinse buffer from 1 wash tray, leaving the microarrays secured.
3. *Immediately* add 4 mL CL blocking buffer/antibody mixture to each microarray.

IMPORTANT! Complete step 3 for all microarrays within the tray in 2 minutes. Do not allow the microarrays to dry out.

4. Repeat for all trays.
5. Cover the arrays, then rock on the rocking platform for 20 minutes at room temperature.

Performing Antibody Washes

IMPORTANT! Do not allow the microarrays to dry out. After completing a step, *immediately* proceed to the next step.

1. Perform CL rinse 1:
   a. Decant the CL blocking buffer/antibody mixture by tilting the wash tray.
   b. Add 300 mL CL rinse buffer to the wash tray.
   c. Place the wash tray on the rocker with the arrays vertically arranged in the wash tray. Agitate on the rocking platform for 10 minutes.
   d. Decant the buffer by tilting the wash tray.
2. Perform CL rinse 2:
   a. Add 300 mL CL rinse buffer to the wash tray, then make sure that all microarrays are submerged in buffer.
   b. Place the wash tray on the rocker with the arrays vertically arranged in the wash tray. Agitate on the rocking platform for 10 minutes.
   c. Decant the buffer by tilting the wash tray.
3. Perform CL rinse 3:
   a. Add 300 mL CL rinse buffer to the wash tray, then make sure that all microarrays are submerged in buffer.

b. Place the wash tray on the rocker with the arrays vertically arranged in the wash tray. Agitate on the rocking platform for 10 minutes.

Performing the CL Reaction

1. Power on the Applied Biosystems 1700 Chemiluminescent Analyzer. The Analyzer must be on at least 10 minutes before using it.
2. Perform a CL enhancing rinse:
   a. Decant the CL rinse buffer by tilting the wash tray.
   b. *Immediately* add 300 mL of CL enhancing rinse buffer to the wash tray.
   c. Place the wash tray on the rocker with the arrays vertically arranged in the wash tray. Agitate on the rocking platform for 10 minutes.
   d. Stop the agitation of the rocking platform .
3. Perform the enhancing step:
   a. Decant the CL enhancing rinse buffer from 1 wash tray leaving the microarrays secure.
   b. *Immediately* add 4 mL of **Chemiluminescence Enhancing Solution** to each microarray.
   c. Place the wash tray on the rocker with the arrays vertically arranged in the wash tray. Agitate on the rocking platform for 20 minutes.
4. Perform a CL enhancing rinse:
   a. Decant the CL Enhancing Solution by tilting the wash tray.
   b. Add 300 mL CL enhancing rinse buffer to the wash tray.
   c. Place the wash tray on the rocker with the arrays vertically arranged in the wash tray. Agitate on the rocking platform for 5 minutes.
   d. Remove the wash tray from the rocking platform shaker.

Note: You may leave the microarrays in the CL enhancing rinse buffer at room temperature for up to 3 hours.

Note: The chemiluminescent reaction is time-dependent. Perform the following steps with only one microarray at a time.

5. Add substrate:
   a. Remove one microarray from the wash tray, decant the CL enhancing rinse buffer, then shake and tap the microarray gently.
   b. Wipe the bottom of the microarray with lint-free tissue.
   c. Add 3.5 mL of **Chemiluminescence Substrate** to the microarray.

IMPORTANT! The chemiluminescent reaction is time-dependent. After you perform this step, proceed with performing CL detection immediately.

Performing CL Detection

1. Load the microarray into the instrument:
   a. Dry any excess liquid from the bottom of the microarray with lint-free tissue.
   b. Open the instrument door.
   c. Orient the microarray so that the end bar code is at the top and the side bar code is on the right, then place the microarray on the heated stage of the instrument.
   d. Lock the bar, then close the door.
2. Perform the read:
   a. On the computer, select **Start > Programs > Applied Biosystems > Expression Array System Software** to start the software.
   b. Enter the Login Name and Password.
   c. Select cartridge settings: Microarray Type, Chemistry Method, Reader Method, Analysis Method, Folder, Sample Name/ID
   d. Click **Start** and wait for the instrument to obtain the images.
3. Analyze the data, using the following documents:
   - *Applied Biosystems 1700 Chemiluminescent Analyzer User Guide* (PN 4338852)
   - *Applied Biosystems 1700 Chemiluminescent Analyzer Chemistry Guide* (PN 4338853)