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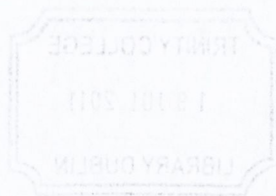
Trinity College, University of Dublin

MD Thesis

Molecular and Radiological Prediction of Response to Neo-adjuvant Chemoradiation in Patients with Oesophageal Cancer



Charles Martin Gillham

BSc, MBBS, MRCP, FRCR

2009

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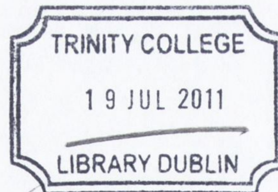
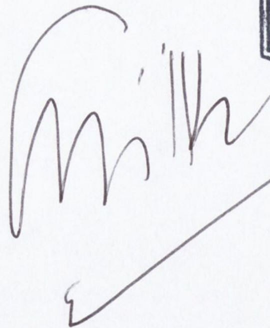


Signed: 

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Summary

The initial phase of the project's laboratory component involved optimising techniques that would become necessary when analysing fresh oesophageal tissue. There are a number of commercially available techniques for tissue collection, storage and homogenisation as well as RNA extraction. Some of the more common techniques, as identified in the literature and available within the Trinity Laboratories, were studied. A number of experiments were performed on fragments of tissue removed following a routine oesophagectomy.

It was identified that, in order to maximise the chances of obtaining good quality RNA in sufficient quantity, tissue should be immediately placed into the fixative medium RNA*later*. It should then be refrigerated overnight before being snap-frozen at -80℃. The handheld homogeniser (Ultra-Turrax T8, IKA® Works, UK) was found to be the most convenient and successful for tissue disruption prior to RNA extraction.The most appropriate technique for extracting RNA was found to be the RNeasy™ Fibrous Tissue kit available from Qiagen, USA.

The use of cDNA microarrays is a powerful method for the quantitative analysis of disease-specific gene expression. They can detect altered expression profiles associated with the pathology or altered biology of a disease entity. This study was performed to identify different genetic profiles associated not only with benign and malignant oesophageal tissue but also with response to neo-adjuvant chemoradiation in the multi-modal management of the disease.

Gene expression profiles for 13 malignant and 11 benign oesophageal samples were compared using a whole genome microarray system from Applied Biosystems. The malignant samples were then sub-divided according to the response to chemoradiation and a further analysis undertaken.

Validation of the microarray data was performed using Real Time Quantitative PCR. In order to select the most appropriate endogenous control for oesophageal samples the Applied Biosystems TaqMan® Human Endogenous Control Plate was used.

12 targets were then chosen from the microarray data. This was done by selecting some of those genes that were most differentially expressed between responders and non-responders at the $p<0.01$ level.

Sufficient RNA remained from 10 of the initial 13 tumour samples. RNA was reverse-transcribed using a Superscript III kit from Invitrogen, UK. Primers and probes were obtained by using Applied Biosystems' pre-designed TaqMan® Gene Expression Assays. PCR was carried out using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, CA, USA). Analysis of relative gene expression data was performed using the $\Delta\Delta C_T$ method[1] with 18s ribosomal RNA as an endogenous control/reference assay.

In an attempt to identify the clinical utility of ^{18}FDG PET as a predictive tool in the management of localised oesophageal cancer scans were performed at diagnosis and following the first week of neoadjuvant chemoradiation. The initial value of two parameters; the mean maximum standardised uptake value (SUV) and the volume of metabolically active tissue were recorded. The change between these and those taken following one week of pre-operative therapy was compared with the final tumour regression grade (TRG). Pathological responders to treatment were deemed those who achieved a TRG of 1-2, whilst non-responders were those with a score of 3-5.
Neither the diagnostic values nor the change following treatment significantly predicted the response of the tumour to chemoradiation.

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Last, but by no means least, I would like to thank my gorgeous wife - Alana. Only through her unfailing support and encouragement has this MD been possible.

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CHAPTER 1

1: INTRODUCTION

Cancer medicine is in the midst of a technological revolution and the way the disease is managed is undergoing enormous change. For the very first time it is becoming increasingly possible to individualise a patient's treatment by predicting those that will and those that will not respond to a chosen therapy. This is being achieved through rapid developments in both advanced diagnostic imaging and translational medicine. The benefits are already being experienced in some of the more common tumour sites, such as breast and lung. As a result the foundations have been laid for some of the less frequent, but by no means less serious, pathological types.

1.2: OESOPHAGEAL CANCER

Oesophageal cancer is the eighth most common cancer worldwide[2]. There are approximately 14,520 people diagnosed with oesophageal cancer each year in the United States, and 13,570 deaths from the disease[3]. Survival correlates with stage. Five-year survival rates range from 40 to 62 percent for patients treated for localized cancer (stage I and IIA), and from 18 to 25 percent for those with involvement of regional nodes (stage IIB and III)[4]. According to data from the National Cancer Institute Surveillance, Epidemiology and End Results (SEER) Program, the five-year survival rate for all patients with oesophageal cancer improved modestly over the last 30 years, from 5 percent in the years 1974 to 1976, to 13 percent during the period 1992 to 1998[3]. These dismal figures are indicative of the advanced stage of disease (loco-regional or metastatic, stages IIB, III and IV) at diagnosis in most individuals[5].

In Ireland oesophageal cancer ranks twelfth in incidence and is the seventh most common cause of cancer death[6]. Although that of squamous cell carcinoma has been declining over the past two decades, the incidence of adenocarcinoma in Ireland and the West has increased markedly. In the US adenocarcinoma now accounts for more than 50% of oesophageal cancer cases[7]. In Ireland

approximately 450 people are diagnosed each year and a similar number die from the disease. Compared to the EU and the US the incidence rate for Irish men is almost 1.2 to 1.5 times higher and for women it is 2.5 to 3 times higher. The mortality rate is similar. The high mortality rate reflects early lymphatic and haematogenous spread as well as the lack of effective treatments and early therapeutic options.

For many years the standard therapy for localised oesophageal carcinoma has been surgical resection[8]. However local control and overall survival remain poor, and even after radical resection and lymphadenectomy the 5 year survival is at best approximately 40 per cent[9,10]. In an effort to improve these figures the management of loco-regional oesophageal cancer has undergone a major evolution over the past 15 years. Numerous strategies employing various pre- and post-operative therapies have been studied as well as trials where surgery has been omitted altogether.

To date the optimal therapy for potentially resectable oesophageal cancer remains unclear.

1.2.1: Radiotherapy Alone

Radiotherapy alone may result in long-term survival in a minority of patients. Before the era of modern chemotherapy and combined chemoradiation (CRT), radiation alone (60 to 66 Gy in 30 to 33 fractions over a period of 6 to 6.6 weeks) was associated with five-year survival rates of 5 to 20 percent, depending upon the tumour extent[11]. In a review of 49 early series involving more than 8400 patients treated with radiation alone, survival rates at one, two, and five years were 18, 8, and 6 percent, respectively[12].

There have been better results reported in later studies from single institutions in well-defined patient populations using specific radiation protocols. For example three and five-year survival rates of 27 and 21 percent, respectively, were observed in a series that included 101 selected patients with clinically localised oesophageal cancer treated with radiation alone (45 to 52.5 Gy in 15 or 16 fractions over three weeks)[11].

Modern radiation techniques are associated with more favourable toxicity profiles than those used in earlier years. More recently, the role of radiation alone has been supplanted by combined CRT in the majority of patients, albeit with a higher rate of treatment-related toxicity.

1.2.2: Chemoradiation Alone

The addition of cisplatin-based chemotherapy to radiation has been shown to provide a significant survival benefit compared to treatment with radiotherapy alone[13,14,15]. However, the available randomized trial data are almost exclusively in squamous cell oesophageal cancer, and none of the trials have performed adequate pre-treatment staging to reliably correlate outcome with loco-regional tumour extent.

The Radiation Therapy Oncology Group (RTOG) study published in 1992 has had a very significant impact on international clinical practice. It compared radiotherapy alone (64 Gy in 32 fractions over 6.5 weeks) with concurrent chemoradiation (four cycles of 5-FU [1000 mg/m^2 by continuous infusion for the first 4 days of weeks 1, 5, 8, and 11] plus cisplatin (75 mg/m^2 day 1, every four weeks) and radiotherapy (50 Gy in 25 fractions over five weeks) in patients with loco-regional thoracic oesophageal cancer[13]. Ninety percent of the trial participants had squamous cell carcinoma. The trial was closed prematurely when an interim analysis showed a significant survival advantage in the CRT arm. In the most recent update of this study, combined treatment was associated with a significantly better median survival (14 versus 9.3 months) and five year survival (27 versus 0 percent)[14]. Analysis of patterns of failure showed a significant reduction in both loco-regional and distant failure for the CRT arm. As a consequence combined CRT became the standard of care for patients with inoperable disease. However, despite this apparent benefit, 46 percent of patients in the experimental treatment group had recurrence or persistence of disease in the oesophagus at 12 months.

The issue of the worryingly high loco-regional failure rate was addressed in a follow-up trial; INT 0123.

All 236 patients in this study received concurrent cisplatin and 5-FU (as in RTOG 85-01) chemotherapy, but they were randomly assigned to one of two different

radiation doses: 50.4 Gy in 28 fractions over 5.5 weeks or 64.8 Gy in 36 fractions over 7 weeks[16]. Patients without evidence of distant metastatic squamous cell carcinoma or adenocarcinoma of the thoracic oesophagus were eligible. The use of the higher radiation dose was not associated with a higher median survival (13 versus 18 months), two year survival rate (31 versus 40 percent), or incidence of loco-regional persistent or recurrent disease (56 versus 52 percent for the high dose and control groups, respectively. In addition, the high radiation dose arm was associated with significantly more toxicity.

The reason for failing to demonstrate improved survival or local-regional control with higher radiation doses is unclear. At present, 50 Gy of radiation therapy plus four courses of concurrent cisplatin and 5-FU (as in RTOG 85-10) remains the standard of care in most centres where surgery is not considered feasible.

More recent studies are investigating the role of newer chemotherapy drugs given in combination with radiotherapy. As an example, in a French phase I study, 33 patients with inoperable, locally advanced squamous cell carcinoma and adenocarcinoma were treated with escalating doses of oxaliplatin combined with infusional 5-FU and folinic acid (FOLFOX) plus radiation therapy (50 Gy) followed by three additional cycles of FOLFOX[17]. Oxaliplatin doses of 85 mg/m^2 were well tolerated and this regimen resulted in a median time to progression of 5 months (95% CI 3-6 months) and an overall survival of 9 months (95% CI 5-13 months).

1.2.3: Pre-operative Chemotherapy

The rationale for this approach relies on three hypotheses: neo-adjuvant therapy may lead to tumour down-staging and thus allow the surgeon to perform a complete (R0) resection. In addition, systemic therapy is delivered while the tumour blood supply is intact and therefore better oxygenated. This enhances the benefit of therapy. Finally treatment is given at the earliest time-point thereby addressing micrometastatic disease.

Multiple randomized trials have evaluated the role of chemotherapy given prior to resection in patients with localised (limited to the primary and regional nodes by clinical assessment) oesophageal cancer[18,19,20,21,22,23,24]. Only two of these studies, one of which has never been published, demonstrated a survival benefit[21,24].

The US Intergroup trial (0113) was one of the larger randomized studies. Four hundred and sixty seven patients with potentially resectable oesophageal cancer were randomly assigned to surgery alone, or three cycles of preoperative chemotherapy consisting of cisplatin (100 mg/m^2, on days 1, 29 and 58), and 5-fluorouracil (5-FU, 1000 mg/m^2 by continuous infusion, days 1 to 5 of each cycle), followed by surgery[18]. Patients with chemotherapy-responsive disease, who underwent potentially curative resection, received two further courses of post-operative chemotherapy with a reduced dose of cisplatin (75 mg/m^2) plus 5-FU.

The majority of patients had adenocarcinoma (55%) and outcomes were similar for both histological types. The clinical response rate to pre-operative chemotherapy was 19%. A complete pathologic response (pCR) was noted in five (2.5%) of 202 patients who received at least one cycle of chemotherapy. There were no differences between the groups in terms of complete resection rate (65 versus 66%), treatment-related mortality (6.4 versus 4.0%), median survival (14.9 versus 16.1 months), or survival at one, two, or three years (59, 35, and 23 versus 60, 37, and 26%), respectively.

In contrast, one of the other large randomized studies, suggested a survival *benefit* for pre-operative chemotherapy compared to surgery alone. This multi-centre trial from the Medical Research Council, UK, randomly assigned 802 patients with operable oesophageal cancer (67% adenocarcinoma) to resection alone, or resection preceded by two courses of cisplatin (80 mg/m^2 on day 1) and 5-FU (1000 mg/m^2 by continuous infusion days 1 to 4) given three weeks apart[24]. Pre-operative radiotherapy was administered at the discretion of the treating clinician and was received by 9% of the patients in each group. The number of patients who proceeded to surgery (92 versus 97%) and the curative resection rate (60 versus 54%) was similar for both groups.

Pre-operative chemotherapy was associated with significantly greater overall survival (hazard ratio 0.79, 95 percent confidence interval, 0.67 to 0.93), two year survival (43 versus 34%) and median survival (16.8 versus 13.3 months). The frequency of post-operative deaths and non-fatal complications were similar in the two groups. On examination of the resected specimen, tumours in the chemotherapy group were significantly smaller, extended less frequently into surrounding tissue, and showed less lymph node involvement. The frequency of

local recurrence as a component of failure was similar in patients undergoing chemotherapy compared to surgery alone (12 versus 11%).

Similar benefit was suggested in a Dutch multi-centre study, in which 160 patients with operable oesophageal squamous cell carcinoma were randomly assigned to surgery alone, or surgery preceded by two or four cycles of cisplatin (80 mg/m^2 on day 1) and etoposide (100 mg IV on days 1 and 2 and 200 mg/m^2 orally on days 3 and 5)[21]. Patients with a major tumour response received four cycles of chemotherapy before surgery, while non-responders underwent surgery after two courses. In a preliminary report, the clinical response rate in those who received pre-operative chemotherapy was 36% (25 of 69). Multimodal therapy was associated with a significantly longer median survival (18.5 versus 11 months). However, average follow-up was only 15 months, and the study has never been published in final form.

Not only have individual trials demonstrated a conflicting benefit for neo-adjuvant chemotherapy but so too have two meta-analyses. One detected no survival benefit at 1, 2 or 3 years[25]. The other reported a survival advantage, but this only reached statistical significance at 5 years (p=0.02)[26].

Despite this confusion it does, however, seem that neo-adjuvant chemotherapy may improve the long term outcome in a sub-group of patients; specifically those that respond well. There are currently no reliable means of identifying such patients at diagnosis.

1.2.4: Postoperative Chemotherapy

Unlike for many other solid tumour types, there have been no randomized trials using chemotherapy for patients with oesophageal adenocarcinoma. The one randomized study that administered chemotherapy to patients with squamous cell oesophageal cancer following surgery showed no improvement in outcome[27].

1.2.5: Pre-operative Radiotherapy

There have been several pre-operative studies, involving 100 or more patients, comparing pre-operative radiotherapy with immediate surgery. The total dose

employed varied from 20 to 40Gy and the majority of patients had squamous cell carcinoma. It is generally concluded that this approach confers no survival advantage[28].

1.2.6: Post-operative Radiotherapy

No survival advantage has been identified when radiotherapy has been administered following surgery[29,30].

1.2.7: Post-operative CRT

Post-operative has been offered to patients whose tumour extended to the surgical margin but there have been no large randomized studies to support this approach.

1.2.8: Pre-operative CRT

The radio-sensitising effect of administering chemotherapy and radiotherapy concurrently provided the impetus to evaluate CRT prior to resection, both to improve loco-regional control and affect distant micro-metastatic disease. At least six trials have directly compared pre-operative CRT followed by surgery and surgery alone for patients with potentially resectable oesophageal carcinoma; only two have demonstrated a significant survival benefit from combined modality therapy[31,32]. Two general approaches have been used: concurrent and sequential CRT.

1.2.8.1: Concurrent CRT

Two completed randomized trials have compared pre-operative concurrent CRT with surgery alone. In an Irish study, 113 patients with oesophageal adenocarcinoma were randomly assigned to surgery alone or surgery preceded by CRT[32]. Pre-operative treatment consisted of two courses of 5-FU (15 mg/kg by bolus days 1 to 5), and cisplatin (75 mg/m^2, on day 7 of each cycle), both administered during weeks one and six of concurrent radiotherapy (40 Gy in 15 fractions over three weeks). A complete pathologic response (pCR) was noted in 25% of patients treated with pre-operative CRT, and when the surgical specimens

were compared, regional nodal involvement was less frequent in this group (42 versus 82%). Combined modality therapy was associated with significantly longer median survival (16 versus 11 months) and three year survival (32 versus 6%). However, the results of surgery alone in this trial were inferior to other contemporary series[18].

In a second study from Michigan, 100 patients with loco-regional oesophageal cancer (25 squamous cell, 75 adenocarcinoma) were randomly assigned to trans-hiatal oesophagectomy with or without pre-operative CRT[31]. Neo-adjuvant treatment consisted of cisplatin (20 mg/m^2 per day, by continuous infusion days 1 to 5, and 17 to 21), 5-FU (300 mg/m^2 per day by continuous infusion on days 1 to 4 and 17 to 20), and vinblastine (1 mg/m^2 per day, by IV bolus, on days 1 to 4, and 17 to 20) plus concurrent radiotherapy (45 Gy in 1.5 Gy fractions, given twice daily for three weeks). Radiotherapy was administered using a three-dimensional (3D) conformal treatment planning technique. Surgery was performed on day 42, following a three week rest after the CRT.

A pCR was observed in 28% of patients receiving neo-adjuvant treatment. There was nearly a two-fold higher, but non-significant, improvement in three year survival for the combined treatment group (30 versus 16%). Despite this, at a median follow-up of 8.2 years, the median survival was similar for both treatments (16.9 versus 17.6 months for multimodality therapy and surgery respectively). Patients achieving a pCR had improved three-year survival compared to those with residual tumour in the resected specimen (64 versus 19%, respectively). Although the combined therapy group had a significantly lower loco-regional recurrence rate (19 versus 42%) pre-operative CRT had no effect on the distant metastatic rate (65 versus 60%). The study was statistically powered to detect a doubling of median survival from 12 to 24 months. Consequently, although this was a negative study, it was insufficiently powered to detect a lesser survival difference.

1.2.8.2: Sequential CRT

Two other completed trials compared sequentially administered chemotherapy and radiotherapy followed by surgery to surgery alone[19,33].

A Scandinavian study randomly assigned 187 patients with potentially resectable squamous cell oesophageal cancer to one of four arms: surgery alone, pre-operative chemotherapy (cisplatin and bleomycin), pre-operative radiotherapy, or pre-operative chemotherapy followed by radiotherapy and then surgery[19]. Three year survival was significantly higher in the two pooled groups receiving radiotherapy compared to those treated without radiation. However, when compared to the surgery alone arm, there was no difference in survival with sequential CRT followed by surgery.

Similar outcomes were noted in a trial from France, in which 86 patients with localised squamous cell oesophageal cancer were randomly assigned to sequential cisplatin plus 5-FU followed by radiotherapy and surgery, or surgery alone[33]. Although the median survival was no different between the groups, the dose of radiation was only 20 Gy, less than one-half of current standard doses.

A meta-analysis of the randomized trials comparing neo-adjuvant CRT followed by surgery with surgery alone included 1,116 patients enrolled on nine trials[34]. When compared to surgery alone, the odds ratios showed a non-significant trend towards improved survival with neoadjuvant CRT (0.79, 0.77, and 0.66 for one, two, and three-year survival, respectively); however, the improvement in three-year survival reached the level of statistical significance only when the analysis was restricted to those trials using concurrent chemotherapy and radiotherapy (OR 0.45, 95 % CI, 0.26 to 0.79). Although patients treated with surgery alone were significantly more likely to undergo resection, those receiving pre-operative CRT were more likely to undergo complete (R0) resection (OR 0.53, 95 % CI 0.33 to 0.84).

A second meta-analysis incorporated six randomized controlled trials (764 patients), all of which were included in the above analysis, comparing pre-operative CRT plus surgery versus surgery alone[35]. Most patients had squamous cell carcinoma, and in at least four of the six trials, radiotherapy and chemotherapy were given concurrently. When compared to surgery alone, pre-operative CRT again significantly improved three-year survival (OR 0.53, 95 % CI 0.31-0.93).

In the absence of a definitive trial, but with two small trials[31,32] demonstrating enhanced survival with neo-adjuvant concurrent CRT, investigators have more

recently focused on ways to intensify treatment. This has consisted of adding several cycles of induction chemotherapy prior to pre-operative chemoradiation[36], increasing the number of cytotoxic agents administered concurrent with radiation therapy[37], and adding adjuvant chemotherapy[38].

Although somewhat controversial, the use of this multimodal approach has increased outside of clinical trials, and the Patterns of Care studies in the US showed that preoperative CRT therapy increased from 10.4% during 1992-1994 to 26.6% in 1996-1999 for patients with stage IIb and III oesophageal cancer[39]. The same is now true in many European Centres, including Ireland.

1.3: The Pathological Response to Therapy

Many patients, however, do *not* benefit from such an approach and there are now evolving strategies to identify predictive response markers. Several analyses suggest that it is the *response* to preoperative therapy (particularly the absence of residual disease (pCR) in the surgical specimen) that best predicts disease-free and overall survival[20,31]. A pCR occurs in approximately 15–30% of cases, and three-year survival rates of approximately 60% irrespective of the applied protocol, type of histology and tumour stage are achieved[40]. A further subdivision of pathological response to neoadjuvant regimens, the tumour regression grade (TRG), may also identify patterns of incomplete response that may impact on treatment outcome[41]. Regression grading stratifies response based on the biological effect of radiation on tumours, dividing it into 5 different grades related to the ratio of fibrosis to tumour (Fig 1.i). Mandard et al described complete response as histologic fibrosis with or without inflammation extending through the different layers of the esophageal wall, but with no viable residual tumour cells (tumour regression grade (TRG) 1). Subtotal response (TRG 2) was characterised by the presence of rare residual cancer cells scattered through the fibrosis. An increase in the number of residual cancer cells, but with fibrosis predominating was termed a partial response (TRG 3). Minimal remission (TRG 4) showed residual cancer outgrowing fibrosis. Absence of any regressive changes (TRG 5) defined no change.

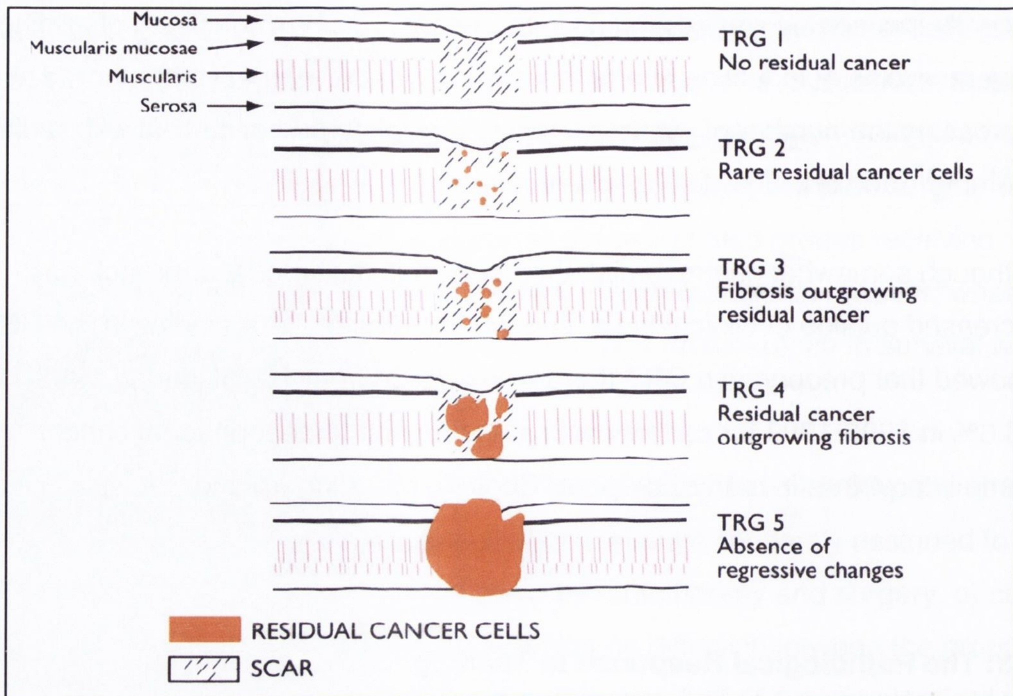


Figure 1.i: Tumour regression grading system (adapted from Mandard et al, 1994[41])

The addition of the pathologic response to standard pTNM has been recently advocated[42]. Where a cohort of patients may benefit from neoadjuvant CRT, with pCR and TRG the surrogate markers, many patients will not be helped, and their prognosis may be worsened by delay in surgery and by the added risks of surgery in patients on multimodal protocols[20,42]. A predictor of response or resistance based on pre-treatment demographics, imaging, histopathologic, molecular, or genetic information would have potentially enormous application in optimising outcomes and in the design of clinical trials. However despite numerous studies to date no clear candidate markers that predict pathological response have emerged.

1.4: PREDICTING RESPONSE

1.4.1: Conventional patient and histological indices

Numerous clinical and pathological parameters have been analyzed in a small number of oesophageal cancer studies with regard to their utility in the prediction of the response to pre-operative treatment. Mandard et al found that the larger the

initial primary tumour the poorer the overall response to neo-adjuvant treatment[41]. Pre-treatment performance status, primary location and age are clearly important factors in terms of tolerating therapy, but they are not known to be associated with the pathological response[43].

Other pre-therapy parameters that some have identified as potentially useful include the patient's nutritional status[44], tumour cell aneuploidy[45] and tumour differentiation[46].

In general, however, many of these factors tend to be relatively crude determinants of the overall management approach, i.e. curative or palliative, rather than being predictors of the molecular *response* to treatment. It would seem that it is the post-therapy pathological stage that best predicts the survival of patients who receive neo-adjuvant chemoradiation[43] and more precise markers are required in order to determine the most appropriate therapeutic strategy.

1.4.2: Tissue Markers

Most studies have correlated the expression of molecular markers in the pre-treatment biopsy with either the biological response to treatment in the oesophagectomy specimen or to survival/recurrence data following treatment. Markers have usually been identified by immuno-histochemical means.

1.4.2.1: Apoptosis

P53

The p53 gene is one of the most widely investigated in human cancer. Several groups have found that the protein it encodes is one of the prognostic indicators in various cancers[47,48]. It is one of the genes responsible for repairing a damaged cells' DNA or triggering apoptosis when this cannot occur (Figure 1.ii) and it is generally accepted that it may be intrinsically involved in the response to chemoradiation[49,50]. Several trials have studied p53 expression as a determinant of response to chemotherapy with or without radiotherapy in oesophageal cancer. In oesophageal adenocarcinoma, Duhaylongsod et al immuno-stained 42 patients for p53 and c-erb B2 protein. All patients received neo-adjuvant CRT followed by

resection[51]. They found that 84% of the p53 positive tumours had residual disease as opposed to 44% of the p53 negative (P=0.01). Similarly in patients with squamous cell carcinoma Seitz et al identified immuno-histochemically a significant association between p53 over-expression and a lower complete response[45]. To counter these results other groups have found no such an association[52]. It may be the small sample sizes or the differences in immuno-histochemical staining methods that explain these discrepancies. Equally it has been postulated that p53 over-expression is not necessarily synonymous with p53 mutations[53]. Furthermore absence of p53 staining may occur with gene deletion, failure of transcription, or a non-stabilizing mutation, all of which may be associated with loss of p53 function[54].

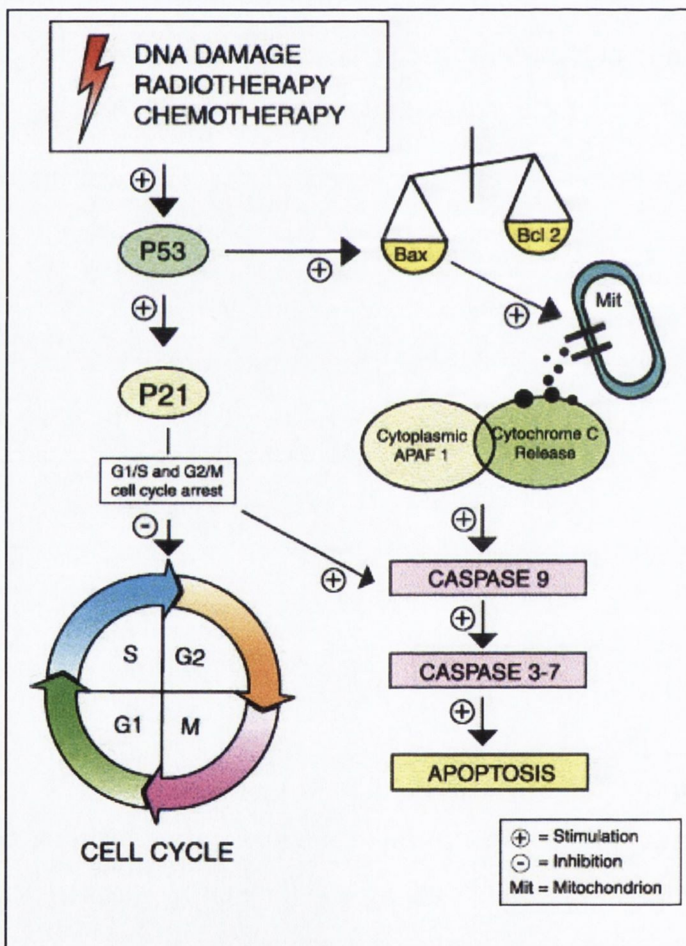


Figure 1.ii. Flow diagram of the P53/apoptosis pathway. Constituents of this pathway are the most commonly assessed predictive markers in oesophageal cancer.

P21

As illustrated in figure 2 the p21 protein is a key member of the p53 signalling pathway. It is transcriptionally activated by p53 following DNA damage by ionising radiation which in turn causes cell cycle arrest and apoptosis[55,56]. It has been studied as a response predictor because it disrupts regulatory networks, in particular those involved in cell death signaling. It may therefore be a causative factor of radio-resistance.

Nakamura et al found that the survival of patients with p21 positive oesophageal tumours treated with definitive CRT was significantly better than those where no such expression existed (p=0.0013)[57]. They also identified that the survival of those patients with p53 negative tumours was significantly higher if they were p21 positive than negative (P = 0.0452).

Conversely another Japanese group found that whilst p21 positive expression in the absence of p53 was associated with favourable effects from preoperative *chemotherapy* there was no such correlation between p21 expression and the clinical effects following *CRT*[58,59]

Bcl 2 and Bax

The Bcl 2 family (Figure 2.i), 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[60]. 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[61]. 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, culminating in 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 in oesophageal cancer. Despite some in vitro evidence suggesting that Bax expression may confer enhanced radio-sensitivity[62] and loss of Bcl-2

expression is associated with a worse survival[63] no other studies have demonstrated their value with respect to response prediction.

Survivin

Survivin is a member of the inhibitor of apoptosis family and is known to be involved in resistance to chemo- and radiation therapy. It is expressed in several cancers including oesophageal[64]. Kato et al found that that high survivin expression predicted a significantly reduced median survival (9.0 vs 30.0 months, p=0.0023) in patients receiving pre-operative chemotherapy. Whilst not correlated with any particular treatment, Grabowski et al also found that survivin expression was associated with a poorer survival[65]. Conversely other groups have found the reverse, with significantly better survival in those whose tumours over-expressed survivin[66].

1.4.2.2: Tumour Hypoxia

Hypoxic regions within tumours may lead to chemo- and radio-resistance by depriving cells of oxygen necessary for the cytotoxic activities of these agents[67]. Furthermore, tumour hypoxia promotes up-regulation of angiogenic and tumour cell survival factors resulting in increased proliferation, radio-resistance and angiogenesis. Angiogenesis has an important role in solid tumour growth and metastasis[68]. Vascular endothelial cell growth factor (VEGF) is the main angiogenic factor known to be involved in pathological angiogenesis. Its induction in several solid tumours is thought to be important with respect to the chemotherapy and radiotherapy response[69]. In one study CRT was administered to 52 patients with oesophageal squamous cell carcinoma[70]. Expression of p53, thymidine phosphorylase and VEGF was analysed by immuno-histochemistry. Sixty percent then underwent radical surgery and from these multivariate analysis identified that only VEGF was a significant prognostic indicator (p=0.0147). Its expression was associated with a high incidence of treatment failure and a significantly worse 5 year survival rate (p=0.037). These results are further supported by Gorski et al, who found that the anti-tumour effects of ionizing radiation could be enhanced if VEGF activity was blocked[71]. It remains unclear,

however, as to whether the expression of VEGF by itself directly or indirectly determines whether a tumour responds to CRT[72].

1.4.2.3: Growth Regulation

Epidermal Growth Factor Receptor (EGFR)

In squamous cell carcinoma Hickey et al compared tumour response with expression of EGFR and proliferating cell nuclear antigen[73]. There was a significant survival advantage in those staining negative for one or both markers, while those who stained positive responded poorly. This was further supported by Gibson et al who studied EGFR expression in both adenocarcinomas and SCC[74]. Increased EGFR expression predicted a poor overall survival ($p=0.009$)
c-erb B2

c-erb B2 protein is a 185kD transmembrane protein and a member of the EGFR family. It has been associated with the pathogenesis of several human cancers and in recent years extensively studied in breast cancer. Its over-expression in this site is associated with a poorer prognosis[75]. In oesophageal cancer the data specifically looking for its predictive value is limited. In adenocarcinoma Duhaylongsod et al found that over-expression predicted a favourable response to CRT and a 5 year actuarial survival of 60%[51]. In squamous cell carcinoma Akamatsu et al, however, found that immunostaining was useful for predicting chemoradioresisitance but this did not correlate with survival[76].

1.4.2.4: Cell Cycle Control

Cyclin D1

Sabria et al assessed cyclin D1 expression by immuno-histochemistry in squamous cell oesophageal cancer[77]. They identified that in patients treated with multi-modal therapy cyclin D1 expression correlated with a poor response to treatment but not to overall survival.

1.4.2.5: Markers of resistance to commonly used chemotherapy agents

Metallothionein (MT)

MT is a small protein with a high affinity for divalent heavy metal ions. It is involved in many patho-physiological processes, like metal homeostasis and detoxification, cell proliferation, apoptosis, therapy resistance, and protection against oxidative damage. Alterations in the immuno-histochemical expression of MT have been reported for various human tumours, and a high expression has been found to be associated with a poor clinical outcome[78]. Much of the work in squamous cell oesophageal cancer comes from Japan. MT-positivity in patients treated with neoadjuvant chemotherapy with or without radiotherapy has usually been associated with a worse prognosis[79]. Some studies have, however, shown no such association[80]. There is no data on the effects of MT-positivity and the response to treatment in oesophageal adenocarcinoma, but it is implicated in the malignant transformation of Barrett's epithelium[81] .

P glycoprotein (P-gp)

Several transmembrane proteins exist that act as an energy-dependent drug efflux pump. One family of these proteins includes the multi-drug resistance protein P-gp. Over-expression has been implicated in resistance to platinum and taxane chemotherapy and consequent decreased survival[80]. These agents are frequently used in the treatment of oesophageal cancer.

Nuclear factor-kappa B (NF-KB)

NF-KB regulates several genes involved in inflammatory, immune and apoptotic responses. In patients treated with neoadjuvant CRT for oesophageal adenocarcinoma Abdel-Latif et al identified that its' expression was inversely related to a major or complete pathological response. 75% of those that did not respond were NFKB negative, whilst only 18% of the responders were positive ($p<0.00001$)[82].

1.4.3: Serum Markers

Serum markers have not proved particularly useful in predicting the response of oesophageal cancer to neo-adjuvant therapy.
Kim et al evaluated serial CEA levels in 90 patients with potentially resectable oesophageal and gastric adenocarcinoma treated with preoperative chemotherapy[83]. Measurements were taken before treatment and serially thereafter. An increasing CEA level predicted relapse and correlated well with visceral involvement and clinical responses correlated with declining levels of CEA. However the levels did not predict resectability or survival.

Another group analysed serum VEGF levels in patients with oesophageal cancer before, during and after chemoradiation. Levels did not decline during therapy. They fell following resection but then rose to pre-operative values before falling to normal at three months. They postulated that the tumours were not generating VEGF and therefore levels could not be used as response markers[84].

Quillien et al examined the serum markers CYFRA 21–1, TPA and SCC in 96 patients with squamous cell oesophageal carcinoma. CYFRA 21–1 was the only marker whose pre-treatment levels significantly correlated with pathological response, but on multivariate analysis treatment was the only independent factor[85].

Nakamura et al assessed the clinical value of CYFRA 21–1 in comparison to SCC-Ag, CEA and CA19-9 in 112 patients with squamous cell carcinoma. Levels of CYFRA 21–1 correlated closely with stage and with clinical response to both chemotherapy and CRT[86].

These reports suggest that CYFRA 21–1 may be the most useful serum marker currently available, but this has not become widely adopted.

1.4.4: Gene Expression Arrays

Patients diagnosed with the same stage of cancer by conventional clinical and histopathological criteria may have a completely different course of disease. Since cancer is fundamentally a malfunction of gene expression giving rise to aberrant malignant growth, the most direct classification approach would be to analyse gene expression patterns. To find the relatively small number of genes that are characteristically de-regulated in a given cancer cell, among thousands of genes that are normally expressed, requires high-throughput technologies and sophisticated computational tools.

The first high density microarrays were developed to analyse gene expression by quantitating thousands of mRNAs present in a cell or tissue sample (DNA arrays). Other microarray approaches include the quantitation of proteins (protein arrays), or the analysis of a large number of tissue samples in parallel (tissue arrays). It was clear early on that arrays could be very useful tools in molecular profiling of cancer cells, thus revealing information that cannot be obtained by traditional histological assessment[87].

In recent years there have been numerous gene expression studies that have enhanced our understanding of the biology of oesophageal cancer[88,89,90,91,92,93,94]. It was hoped that these might identify potential biomarkers for therapeutic targeting. However, the studies did not specifically address treatment and pathological outcome data and so their clinical value is limited. There have been only two groups that have used gene expression analysis to identify oesophageal cancers with different outcomes and at the initiation of this project there were no expression profiling studies that focused on identifying predictive markers to CRT[95,96]. The first, from Japan, used microarrays to predict prognosis in 20 patients with squamous cell carcinoma who underwent surgery followed by adjuvant chemotherapy. Gene expression analyses identified 52 genes that were likely to be correlated with survival and possibly with sensitivity/resistance to cytotoxic therapy. Their data was validated using RQ-PCR. The other study from MD Anderson performed gene expression analyses on 19 patients prior to neo-adjuvant CRT and correlated their findings with the final histopathological response. Unsupervised hierarchical cluster analysis of the cancer biopsies

segregated them in to two molecular subtypes. Amongst the adenocarcinomas, most that achieved a complete response clustered in one group and all but one of the poorer responders in the other. They identified a number of genes that were differentially expressed between the two molecular sub-types.

1.4.5: Molecular Imaging

Alterations in tissue metabolism often precede anatomical changes and this forms the basis of ^{18}fluorodeoxyglucose (FDG) positron emission tomography (PET) scanning. The utility of PET in detecting and staging cancer is well established, and increasingly, it is being used to monitor treatment[97]. In 22 patients with advanced breast cancer, changes in FDG uptake was able to predict the eventual histopathologic response with an accuracy of 88% after the first course of drugs and 91% after the second course[98]. In another series of 30 women with large or locally advanced breast cancers, the mean pre-treatment dose uptake ratio (DUR) was significantly higher in the tumours that responded completely to chemotherapy, and there was a correlation between the amount of decline in the DUR and the extent of the tumour response. In this series PET scans, after the first course, were able to predict the eventual response with a sensitivity of 90% and a specificity of 74%[99].

Similar value for PET has been demonstrated in other cancers. A Pennsylvania group used PET to identify persistent head and neck cancer after radiotherapy[100] and Sakamoto et al from Osaka found that PET was more accurate in identifying responses of this cancer to radiotherapy with or without chemotherapy than were CT and MRI[101]. Similar utility has been described in cancers of the lung[102] and colon[103], as well as Hodgkin's[104] and non-Hodgkin's lymphoma[105]. In evaluating patients with lymphoma, PET may also have prognostic value in that parameters such as the tumour:normal-tissue contrast ratio correlate with the proliferative index[106]. In prostate cancer, in which PET generally is not useful because of the cancer's location Morris et al were able to distinguish active bony metastases from other types of bone lesions[107].

There has been comparable work in oesophageal cancer. Studies evaluating tumour response with PET during and at the completion of neo-adjuvant therapy have yielded encouraging results (Table 1.i). These studies suggest that changes

in FDG uptake in response to therapy correlate with the pathological response as well as predict the risk of local recurrence and survival. However many of these studies have small numbers and, in general, the second PET has been performed *after* the neo-adjuvant phase of treatment. Earlier response prediction could potentially differentiate responders from non-responders, minimise the inherent toxicity associated with current regimens and direct non-responders towards alternative therapies. At the initiation of this study no group had performed the second PET during CRT and until now there have been no similar studies in oesophageal adenocarcinoma.

Table 1.i: PET evaluation of response to pre-operative therapy for oesophageal cancer

Study + Reference	Pathology	Chemo	Radiotherapy	Second PET	Main Results
Couper et al, 1998[108]	14 AD	cis/5FU/ LV	No	During	67% with CT evidence of response had a reduction of >30% in FDG tumour:liver uptake ratios.
Brucher et al, 2001[109]	24 SCC	5FU	30Gy/15#	3 weeks after CRT	SUV reduction of >52% led to sensitivity, specificity, positive and negative predictive values of 100%, 55%, 72% and 100% respectively
Weber et al, 2001[110]	37 AD	cis/5FU/ LV	No	During	FDG uptake significantly different between responders and non-responders. When 35% reduction in uptake used to define response sensitivity 0f 93% and specificity of 95%
Kato et al, 2002[111]	10 SCC	cis/5FU	40Gy/20#	2 weeks after CRT	Pathological response did not correlate with rate of reduction of SUV
Arslan et al, 2002[112]	22 AD 2 SCC	See notes‡	40-50.4Gy/20-28#	4 wks after CRT	Change in volume identified responders. Quantitative evaluation of primary tumour pre and post therapy could not separate post therapy inflammation from residual tumour
Flamen et al, 2002[113]	27 SCC 9 AD	cis/5FU	40Gy/20#	4-6 weeks after CRT	When >80% reduction in FDG tumour:liver uptake ratios used to define response sensitivity 71% and specificity 82%
Downey et al, 2003[114]	26 AD 13 SCC	cis/taxol	50.4Gy/28# (2 had no RT)	After CRT (not specified)	SUV reduction >60% associated with non-significant disease-free and survival advantage compared to when reduction <60%
Kroep et al, 2003[115]	13 AD	cis/gem	No	During and at end (not specified)	FDG significantly reduced in responders vs non-responders. Early and late response evaluation showed a specificity of 86% and 100% respectively and a sensitivity of 100%
Brink et al, 2004[116]	13 AD 7 SCC	cis/5FU	36Gy/20#	2-3 weeks after CRT	No correlation
Swisher et al, 2004[117]	73 AD 10 SCC	See notes*	50.4Gy/28#	After CRT (not specified)	Pathological response correlated with post therapy SUV. Post therapy SUV >4 was only pre-operative factor to correlate with decreased survival
Wieder et al, 2004[118]	38 SCC	5FU	40Gy/20#	During CRT in 27	Changes in SUV were significantly different between 2 groups
Song et al, 2005[119]	32 SCC	cis/cape	45.6/38#(BID) + 46Gy/23#/5wks	4 weeks after CRT	Pathological response could be predicted when analysis limited to initial highly metabolic tumours

Key: AD – adenocarcinoma, SCC – squamous cell carcinoma, CT – computed tomography, 5FU – 5-fluorouracil, Gy – Gray, # - fractions of radiotherapy, cis – cisplatin, LV – leucovorin, carbo – carboplatin, cape – capecitabine, BID – twice daily radiotherapy fractionation, gem - gemcitabine

‡ Received cisplatin/5 fluorouracil or cisplatin/taxol or carboplatin/5 fluorouracil in combination with radiotherapy

*Patients received either irinotecan/5FU/docetaxol (up to 2 cycles) prior to CRT with same drugs (reduced dose) or same RT with cisplatin/5FU or taxol/carboplatin (no pre-CRT chemotherapy)

1.5: Conclusions

The management of patients with localised oesophageal cancer would be greatly enhanced if predictors of response could be identified, but it would seem there is little to be gained by studying conventional patient and histological indices.

At present none of the tissue or serum markers of response to neo-adjuvant treatment are sufficiently accurate on their own to be used to predict response in an individual patient. This is probably partially explained by the multi-factorial nature of carcinogenesis and the limited impact on the process caused by the presence or absence of a single molecular marker. Advances in microarray technology may mean that, by assessing the transcriptional activity of a large number of genes, the complex gene expression profile may contain more information than any individual molecule that contributes to it.

Molecular imaging is another evolving science. The cumulative data suggests that changes seen on serial PET scans after neo-adjuvant therapy correlate with the final pathological response and survival. However larger numbers are required and it would be more clinically beneficial if such imaging proved predictive earlier in the course of treatment.

A further level of uncertainty lies in the fact that the ability to predict response does not necessarily equate to an ability to predict prognosis in terms of local control or overall survival. This, however, is beyond the scope of the thesis.

CHAPTER 2

Optimising Methods for Oesophageal Tissue Collection, Storage and Homogenisation and RNA Extraction

2.1: SUMMARY

The initial phase of the project's laboratory component involved optimising techniques that would become necessary when analysing fresh oesophageal tissue. There are a number of commercially available techniques for tissue collection, storage and homogenisation as well as RNA extraction. Some of the more common techniques, as identified in the literature and available within the Trinity Laboratories, were studied. A number of experiments were performed on fragments of tissue removed following a routine oesophagectomy.

It was identified that, in order to maximise the chances of obtaining good quality RNA in sufficient quantity, tissue should be immediately placed into the fixative medium RNA*later*. It should then be refrigerated overnight before being snap-frozen at -80℃. The handheld homogeniser (Ultra-Turrax T8, IKA® Works, UK) was found to be the most convenient and successful for tissue disruption prior to RNA extraction.The most appropriate technique for extracting RNA was found to be the RNeasy™ Fibrous Tissue kit available from Qiagen, USA.

2.2: AIMS

Optimise Techniques for Tissue Collection, Storage and Homogenisation and RNA extraction

- Verify that high quality RNA could be extracted from pre-treatment biopsies
- Assess different means of tissue storage and their effects on RNA quality
- Assess different commercially available methods for RNA extraction
- Assess different methods available for tissue homogenisation

2.3: INTRODUCTION

Obtaining pure, intact RNA is important in a number of molecular biological techniques, and is fundamental for gene expression analyses. The main challenge in the isolation of high molecular weight RNA is that it is extremely labile and subject to degradation by ubiquitously expressed ribonucleases. These are stable, active enzymes that require no co-factors for enzymatic activity. Sample disruption and cell lysis in an environment that causes denaturation of ribonucleases is therefore essential. Of equal importance is appropriate decontamination of work surface areas, solutions and disposables.

Before proceeding with molecular analysis of the samples it was essential to optimise the means by which tissue was stored and homogenised. Furthermore it was necessary to assess the different commercially available means of RNA extraction in terms of resultant RNA quality and quantity.

2.4: METHODS

After obtaining written, informed consent a surgically resected oesophageal tumour was immediately transported from the operating theatre to the Department of Pathology, St James' Hospital. Under the supervision of a lead pathologist, 12 tumour biopsies (50-150mg) were taken.

2.4.1: Tissue Storage

Nine of the samples were divided into 3 groups consisting of 3 samples each and stored in one of the following ways:

Method 1: Samples were immediately snap-frozen in liquid nitrogen.

Method 2: Samples were immersed in an OCT^{TM}-filled cryo-mould before being snap-frozen.
OCT^{TM} (optimal cutting temperature) is a commercially available cryo-embedding media (Sakura Finetek, Torrance CA)

Method 3: Samples were immersed in RNA*later,* refrigerated, then snap-frozen after 24-48 hrs.
RNA*later* (Ambion, UK) is an aqueous, non-toxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA[120].

All snap frozen tumour samples were then stored at –80°C under a designated code in the department's BioBank.

2.4.2: RNA Extraction

Three commercially available kits were assessed:
Kit 1: Versagene™ RNA Purification Kit (Gentra Systems, MN, USA)
Kit 2: Qiazol™ Lysis Reagent Kit (**Qiagen, West Sussex, UK**)
Kit 3: RNeasy™ Fibrous Tissue Kit (Qiagen, West Sussex, UK)

Procedure

Versagene™ Kit
One sample from each storage method group underwent RNA extraction using the Versagene™ kit. Briefly, samples were weighed and added to proprietary lysis solution (404µl per 0.5-40 mg tissue). They were then homogenised by ball bearings in a Mixer Mill (MM300 Qiagen, USA) for 2 minutes. The homogenate was passed through a clear column to remove large particles and subsequently through a series of on-column RNA-binding, centrifugation and wash steps. Finally, extracted RNA was eluted from the column and stored under a designated code in the –80°C BioBank.

QIAzol™ Kit
One sample from each storage method group underwent RNA extraction using the QIAzol kit. Briefly, 50-100mg of tissue was weighed then added to 1ml of TRI reagent supplied with the kit. This was then homogenised by ball bearings in a Mixer Mill (MM300, Qiagen, UK) for 2 minutes. The homogenate was transferred to a new tube and 0.5ml isopropanol per 1ml of TRI reagent was added to it. After

standing for 10 minutes at 4^0C, centrifugation was performed for 10 minutes at 12,000 g. This resulted in the formation of a pellet of pure RNA. Excess liquid was decanted leaving the RNA pellet in situ. The tube was then air-dried for a further 5 minutes before being re-suspended and stored under a designated code in the BioBank.

RNeasy™ Fibrous Tissue Kit

One sample from each storage method group underwent RNA extraction using the RNeasy™ Fibrous Tissue Kit. Approximately 30mg of tissue was weighed before being homogenised by ball bearings in a Mixer Mill (MM300 Qiagen, UK) for 2 minutes in 300µl proprietary buffer RLT. 10 µl proteinase K and 590 µl RNAse-free water were then added to the homogenate and incubated at 55^0C for 10 minutes. Subsequently on-column RNA-binding was performed before DNAse (10µl DNAse in 70µl Buffer RDD) was applied directly onto the column membrane for 15 minutes at room temperature. Subsequently centrifugation, wash and RNA elution steps were performed. Finally, extracted RNA was diluted in 50µl RNAse-free water and stored under a designated code in the BioBank.

The extracted RNA was analysed using both the NanoDrop® ND-100 v3.0.1 Spectrophotometer (NanoDrop Technologies, Montchanin, USA) and the Agilent 2100 Bioanalyzer/RNA 6000 LabChip® kit (Agilent Technologies, Waldbrann, Germany).

RNA quality was deemed good if the 260:280[a] ratio was ≥2.0 and the 260:230[b] ratio was 1.8-2.2 on the NanoDrop® spectrophotometer and if there was minimal degradation and a 28s:18s ribosomal RNA band ratio of ≥1.3 as measured by the Agilent® Bioanalyzer. Any result deviating from this was deemed poor.

[a]260/280: This is the ratio of sample absorbance at 260 and 280 nm. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA and RNA. A ratio of ~1.8 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA[121]. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

[b]260/230: ratio of sample absorbance at 260 and 230 nm. This is a secondary measure of nucleic acid purity. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. They are commonly in the range of 1.8-2.2. If the ratio is appreciably lower, this may indicate the presence of co-purified contaminants.

2.4.3: Homogenisation

The 3 remaining biopsies were homogenised by one of the following means:

Sample 1: Using ball bearings in a Mixer Mill (MM300 Qiagen, USA)
Sample 2: Using a pestle and mortar.
Sample 3: Using a handheld homogeniser (Ultra-Turrax T8, IKA® Works, UK).

Procedure
All 3 techniques were carried out under a laminate flow hood.

Mixer Mill

Tissue samples were placed into a 1.5ml flat-bottomed tube containing a sterile, stainless steel ball bearing and 1ml of QIAzol lysis reagent. The tube was closed and homogenised at full speed for 2 minutes. Once settled, the bottom of the tube was examined for remaining, undisrupted tissue. If present, a further 2 minutes of homogenisation was performed.

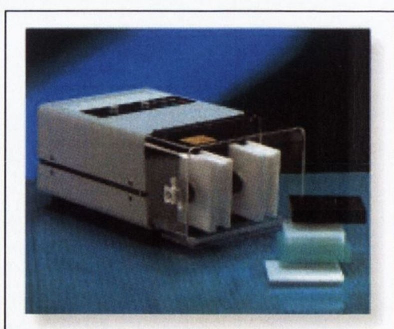


Figure 2.i: The Mixer Mill 300

Pestle and mortar

This technique was performed under a laminate flow hood. Before use, the porcelain pestle and mortar were thoroughly cleaned using RNAzap solution (Qiagen, USA) and cooled with liquid nitrogen. The tissue fragments were then added and maximally ground with the mortar. All ground tissue was then placed directly into a spin column.

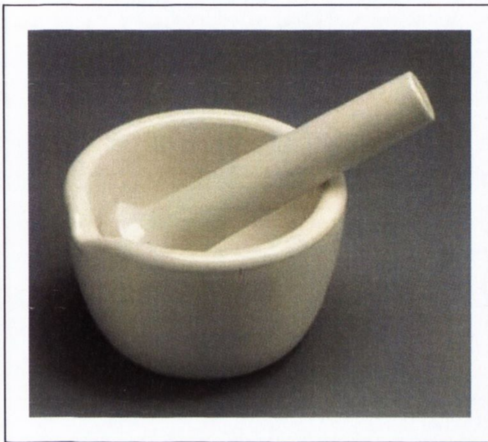


Figure 2.ii: The Pestle and Mortar

Hand held homogeniser (HHH)

This technique was performed under a laminate flow hood. The HHH was taken through a sequence of washes in 100% ethanol, RNaseZap (Ambion) and distilled water. The tissue fragments were then placed into a flat-bottomed tube containing 1ml of QIAzol lysis solution, which had been pre chilled for 5 minutes on ice. Homogenisation was performed for 30 seconds, ensuring that the tube was kept immersed in ice throughout. The contents of the tube were allowed to settle and the base of the tube examined for residual tissue fragments. If present, a further 30 second homogenisation was performed. Once complete, 200µl chloroform was added to the tube, agitated and left at room temperature for 5 minutes before being centrifuged at 16000 g for 10 minutes. Clear lysate was then added to the spin column.

RNA was subsequently extracted using the most reliable method identified in earlier experiments and analysed using both the NanoDrop® ND-100 v3.0.1 Spectrophotometer (NanoDrop Technologies, Montchanin, USA) and the Agilent 2100 Bioanalyzer/RNA 6000 LabChip® kit (Agilent Technologies, Waldbrann, Germany).The same quality criteria as before (section 2.4.2) were applied.

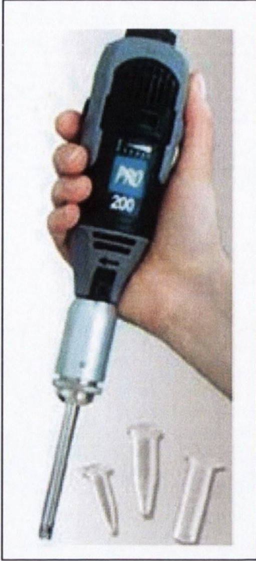


Figure 2.iii: The Hand Held Homogenizer (HHH)

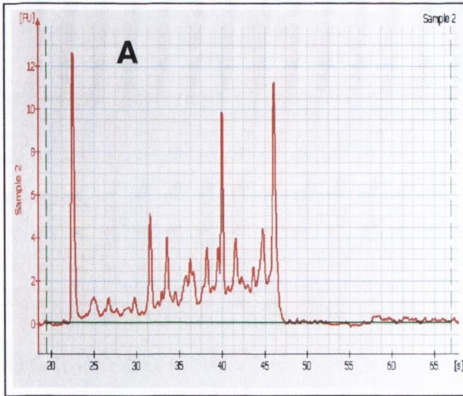
2.5: RESULTS

2.5.1: Tissue Storage and RNA Extraction Techniques

For samples that were snap frozen in liquid nitrogen only the Versagene™ extraction kit resulted in good quality RNA. It was not possible to obtain good quality RNA from any of the tissue that had been stored in OCT. Good quality RNA was extracted from all the samples that had been collected in RNA*later* and refrigerated overnight (Table 2.i).

Table 2.i: RNA quality comparisons for 3 different storage techniques and extraction methods

	Versagene™	**Qiazol™**	**RNeasy™**
Liquid Nitrogen	GOOD	POOR	POOR
OCT	POOR	POOR	POOR
RNA*later*	GOOD	GOOD	GOOD



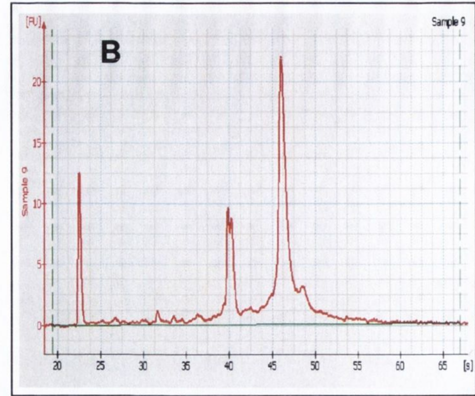


Figure 3.iv: Poor (A) and Good (B) quality RNA traces from Agilent® 2100 Bioanalyzer

2.5.2: Tissue Homogenisation

Although the quality of RNA extracted by each technique was comparable, there was an almost two-fold increase in the concentration of RNA extracted by pestle and mortar or HHH compared to the Mixer Mill (Table ii).

Table 2.ii: RNA quality/quantity comparisons for 3 different homogenisation techniques

	Mixer Mill	**Pestle and mortar**	**Hand held homogenizer (HHH)**
RNA Quality	GOOD	GOOD	GOOD
RNA Quantity	124 ng/µl	218 ng/µl	238 ng/µl

2.6: DISCUSSION

It is appreciated that the optimisation experiments were performed with a small number of samples. It is possible that had these steps been performed on multiple samples different results may have been obtained. However the primary objective of these studies was to define standardised operating procedures. These would become essential for later gene expression array studies. Furthermore, in the absence of previous experience, each step served to provide a useful introduction to basic laboratory techniques as well as an understanding of some of the difficulties associated with RNA extraction. The methods used for each step were chosen because of their commercial availability and because they appeared to be the most frequently used in similar previously published studies.

2.6.1: Tissue Storage and RNA Extraction Techniques

RNA*later* was the most reliable means of storing tissue designated for RNA extraction and this became the medium in which all such tissue was collected. It's only drawback to use in routine practice is that it would ideally needed to be available in the theatre/endoscopy suite and used before its expiry date. OCT was clearly the least useful and subsequently altered departmental practice. Whilst the Versagene™ kit proved successful in generating good quality RNA from both liquid nitrogen and RNA*later*-stored samples it was far less user-friendly than the RNeasy™ kit. It involved a number of complex steps and the potential for human error was greater. In any case the RNeasy™ kit was being used widely in other laboratories locally as well as in seminal microarray publications[89].

2.6.2: Homogenisation

There was little difference, in terms of RNA quality between the three tools assessed. The Mixer Mill technique, however, generated much less RNA and was not adopted for this reason. The HHH was easier to use than the pestle and mortar. The latter technique was both labour intensive and potentially hazardous; liquid nitrogen had to be added to the biopsy material on several occasions and occasionally tissue fragments were expelled from the mortar. As a consequence the HHH method was adopted for oesophageal tissue homogenisation.

CHAPTER 3

Gene Expression Analysis and Correlation with Pathological Outcome to Neoadjuvant Chemoradiation in Oesophageal Cancer

3.1: SUMMARY

The use of cDNA microarrays is a powerful method for the quantitative analysis of disease-specific gene expression. They can detect altered expression profiles associated with the pathology or altered biology of a disease entity. This study was performed to identify different genetic profiles associated not only with benign and malignant oesophageal tissue but also with response to neo-adjuvant chemoradiation in the multi-modal management of the disease.

Gene expression profiles for 13 malignant and 11 benign oesophageal samples were compared using a whole genome microarray system from Applied Biosystems. The malignant samples were then sub-divided according to the response to chemoradiation and a further analysis undertaken.

3.2: INTRODUCTION

3.2.1: Microarrays

DNA microarrays represent an important new tool for analyzing human tissue. It is generally believed that thousands of genes and their products in every living organism function in a complex and orchestrated way. An understanding of this would provide the potential for improving a wealth of diagnostic and therapeutic modalities. Traditional methods in molecular biology generally work on a "one target one experiment" basis, which means that the throughput is very limited and the whole picture of gene function difficult to ascertain. Microarray technology enables investigators to measure the expression of several thousand mRNAs simultaneously in a biological specimen. The gene density of microarrays in current use ranges from several thousand to over 30,000 unique human sequences[122] and represents a dramatic increase in throughput[123].

The development of the first array is credited to Mark Schena and colleagues at Stanford University in the early 1990s[123]. While studying plant transcription factors they conceived a strategy to manufacture microscopic arrays containing plant gene sequences attached to a glass substrate and used these “microarrays” to quantify gene expression in hybridization experiments with fluorescently-labeled plant mRNA samples.

A microarray is an ordered array of microscopic elements on a planar substrate that allows the specific binding of genes or gene products. It provides a medium for matching known and unknown DNA samples based on base-pairing rules and automating the process of identifying the unknowns. DNA microarrays are made by high-speed robotics on a solid matrix/substrate, for which probes with known identity are used to determine complementary binding, thus allowing parallel gene expression and gene discovery studies. The sample spot sizes in the original experiments were less than 200µm in diameter and these arrays contained several thousand spots. Current diameters can be as small as 4µm.

The early experiments were performed using cDNA microarrays. These are made by spotting cDNAs (usually 500-2500 base pairs) onto glass slides. The mRNA samples are reverse transcribed or, if the starting amount of RNA is low, one or more rounds of in vitro transcription/reverse transcription amplification can be performed. During the reverse transcription step fluorescence-labeled nucleotides are incorporated. Different dyes are used to distinguish between the sample of interest (tumour mRNA) and the control sample (e.g. normal tissue mRNA). Both are mixed together and hybridised to the cDNAs on the array. The ratio of fluorescence intensities between the two dyes in one spot determines the extent of up or down-regulation of a gene. The main advantages of cDNA arrays are their low cost and the flexibility in the design of the chips. However in view of the large number of base pairs cDNA arrays are susceptible to unspecific hybridization and cross-talk between similar genes. There has subsequently been a trend towards the use of oligonucleotide applications. Oligonucleotides are single-stranded 15-70 nucleotide molecules and are produced by chemical synthesis. These synthetic targets provide for high levels of specificity during hybridization reactions without overtly decreasing signal strength.

In the last 10 years a plethora of microarray devices has become available. With respect to expression analysis the field has been dominated by two major technologies: pre-fabricated oligonucleotide arrays and spotted oligonucleotide arrays.

3.2.1.1: Pre-fabricated arrays (Affymetrix™ GeneChips)

Affymetrix™ Incorporated GeneChip technology have the monopoly in this area. The gene array process combines photolithography and combinatorial chemistry (Figure 3.i). Up to 40 separate oligonucleotides are used for the detection of each gene. They include both perfect match and "mismatch" oligonucleotides. The mismatch probe is used to detect and eliminate any false or contaminating fluorescence within that measurement. The mismatch probe serves as an internal control for its perfect match partner because it hybridizes to nonspecific sequences allowing spurious signals, from cross hybridization for example, to be efficiently quantified and subtracted from a gene expression measurement or genotype call.

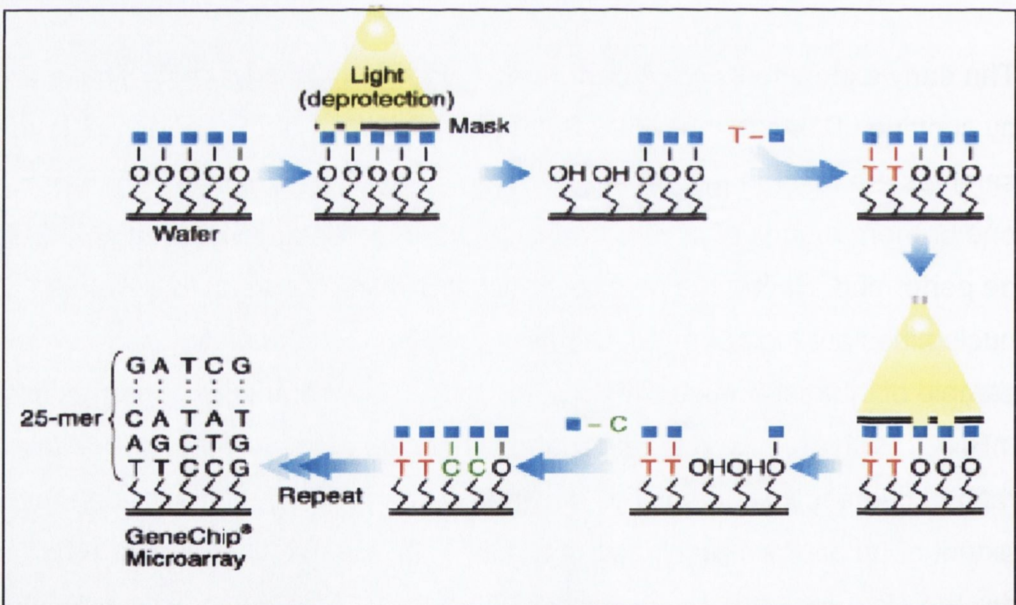


Figure 3.i: Photolithographic generation of oligonucleotide microarray

In order to detect hybridized target mRNA on an array it is necessary to label it with a fluorochrome. Early array design required control and sample RNA to be differentially labeled with two different fluorochromes and hybridized on the same array. The Affymetrix system, however, uses an RT-IVT amplification and labeling step to incorporate biotin-NTPs into the resultant cRNA. Following hybridisation

and appropriate washing steps the array is stained with Streptavidin-Phycoerythrin. The signal can then be amplified using goat IgG and biotinylated antibody.

3.2.1.2: Spotted Arrays

The other most commonly used microarrays are spotted microarrays. The probes are manufactured separately from the arrays and may consist of cDNA, PCR products or oligonucleotides. Microscopic quantities of probe are typically spotted onto the arrays robotically. Probes are usually fixed to the surface by non-specific binding to polylysine-coated slides. Following robotic spotting, the remaining exposed amines of polylysine are blocked with succinic anhydride and the DNA is denatured by heating.

The advantage with this system is that any designed probe can be spotted on the array. Probes can be quality-controlled prior to spotting meaning it is therefore easier to determine whether they have been correctly synthesized prior to the running of an experiment. It is also able to choose whether to perform the experiment in a single or dual-label format. Spotting will, however, not be as uniform as the *in situ*-synthesised Affymetrix chips and the cost rises as the number of probes increase.

3.2.2: Applied Biosystems Expression Array System

The Applied Biosystems Expression Array System was used in this study. It relies on spotted arrays, but combines chemiluminescent detection with improved probe design to provide an average sensitivity of 0.5 copies per cell. This compares to the one to three copies afforded by most fluorescent platforms. The system has excellent on-line support and experienced users work within the Trinity College laboratories.

The system is based on a design that represents the whole human genome, utilises current transcript data and relies entirely upon gene annotations that have been validated by experts in human curation. Each probe is part of a relational database that includes both Celera Genomics annotations and those in the public

domain. Combined with specially developed chemiluminescent chemistries, this complete system delivers greater probe and detection sensitivity than previous generations of microarray systems. In addition annotation information for all the 29,098 human genes that are represented on the microarray is included in an Oracle® database that is provided with the AB1700 system. The manufacturers claim that the result is a complete system capable of rapid and accurate analysis of microarray data for gene expression research.

The expression array system consists of an analyser (Applied Biosystems 1700 Chemiluminescent Analyser) that can image arrays in chemiluminescence, to survey and measure the gene expression at very low levels and in fluorescence, to locate and auto-grid features. The 1700 is equipped with a high resolution, large format CCD camera (Figure 3.ii). The cooled CCD is back-illuminated for high efficiency and has very low read noise. This, coupled with the low background from chemiluminescence, results in very high sensitivity, compared with other microarray techniques.

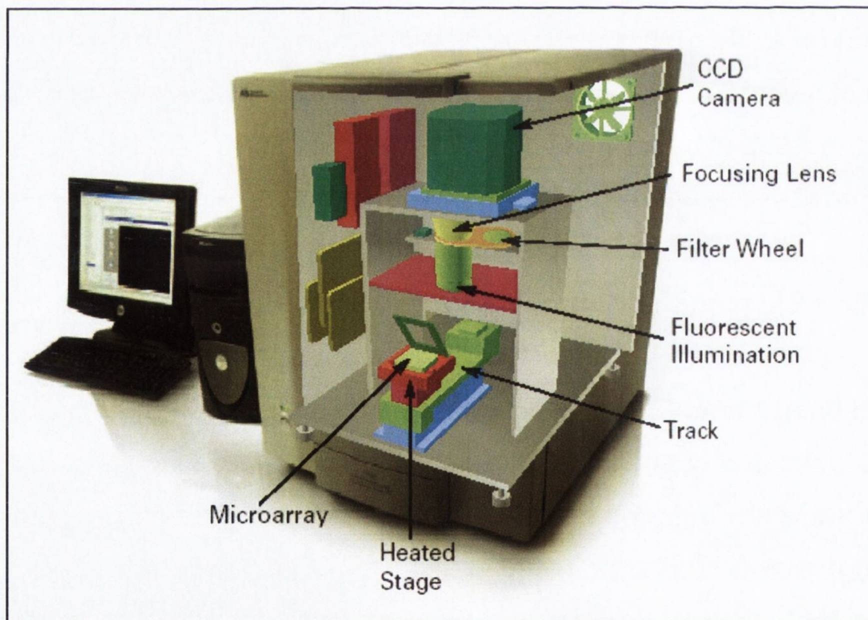


Figure 3.ii: The Applied Biosystems 1700 Chemiluminescent Microarray analyzer

3.2.2.1: Printing of the probes onto the nylon matrix

The microarrays are sealed in pre-assembled cartridges (Figure 3.iii) and contain oligonucleotides with a feature diameter of less than 180 micrometers, and a

space of greater than 45 micrometers (edge-to-edge) between each feature. The oligonucleotides target transcripts in each gene of the human genome. Oligonucleotide probes are synthesized at Applied Biosystems and designed to ensure maximal specificity. Prior to microarray manufacture, all probes undergo analysis by mass spectrometry for quality control. All Applied Biosystems microarrays utilize 60-mer oloigonucleotides (oligos) as DNA probes. Oligos of this length offer the best combination of sensitivity and specificity when compared to microarrays containing either shorter oligos or cDNA probes. 60-mer oligos offer the good single-base hybridization specificity that is expected from shorter oligos, and the strong sensitivity of longer fragments expected from cDNA arrays.

Figure 3.iii: Pre-assembled microarray cartridges

Results may be achieved from as little as 500ng of starting total RNA by using the Applied Biosystems Chemiluminescent RT-IVT Labeling kit. The chemistry of the RT-IVT kit, which exploits the Eberwine[124] linear amplification procedure, increases the yield of cRNA from cDNA by more than a thousand fold. The reverse transcriptase incorporates de-oxynucleotides and digoxigenin-dUTP (DIG-dUTP) in the synthesis of single-stranded cDNA from sample RNA and RT Labeling Control RNA. The reverse transcriptase used in this reaction is a modified version of M-MLV reverse transcriptase. The modified reverse transcriptase has no RNase H activity. It also provides longer cDNA transcripts and higher yields that the wild type enzyme. Multiple transcription rounds result in the production of DIG-labeled cRNA (Figure 3.v). The resultant digoxigenin-labeled cDNA or cRNA is specifically hybridized to the Applied Biosystems microarray.

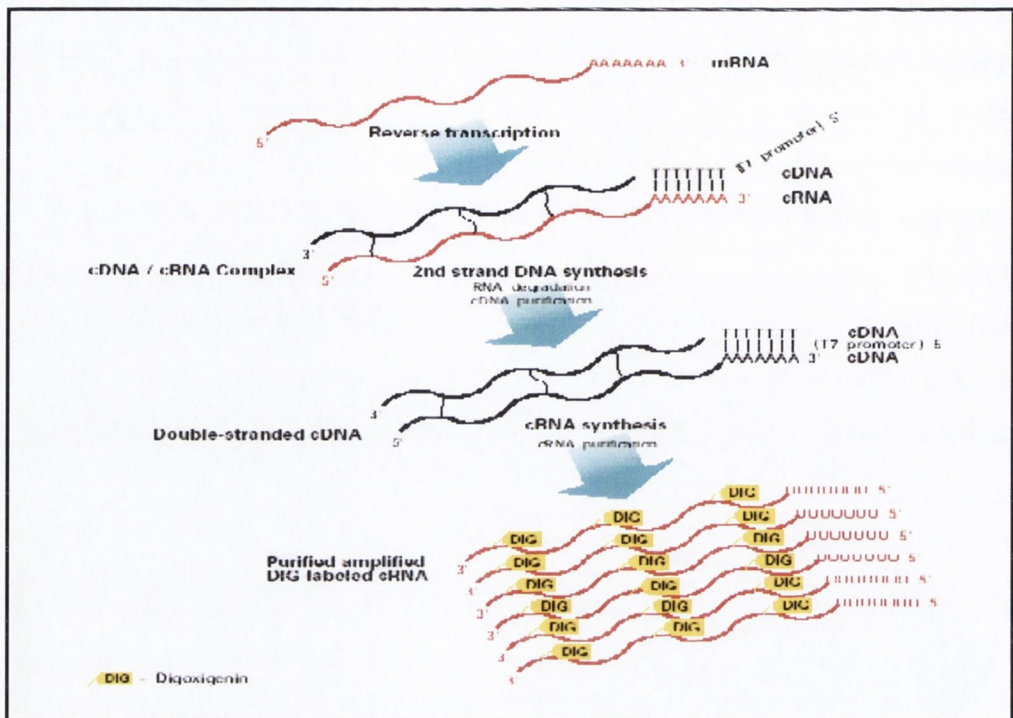


Figure 3.iv: RT-IVT process.

When unbound materials have been washed from the microarray, the Applied Biosystems Chemiluminescence Detection kit is used to visualize features that have digoxignenin-labelled cDNA or cRNA bound to the oligonucleotide probes. Visualization is achieved by incubating the microarray with an anti-digoxigenin alkaline phosphatase conjugate. Alkaline phosphatase hydrolyses a chemiluminescent substrate and emits light at a wavelength of ~458nm. The signal intensity is proportional to the mRNA level expressed in the cells (Figure 3.v)

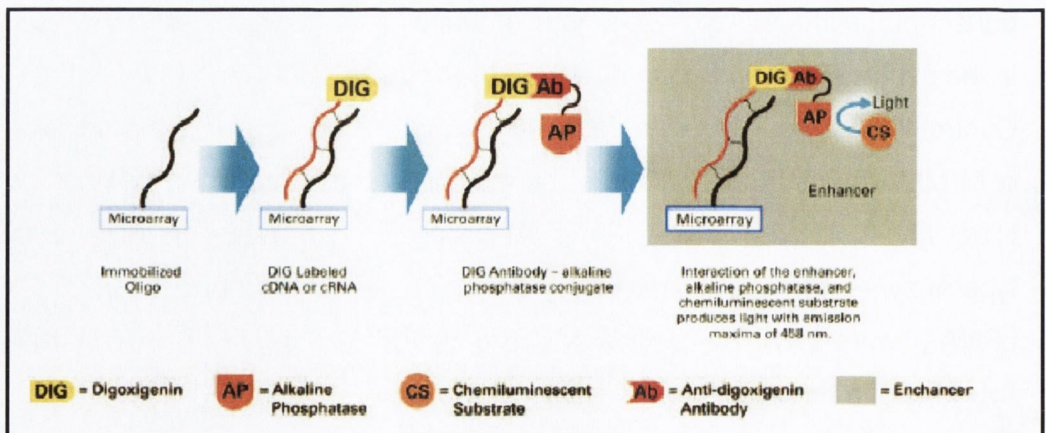


Figure 3.v: Chemiluminescence Detection kit reports the hybridization of DIG-labeled cRNA to probes that are attached to the microarray.

In addition to the 60-mer unique probe an internal control probe (a 24-mer oligo) is co-spotted on the microarray. At the hybridization step a complimentary oligo, pre-labeled with the fluorescent LIZ® dye, is included within the hybridization mixture. The fluorescent signal, which has a close spatial correlation with chemiluminescent signal, locates all features on the microarray, even in the absence of gene expression products (Figure 3.vi).

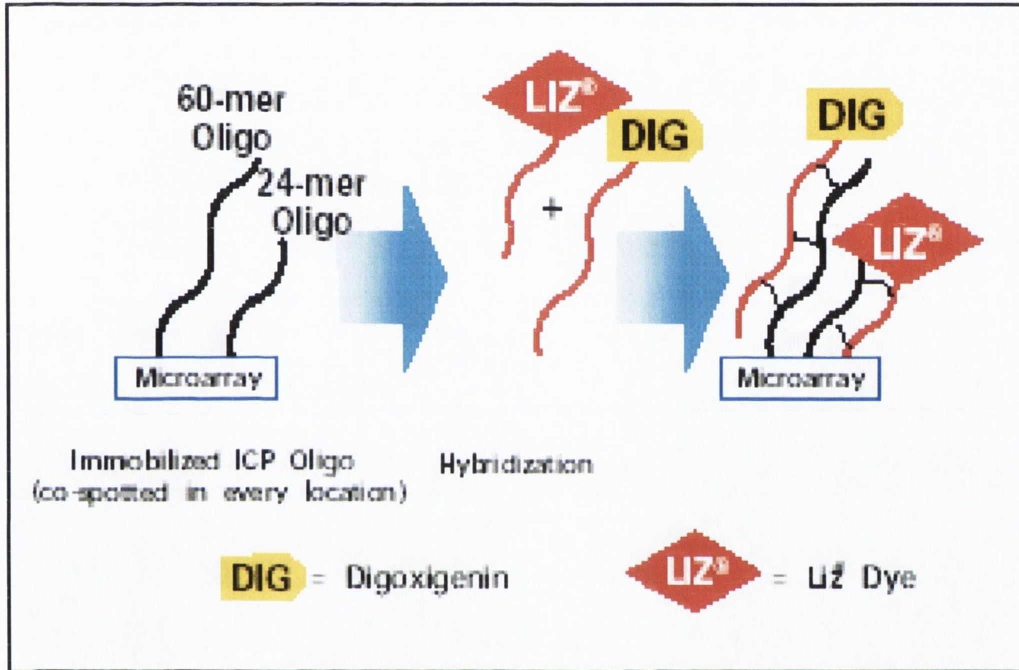


Figure 3.vi: Fluorescence oligos used to image the microarray features

A motorized chassis moves the microarray into the optical path of the analyzer to detect chemiluminescent and fluorescent signals. System software can then relate the resultant intensities to gene expression, accurate feature registration and data quality control. When the microarray is loaded into the 1700 analyzer the temperature is raised to 35°C to allow the enzymatic reaction to reach a steady state. Multiple images are taken to bring the microarray into focus and to measure the chemiluminescent and fluorescent signals.

The microarray is imaged in both short (5 seconds) and long (25 seconds) read times to extend the linear dynamic range of the chemiluminescent signals (>1000 fold). There are two imaging areas for each microarray. The total time required to image a microarray is approximately 12 minutes. Light production on the microarray reaches a steady state within the first five minutes, while the microarray

is being focused and brought up to 35℃. The chemiluminescent reaction emits light at a steady state for at least 60 minutes thereafter. The absence of an excitation background, together with the highly reproducible photon emissions, makes the signal-to-noise ratio produced from equivalent hybridizations superior to that found in alternative array systems.

Chemiluminescent and fluorescent fiducials are features that monitor data quality and analysis, and are built into every Applied Biosystems microarray. Labeled oligonucleotides (DIG and LIZ® dye-labeled oligos) in concentration ladders ranging over 500-fold are deposited on the microarray during manufacture. The resident software is able to recognize these features and uses them to monitor the efficacy of the chemiluminescent chemistries and the efficiency of fluorescent detection (figure 3.vii).

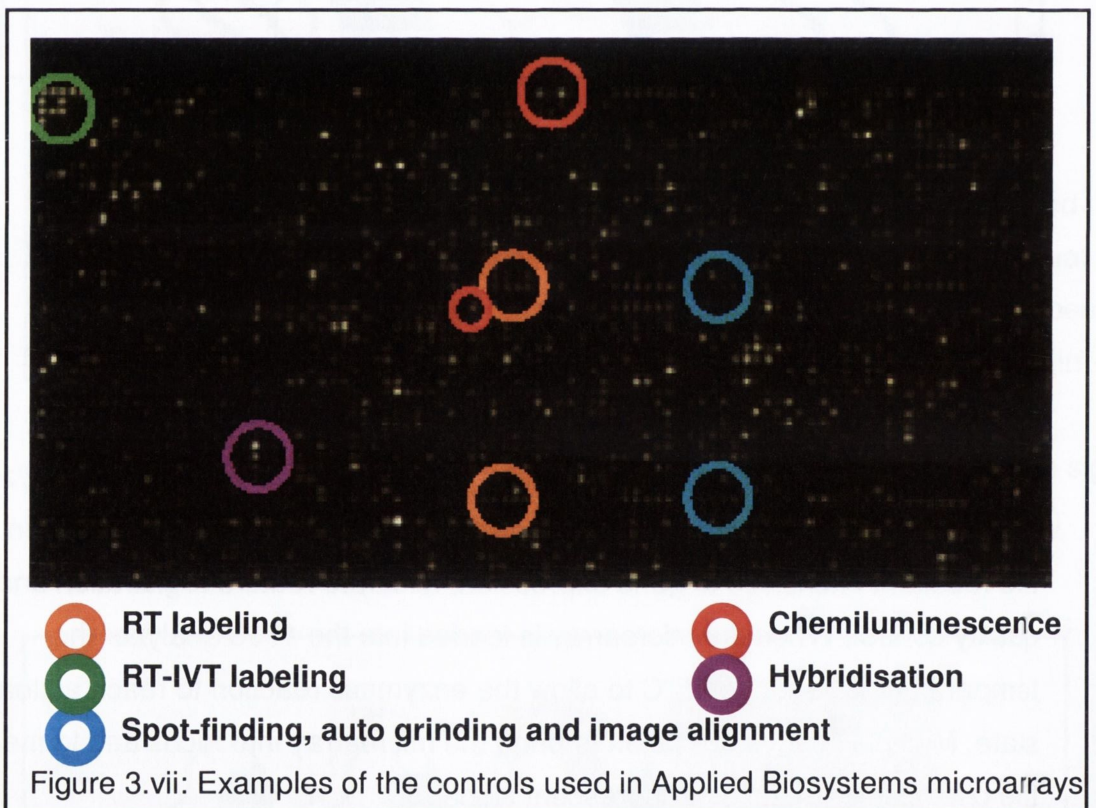


Figure 3.vii: Examples of the controls used in Applied Biosystems microarrays

Labeled oligonucleotides are included with the hybridization controls in the Applied Biosystems Chemiluminescence Detection kit. These oligonucleotides are complementary to those deposited on the microarray during manufacture. They monitor hybridization conditions, such as sample mixing and washing stringency, providing protocol diagnostics and ensuring signal uniformity (Figure 3.vii).

Finally, labeling kit controls are included with both the Applied Biosystems Chemiluminescence RT Labeling kit and the RT-IVT Labeling kit. They are synthetic bacterial control genes (*Dap, Lys, Phe, BioB, BioC* and *BioD*). These controls provide quality information on RT and RT-IVT kit enzyme activity and DIG-label incorporation efficiency for each experiment.

3.3: AIMS

Quality assurance of the materials and methods used

To identify genes differentially expressed by benign and malignant oesophageal tissue

To identify genes that predicted the response to chemoradiation

3.4: MATERIALS and METHODS

3.4.1: Selection of Samples for Gene Expression Analyses

Between July 2003 and June 2005 all patients referred to St James' Hospital, Dublin with a presumptive diagnosis of operable oesophageal cancer provided informed consent. This allowed the department to collect and store representative samples of their tumour and normal tissue. Pre-treatment investigations included computerized tomography of the neck, thorax and abdomen, and oesophago-gastroscopy. The criteria for inclusion in the multimodal protocol was as follows: age < 77; satisfactory performance status and medical fitness for surgery ; a biopsy proven tumour of the oesophagus or oesophagogastric junction; and a staged tumour deemed resectable by the primary surgeon. All patients had, in addition, a leukocyte count greater than 3500 per cubic millimetre, a platelet count above 100,000 per cubic millimetre, serum creatinine less than 1.4 mg per decilitre (124µmol per litre), no previous chemotherapy or radiation therapy, and no previous cancer other than of the skin.

3.4.2: Neo-adjuvant Protocol

The pre-operative treatment protocol was with 3 to 4 weeks of radiation therapy, the first combined with chemotherapy, further chemotherapy alone in week five, and surgery approximately one month later (figure 3.viii). For radiotherapy the planning target volume (PTV) incorporated the gross tumour volume (GTV) plus a 4-5cm margin superiorly and inferiorly with 2cm circumferentially. This was identified using the information gained from the endoscopy and diagnostic CT scans, as well as by the use of a barium swallow during simulation. The dose of radiotherapy was 44 Gray in 22 daily fractions over four and a half weeks prior to 2004. Since then a dose of 40.05Gray in 15 daily fractions over 3 weeks has been utilised. Each dose was prescribed to the mid-plane using 10-15 megavoltage photons. 5-Fluorouracil (5-FU) at 15mg/kg was delivered on days 1 to 5 and cisplatin at $75mg/m^2$ on day 6. If the glomerular filtration rate was less than 60mls/min or if significant nephro-/neurotoxicity developed during treatment, carboplatin (AUC5) was substituted for cisplatin. A similar modification occurred if the patient had significant hearing impairment as assessed by audiometry.

3.4.3: Surgery

Surgery involved trans-thoracic oesophagectomy including en-bloc lymphadenectomy of the abdominal and mediastinal nodes, and it was not undertaken until the neutrophil count was consistently above 2000/µl on three successive occasions in a two week period.

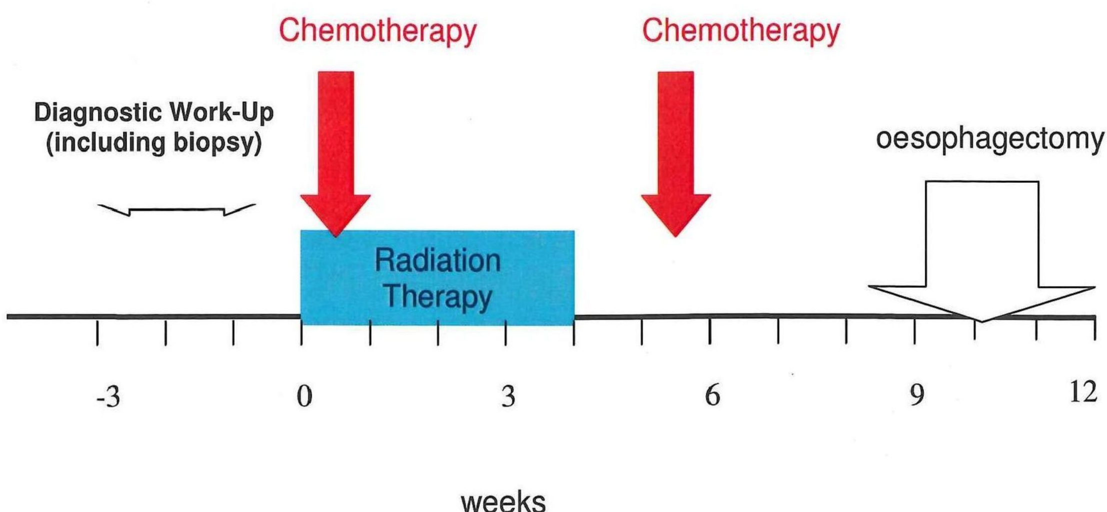


Figure 3.viii: Time-line for the various stages of the multi-modal protocol

3.4.4: Histology

All the surgical specimens were classified by one experienced pathologist who was unaware of the clinical data and who graded and staged the specimens in accordance with the criteria of the International Union Against Cancer and the American Joint Committee on Cancer Staging[125]. Tumour response to treatment was classified according to the criteria described by Mandard et al as complete, subtotal, partial, minimal and no response to treatment[41]. The patients were then subdivided into two groups: Responders – consisting of patients with complete and subtotal responses (TRG 1 and 2), and Non-Responders (TRG 3, 4 and 5) including patients with partial and minimal response and no change.

3.4.5: Collection and Storage of Oesophageal Tissue

Following informed signed consent diagnostic endoscopic biopsies, weighing between 10 and 20 μg, were obtained prior to neo-adjuvant chemoradiation. Immediately adjacent tissue was taken for histological confirmation. Additional fragments of normal mucosa, at a distance greater than 5cm from the macroscopic primary, were also collected. All specimens were immediately immersed in RNAl*ater*®, before storage at -80ºC.

3.4.6: Tissue Disruption

Tissue disruption and sample homogenization is achieved using Trizol® reagant and a handheld homogenizer (HHH) (Ultra-Turrax T8, IKA® Works, UK).

- First clean HHH by switching on and inserting into:

i. distilled water, then
ii. ethanol, then
iii. RNA ZAP and finally
iv. distilled water again

- Pipette 1ml Qiazol® solution into a flat-bottomed tube and leave on ice for approximately 5 minutes
- Add tissue sample
- Homogenise for thirty seconds at the maximum setting to create a uniformly cloudy liquid – ensuring no tissue remains attached to homogenizer
- Clean – as above – between each specimen

3.4.7: RNA Extraction

Total RNA is extracted using the following modified Qiagen RNeasy™ (Qiagen, West Sussex, UK) kit:

- To each tube add 200µl chloroform, shake for 15s and leave at room temperature for 5 mins
- Centrifuge at 16,000g for 10 minutes at 4°C
- Carefully pipette clear supernatant into different tube.
- To the clear supernatant add 500µl isopropanol, shake for 15s and leave at room temperature for 10mins at which point a cloudy precipitate should form
- Centrifuge at 16,000g for 10mins at 4°C
- Carefully decant clear liquid and pipette off remaining clear fluid to leave cloudy white pellet (RNA). Leave open for 1min to allow isopropanol to evaporate further
- To this add 1ml of 75% ethanol
- Centrifuge at 16,000g for 5mins at 4°C
- Carefully decant clear liquid and pipette off remaining clear fluid to leave cloudy white pellet (RNA). Leave open for 1min to allow ethanol to evaporate further
- To this add

  50µl RNAse free water
  350µl buffer RLT
  350µl 70% ethanol
  and gently mix

- Apply up to 750µl of sample to RNeasy® mini column placed in a 2ml collection tube. Centrifuge for 15s at 10000rpm. Discard flow-through.
- Pipet 350µl Buffer RW1 into RNeasy® mini column. Centrifuge for 15s at 10000rpm. Discard flow-through.
- Pipet 80µl DNase I incubation mix (10ml DNase I stock solution and 70µl Buffer RDD) directly onto RNeasy® silica-gel membrane. Place on benchtop (20-30°C) for 15 mins.

- Pipet 350µl Buffer RW1 into RNeasy® mini column. Centrifuge for 15s at 10000rpm. Discard flow-through.
- Transfer RNeasy column into a new 2ml collection tube. Pipet 500µl Buffer RPE onto RNeasy® column. Centrifuge for 15s at 10000rpm. Discard flow-through.
- Add another 500µl Buffer RPE onto RNeasy® column. Centrifuge for 2 mins at 10000rpm. Discard flow-through.
- Transfer RNeasy® column to new 1.5ml collection tube. Pipet 50µl RNase-free water directly onto silica-gel membrane. Centrifuge for 1 min at 10000rpm to elute

Before proceeding to the microarray step assess the quality and quantity of RNA using both the NanoDrop® ND-100 v3.0.1 Spectrophotometer (NanoDrop Technologies, Montchanin, USA) and the Agilent 2100 Bioanalyzer/RNA 6000 LabChip® kit (Agilent Technologies, Waldbrann, Germany).

For the purposes of gene expression studies only the purest RNA samples were subsequently used for generating complementary RNA. The same criteria as before (section 2.4.2) were applied. In addition it was necessary to achieve an RNA concentration >100ng/µl as identified using both technologies

3.4.8: Microarray

1µg of RNA was used in each microarray experiment and the Applied Biosystems Version 2.0 protocol was followed.

3.4.8.1: Reverse Transcription-*in vitro* Transcription (RT-IVT) Labeling

Reverse Transcription

- Pipette the following components into 0.2ml MicroAmp reaction tube on ice:
  2µl T7-Oligo (dT) primer
  4µl Control RNA
  RNA sample (1µg) made up to 10µl with nuclease-free water

- Heat and cool the RNA and primer mixture in a thermal cycler to 70℃ for 5min and them follow with a 4℃ hold.
- After the run, place the tube on ice.
- Add the following reagants to the reaction tube on ice
  1μ RNase inhibitor
  2μl 10X 1st Strand Buffer Mix
  1μl RT Enzyme Mix
- Perform reverse transcription in a thermal cycler:
  25℃ for l0min, 42℃ for 2hrs, 70℃ for 15min and 4℃ hold.
- After the run, place the tube on ice

Second Strand Synthesis

- Add the following components to the cDNA mixture:
  20μl 5X 2nd Strand Buffer Mix
  5μl 2nd Strand Enzyme Mix
  55μl nuclease-free water
- Perform second strand synthesis in a thermal cycler:
  16℃ for 2hrs, 70℃ for l5min and 4℃ hold.

Purification of cDNA

- In a l.5ml nuclease-free microcentrifuge tube, combine:
  100μl DNA Binding and
  100μl 2nd strand synthesis reaction mix
- Insert a DNA purification column into a 2.0-ml receptacle tube.
- Transfer the reaction-DNA Binding Buffer mixture (200μl to the column and centrifuge at 13,000xg for 1 min.
- Remove the column from the tube, discard the liquid, and then reinsert the column into the tube.

- Add 300µl of DNA Wash Buffer to the column and centrifuge at 13,000xg for 1min.
- Remove the column from the tube, discard the liquid, and then reinsert the column into the tube.
- Add 300µl of DNA Wash Buffer to the column and centrifuge at 13,000xg for 1 min.
- Remove the column from the tube, discard the liquid, and then reinsert the column into the tube.
- Close the tube, then centrifuge the empty column and tube at 13,000xg for 1 min.
- Transfer the column to a new 1.5ml elution tube.
- Pipette 10µl of DNA Elution Buffer onto the fibre matrix at the bottom of the column and allow to stand at room temperature for 1 min.
- Centrifuge the column and tube at 13,000xg for 1 min.
- Repeat elution step twice more for a final elution volume of 20µl

In Vitro Transcription Labeling

- Add the following IVT components to the ds cDNA output (made up to 24µl with nuclease-free water)
  8µl 5X IVT Buffer Mix
  4µl DIG-UTP (approximately 14 nmol)
  4µl IVT Enzyme Mix
- Perform IVT in the thermal cycler:
  37°C for 9hrs followed by a 4°C hold.

Purification of cRNA

- In a new 1.5ml nuclease-free microcentrifuge tube, combine and then vortex briefly to mix:

20µl nuclease-free water

40µl entire IVT reaction

- Add and mix by pipetting:

  200µl RNA Binding Buffer

  140µl 100% ethanol

- Insert an RNA purification column into a 2ml receptacle tube, add the IVT reaction-RNA Binding Buffer-ethanol mixture (400µl) to the column, and centrifuge at 13,000xg for 1min
- Discard the flow-through
- Add 500µl of RNA Wash Buffer to the column and centrifuge at l3,000xg for 1min
- Discard the flow-through.
- Add 500µl of RNA Wash Buffer to the column and centrifuge at l0,000xg for 1min
- Discard the flow-through
- Close the tube, then centrifuge the column and tube at l3,000xg for an additional minute
- Transfer the column to a new 1.5ml elution tube
- Pipette 50µl of RNA Elution Buffer onto the fibre matrix at the bottom of the column and incubate at room temperature for 2min
- Centrifuge at 13,000xg for 1min for an elution volume of 50µl
- Pipette 50µl of RNA Elution Buffer onto the fibre matrix at the bottom of the column and incubate at room temperature for 2min
- Centrifuge at 13,000xg for 1min for a final elution volume of 100µl

- Resulting labeled cRNA stored on ice while quantity and quality assessed using Agilent Bioanalyser

- cRNA is then stored at -20℃ for up to 2 months (or -80℃ for long term storage)

3.4.8.2: Chemiluminescence Detection

Pre-hybridising Microarrays

- Prepare pre-hybridisation mixture in a nuclease-free tube and vortex to mix:

  150µl nuclease-free water
  330µl Hybridisation Buffer
  100µl Hybridisation Denaturant
  420µl Blocking Reagent

- Transfer pre-hybridisation mixture into each microarray cartridge and incubate in a 55℃ oven agitating at 100rpm for 1 hr.

Fragmenting cRNA

- Combine the following components in a 0.2mL MicroAmp® reaction tube on ice, then mix by pipetting:
  10µl cRNA Fragmentation Buffer
  10µg DIG-labeled cRNA made up to 90 µl with nuclease-free water

- Heat the tube in a thermal cycler at 60℃ for 30min.

- Add 50µl of cRNA Fragmentation Stop Buffer, mix by pipetting and place on ice.

Hybridisation

- For each microarray, prepare hybridisation mixture in a nuclease-free microcentrifuge tube:

100µl nuclease-free water
170µl Hybridisation Buffer
30µl Hybridisation Controls
50µl Hybridisation Denaturant
150µl fragmented cRNA targets

- Vortex the hybridisation mixture, then centrifuge the tube briefly
- Quickly transfer hybridisation mixture into each microarray cartridge, drying the port with lint-free tissue prior to sealing
- Return cartridges to the oven and incubate at 55°C agitating at 100rpm for 16hrs.

Hybridisation washes

- Equilibrate to 22°C: Hybridisation wash buffer concentrate
  Hybridisation wash detergent concentrate
  Chemiluminescence rinse buffer concentrate
  Chemiluminescence enhancing rinse concentrate
- Heat to 37°C for 30 minutes, mix well and then allow to equilibrate to room temperature: Chemiluminescent enhancing solution
  Blocking reagant
- Then prepare following washes:

Wash Buffer 1	30ml hybridisation wash buffer concentrate 60ml hybridisation wash detergent concentrate 210mls nuclease-free de-ionized water
Wash Buffer 2	1.5ml hybridisation wash buffer concentrate 298.5ml nuclease-free de-ionized water

Chemiluminescence (CL) Rinse Buffer

75mls chemiluminescence rinse buffer concentrate

1425mls nuclease-free de-ionized water

CL enhancing rinse concentrate

15mls chemiluminescence enhancing rinse concentrate

585mls nuclease-free de-ionized water

NB: Each wash volume is suitable for one tray which will house 4 microarrays

- Add 300ml hybridisation wash buffer 1 to a clean wash tray.

- Remove microarrays from their cartridges, decant liquid (but ensure >0.75ml hybridisation volume remains) and submerge in wash buffer.

- Agitate on the rocking platform (tilt angle 10°; tilt speed 30 tilts back and forward per min) for 5min.
- Decant buffer by tilting wash tray

- Add 300ml hybridisation wash buffer 2, making sure that all microarrays are submerged in buffer.

- Agitate on the rocking platform for 5min.

- Decant buffer by tilting wash tray

- Add 300ml CL rinse buffer to the wash tray, making sure that all microarrays are submerged in buffer

- Agitate on the rocking platform shaker for 5min

- Decant buffer by tilting wash tray

- Add 300ml CL rinse buffer to the wash tray, making sure that all microarrays are submerged in buffer

- Agitate on the rocking platform shaker for 5min
- Remove wash tray from the rocking platform shaker
- Microarrays may be left in CL rinse buffer at room temperature for up to 1hr

Antibody Binding

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

(N.B. per microarray):

2.8ml nuclease free water
0.2ml Chemiluminescence Rinse Buffer Concentrate
1ml Blocking Reagent
15μl Anti-digoxigenin-alkaline phosphatase conjugate

- Decant the CL rinse buffer from the wash tray leaving microarrays secured
- Immediately add 4ml CL blocking buffer/antibody mixture to the microarray
- Cover the arrays with the wash tray cover and agitate on the rocking platform for 20min at room temperature.

Antibody washes

- Decant CL blocking buffer/antibody mixture by tilting the wash tray
- Add 300ml CL rinse buffer to the wash tray
- Cover the wash tray and agitate on the rocking platform shaker for 10min.
- Decant the buffer from the wash tray
- Add 300ml CL rinse buffer to the wash tray and agitate on the rocking platform for 10min
- Decant the buffer from the wash tray
- Add 300ml CL rinse buffer to the wash tray and agitate on the rocking platform for 10min

- Remove wash tray from the rocking platform shaker. Microarrays may be left in the CL rinse buffer at room temperature for up to 3hr

Performing Chemiluminescent reaction

- Decant CL rinse buffer by tilting wash tray
- Immediately add 300ml CL enhancing rinse buffer to tray
- Agitate on the rocking platform for 10 min.
- Decant CL enhancing rinse buffer
- Quickly add 4ml of Chemiluminescence Enhancing Solution to each microarray
- Agitate on the rocking platform for 20 min
- Decant CL enhancing rinse buffer
- Add 300ml CL enhancing rinse buffer and agitate on the rocking platform for 5min.

NB: Microarrays may be left in CL enhancing rinse buffer for up to 3hrs

- Remove the microarray from the tray, decant the CL enhancing rinse buffer, then shake and tap the microarray gently.
- Wipe the bottom of the microarray with lint-free tissue
- Add 3.5ml of Chemiluminescence Substrate to the microarray
- The chemiluminescent reaction is time dependent. After performing this step, proceed with performing CL detection immediately on the ABI 1700

Chemiluminescent Detection

- Open instrument door
- Load the microarray
- Close and lock the door

- Then on the computer start the read process and wait for the instrument to obtain images

Image Detection

- Arrays are captured in two halves and processed using the ABI 1700 reader and software system

3.4.9: Statistical analysis

The microarrays were analysed using a combination of Microsoft® Excel (Microsoft® Corporation) and Bioconductor R version 2.0, a free language and environment for statistical computing and graphics (R Development Core Team, 2004).

3.4.9.1: Filtering genes

Flagged genes and those with a signal-to-noise ratio (S/N) of less than 3 were deemed undetectable and removed from further analysis. All the in-built control elements on the array were also removed prior to further analysis.

3.4.9.2: Comparing samples

When comparing sample groups non-parametric t-tests/ANOVA were used to generate unadjusted p-values.

3.4.9.3: Adjusting p-values for multiple comparisons (FDR)

Microarrays involve testing many hypotheses within a single study. As a consequence such testing is likely to produce hundreds of false positives if α-values, that are commonly applied in other types of statistical analyses, are used. For example, the use of a p value of 0.05 would, in many microarray experiments, yield 1,500 false positive genes.

In an attempt to reduce these error rates methodologists introduced very stringent means, such as the family-wise error rate (FWER). The FWER is defined as the probability of at least one false positive conclusion over all the true null hypotheses (a null hypothesis corresponds to the lack of relationship between gene expression measurement and a response variable). The most commonly used methods were the Bonferroni and Sidak correction.

However, many biologists argued that controlling the FWER in multiple testing settings might not always be appropriate. In large scale hypothesis-generating studies such as microarray experiments, this criterion becomes so conservative that the probability of detecting any true association is, in some cases, almost nil. As an alternative and less stringent means of controlling the error rate, Benjamini and Hochberg introduced the false discovery rate (FDR)[126]. The FDR is the expected proportion of erroneously rejected null hypotheses among the rejected ones. The main reason for controlling the FDR is that it controls a quantity that is relevant and leads to more powerful procedures than those relying on the FWER.

In this project FDR corrections are used, where possible, in order to generate manageable results.

3.4.9.4: Hierarchical clustering

Hierarchical clustering was performed on the generated final differential gene lists to determine whether the samples grouped appropriately. Clustering was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) with Euclidian distance as the similarity measure. Average value was used as the ordering function.

3.4.9.5: Analysis of gene lists

Gene lists were identified and manipulated using the R software. This allowed identification of genes that were differentially expressed between groups at several statistical levels and also the degree to which each of those genes was up or down regulated. For secondary analysis these lists were uploaded into Panther®[127]. The Panther® database was designed for the high throughput analysis of protein sequences. One of the key features is a simplified ontology of protein function, which allows browsing of the database by biological functions. Biologist curators have associated the ontology terms with groups of protein sequences rather than individual sequences. Statistical models (Hidden Markov Models) are built from each of these groups. The advantage of this approach is that new sequences can be automatically classified as they become available. To ensure accurate functional classification, Hidden Markov Models are constructed not only for families, but also for functionally distinct subfamilies. Multiple sequence alignments

and phylogenetic trees, including curator-assigned information, are available for each family.

3.5: RESULTS

In total, benign and malignant tissue was obtained from 23 and 25 patients respectively with localised oesophageal cancer.

3.5.1 Quality assurance of the materials and methods used

RNA

For the purposes of gene expression studies only the purest RNA samples were subsequently used for generating complementary RNA. Tables 3.i and 3.ii reveal the necessary information required to determine whether the extracted RNA was appropriate for further analysis. Only those samples highlighted in red were deemed suitable. Of the 25 initial tumour samples the RNA quality was too poor or dilute in 10. Subsequent steps were therefore only performed in 15. Of the initial 23 normal samples the RNA quality was too poor or dilute in 11. Subsequent steps were therefore only performed in 12.

Table 3.i: Nanodrop and Agilent results for RNA extracted from diagnostic *tumour* biopsies

Code	Nano RNA (ng/µl)	260:280	260:230	Agilent RNA (ng/µl)	28s:18s
R04-0003	**482.2**	**2.06**	**2.14**	**437**	**1.4**
R04-0013	38.9	2.27	0.46	45	0
R04-0023	8.7	2.08	0.33	9.2	0
R05-0485	**904**	**2.17**	**1.99**	**960.1**	**1.5**
R04-1357	10.2	1.99	0.37	24.6	1
R05-0321	**279.1**	**2.14**	**1.95**	**296.6**	**1.3**
R04-0599	44.4	2.21	0.49	52	0
R04-1220	**241.8**	**2.11**	**1.93**	**217.8**	**1.6**
R04-1213	418.1	1.87	1.67	411.2	1.1
R04-0213	**341**	**2.09**	**1.81**	**389**	**1.6**
R04-1071	76.1	1.75	0.71	57.6	1.2
R04-0854	**227**	**2.00**	**1.86**	**299.1**	**1.4**
R04-1305	182.3	1.79	0.86	161.4	1.1
R05-0021	**132.6**	**2.14**	**1.86**	**125.2**	**1.3**
R05-0345	**279.1**	**2.12**	**1.83**	**363**	**1.9**
R04-1240	**110.1**	**2.11**	**2.1**	**166.3**	**1.6**
R04-0111	**459.3**	**2.07**	**1.91**	**470.8**	**1.3**
R05-0033	**349.5**	**2.11**	**2.06**	**355.9**	**1.4**
R05-0173	540.3	2.02	2.05	570.3	1.1
R04-0148	**330.8**	**2.08**	**2.04**	**354.7**	**1.5**
R04-0157	**287**	**2.14**	**1.98**	**286.3**	**1.3**
R05-0095	45	1.06	0.75	56.5	0.4
R05-0087	**85.9**	**2.21**	**2.01**	**84.4**	**1.8**
R05-0089	14.2	1.93	0.11	16.8	1.2
R04-0860	**380.2**	**2.09**	**2.08**	**384.5**	**1.8**

Table 3.ii: Nanodrop and Agilent results for RNA extracted from *normal* tissue biopsies

Code	Nano RNA (ng/µl)	260:280	260:230	Agilent RNA (ng/µl)	28s:18s
R04-0013	217.5	2.1	1.99	249.3	0.6
R04-0026	318.6	2.1	0.86	346.7	0.9
R05-0034	286.8	2.10	1.86	275.3	1.1
R04-1358	9.7	1.46	1.00	8.8	0
R05-0325	**119.4**	**2.15**	**1.88**	**109.5**	**1.5**
R04-0602	91.5	1.9	0.98	76.8	0
R04-1220	12.1	1.56	0.44	5.7	0
R04-1214	70.5	2.06	1.23	71.2	0.5
R04-0216	**125.7**	**2.11**	**1.87**	**118.5**	**1.4**
R04-1074	121.5	2.1	2.14	110.5	0.7
R04-0857	**156.8**	**2.13**	**1.89**	**175.4**	**1.6**
R04-1308	**172**	**2.1**	**1.93**	**241**	**1.5**
R05-0024	**174.3**	**2.11**	**2.02**	**152.8**	**1.4**
R05-0349	**204.5**	**2.13**	**1.82**	**200.4**	**1.7**
R04-1243	**512.8**	**2.04**	**2.13**	**488.5**	**1.5**
R04-0114	**222.3**	**2.1**	**2.02**	**201.5**	**1.4**
R05-0036	**235.1**	**2.21**	**2.08**	**248.3**	**1.3**
R05-0179	75.3	2.01	1.02	49.9	0
R04-0150	**135.2**	**2.31**	**1.81**	**148.6**	**1.3**
R05-0098	**257.1**	**2.14**	**1.99**	**241.1**	**1.4**
R05-0088	12.1	1.93	0.12	10.3	0.8
R05-0092	54.6	2.03	1.54	38.7	0
R04-0863	**118.2**	**2.12**	**2.18**	**127.1**	**1.3**

Figure 3.x illustrates a typical reading from the NanoDrop® spectrophotometer. The instrument measure nucleic acid samples up to 3700 ng/ul. It automatically detects the high concentration and utilizes the 0.2mm path-length to calculate the absorbance.

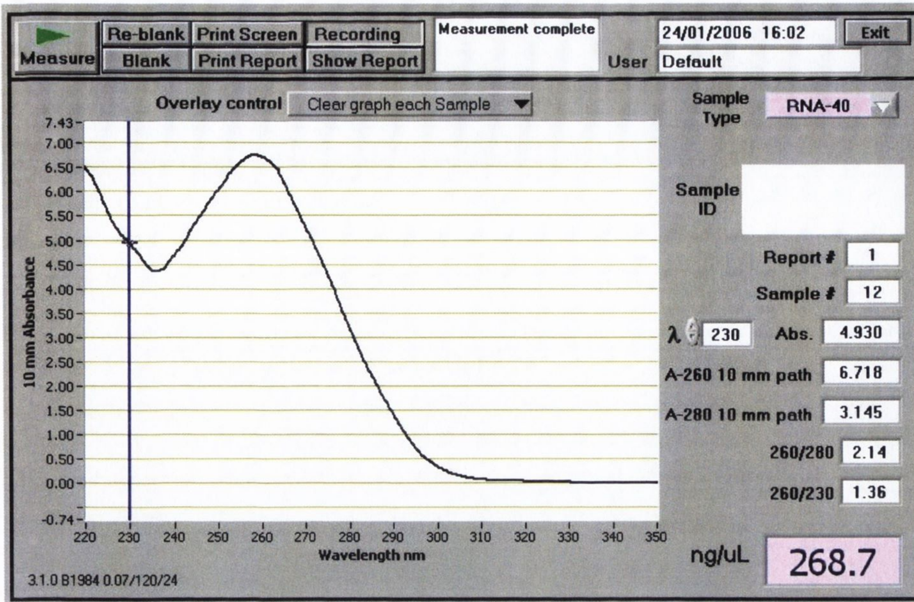


Figure 3.x:

The Agilent 2100 Bioanalyzer provides two visual representatives of each sample, an electropherogram (figure 3.x) and a gel-like image (figure 3.xi A/B). Figure 3xi. A reveals a near perfect sample with 18s (Peak 2) and 28s (Peak 3) ribosomal RNA peaks dominating the picture. There are minimal amounts of small RNA (Peak 1). Figure 3.xi.B reveals significant RNase degradation of total RNA with a shift in RNA size distribution towards smaller fragments and a decrease in the fluorescence signal.

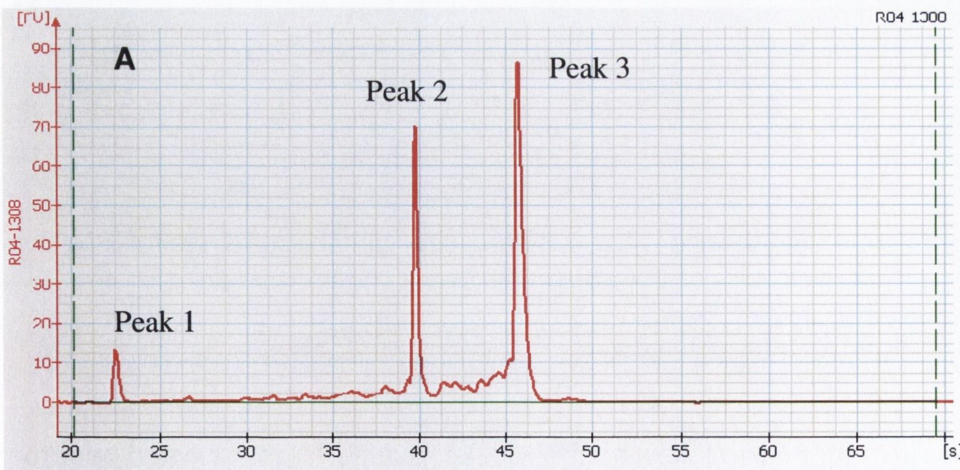


Figure 3.xi

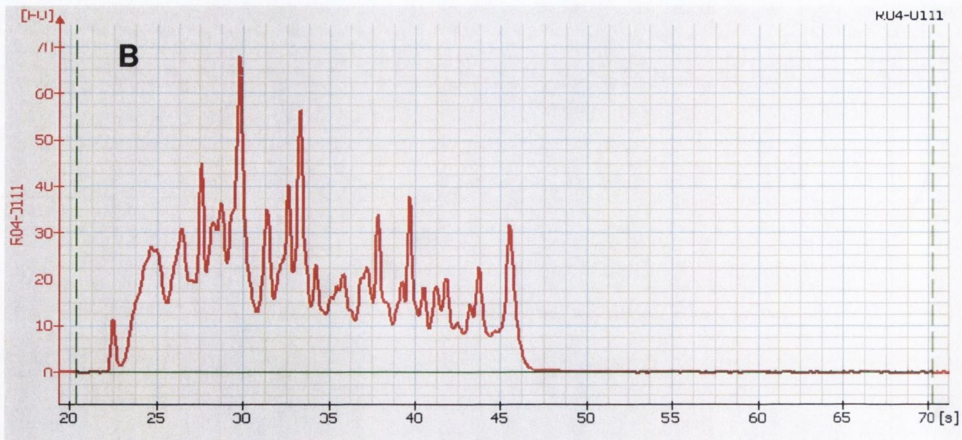


Figure 3.xi: Typical Agilent 2100 Bioanalyzer electropherogram readings for good (A) and poor (B) quality RNA.

The gel-like RNA readings (figure 3.xii) illustrate some of the data. Columns 2 and 12 demonstrate significant RNA degradation with significant levels of small RNA. In columns 3, 4 and 10 the RNA concentrations were particularly low (<50ng/µl). Columns 5, 6, 7, 9 and 11 were satisfactory.

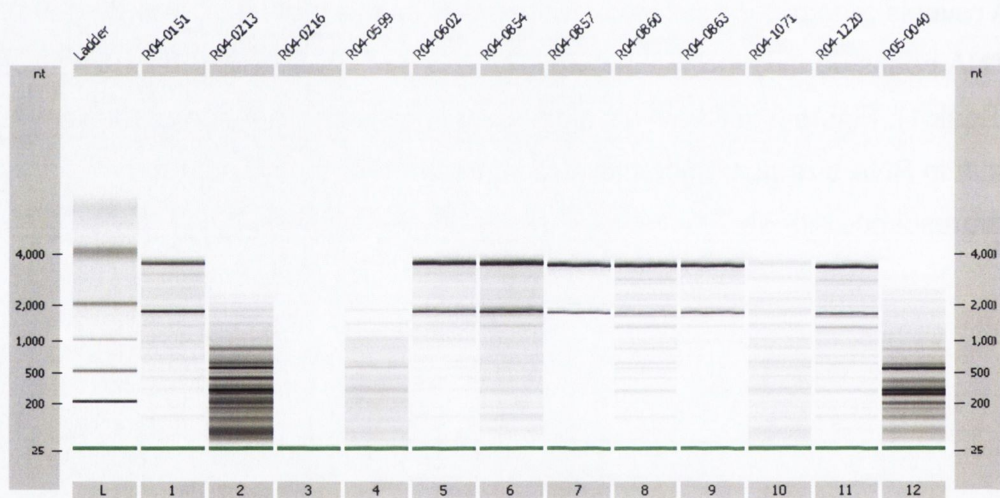


Figure 3.xii: Typical Agilent 2100 Bioanalyzer gel-like readings for RNA.

Post RT-IVT cRNA analysis

The cRNA generated following the RT-IVT reaction was analysed prior to hybridisation. This was again performed using the Agilent 2100 Bioanalyzer/RNA 6000 LabChip® kit (Agilent Technologies, Waldbrann, Germany) and allowed estimation of cRNA concentration (ng/µl) necessary for the fragmenting cRNA step. (see Methods section 3.4.8.2)

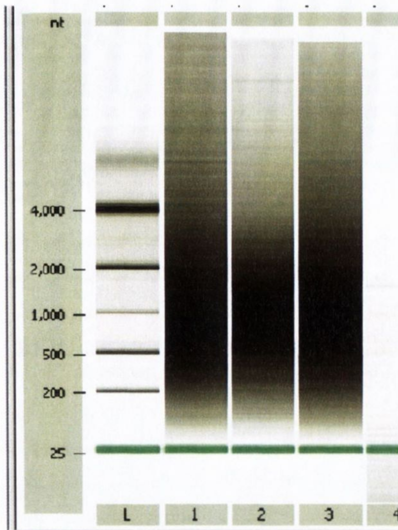


Figure 3.xiii: Typical cRNA outputs on the Agilent 2100 Bioanalyzer (columns 1-3)

Table 3.iii: cRNA concentrations measured by the Agilent Bioanalyzer for tumour and corresponding normal samples, where available

Malignant		Benign	
Code	**cRNA concentration (ng/ul)**	**Code**	**cRNA concentration (ng/ul)**
R04-0003	351	R04-1221	364
R05-0485	202	R04-0216	111
R05-0321	376	R04-1308	416
R04-1220	164	R05-0024	320
R05-0486	111	R05-0349	157
R04-0854	339	R04-1243	328
R05-0021	346	R04-0114	123
R05-0345	201	RO5-0036	165
R04-1240	377	R04-0151	284
R04-0484	144	R05-0098	265
R05-0033	296	R04-0863	144
R04-0148	200	R05-0325	249
R04-0157	227	R05-0487	211
R05-0087	389	None	NA
R04-0860	282	None	NA

Figure 3.xiv:

Two of the 15 tumour samples did not pass all the internal controls. One of the 13 normal samples did not pass all the internal controls.

Table 3.iv: depicts the demographic and pathological data for those 13 patients in whom gene analysis of their tumour samples was subsequently performed.

Table 3.iv: Demographic data: tumour samples

Sample	Sex	Age	Type	Stage	Site	Grade	TRG
1	M	64	AD	T3N0M0	Lower	III	1
2	F	58	SCC	T3N0M0	Mid	III	1
3	M	60	AD	T3N0M0	Lower	NS	2
4	M	54	AD	T3N0M0	Lower	II	2
5	M	59	SCC	T3N0M0	Lower	II	3
6	M	62	AD	T3N0M0	Lower	III	3
7	M	37	AD	T3N0M0	OGJ	II	3
8	M	56	AD	T3N0M0	Lower	II	3
9	M	49	AD	T3N0M0	OGJ	III	3
10	M	66	AD	T3N0M0	Lower	III	4
11	M	58	SCC	T3N0M0	Mid	NS	4
12	M	63	AD	T3N0M0	Lower	III	4
13	M	59	AD	T3N1M0	Lower	II	4

Key: M – male, F – female, AD – adenocarcinoma, SCC – squamous cell carcinoma, Site – region of oesophagus where tumour located (mid third, lower third, oesophagogastric junction), TRG – tumour regression grade

Sample Comparisons

Data was generated on between 14 and 17,000 genes per specimen.

Normalisation was achieved using a 5% trimmed mean. This is more powerful when analysing low signal intensity genes.

3.5.2 Identifying genes differentially expressed by benign and malignant oesophageal tissue

Fold change and t-test were performed between the two samples and Benjamin Hochberg FDR applied to the result. The initial list of genes was filtered to a) Identify those that were most differentially expressed between benign and malignant tissue and b) to generate a list of more manageable size for further pursuing individual genes. This is illustrated in table 3.v.

Table 3.v: Gene filtering: Benign versus malignant tissue

Statistical Value	Fold Change	Differentially Expressed Probes
FDR 0.25	N/A	6776
FDR 0.1	N/A	3685
FDR 0.05	N/A	2286
FDR 0.01	N/A	411
FDR 0.01	>2	363
FDR 0.01	>4	156
FDR 0.01	>10	22

Figure 3.xv: A-D demonstrates the step-wise filtering (performed by Panther[127]) from an FDR of 0.25 to 0.01 in order to generate a list of 411 differentially expressed probes. These were subsequently used to examine differentially expressed biological pathways, processes and molecular functions that were differentially regulated when compare to the reference human genome.

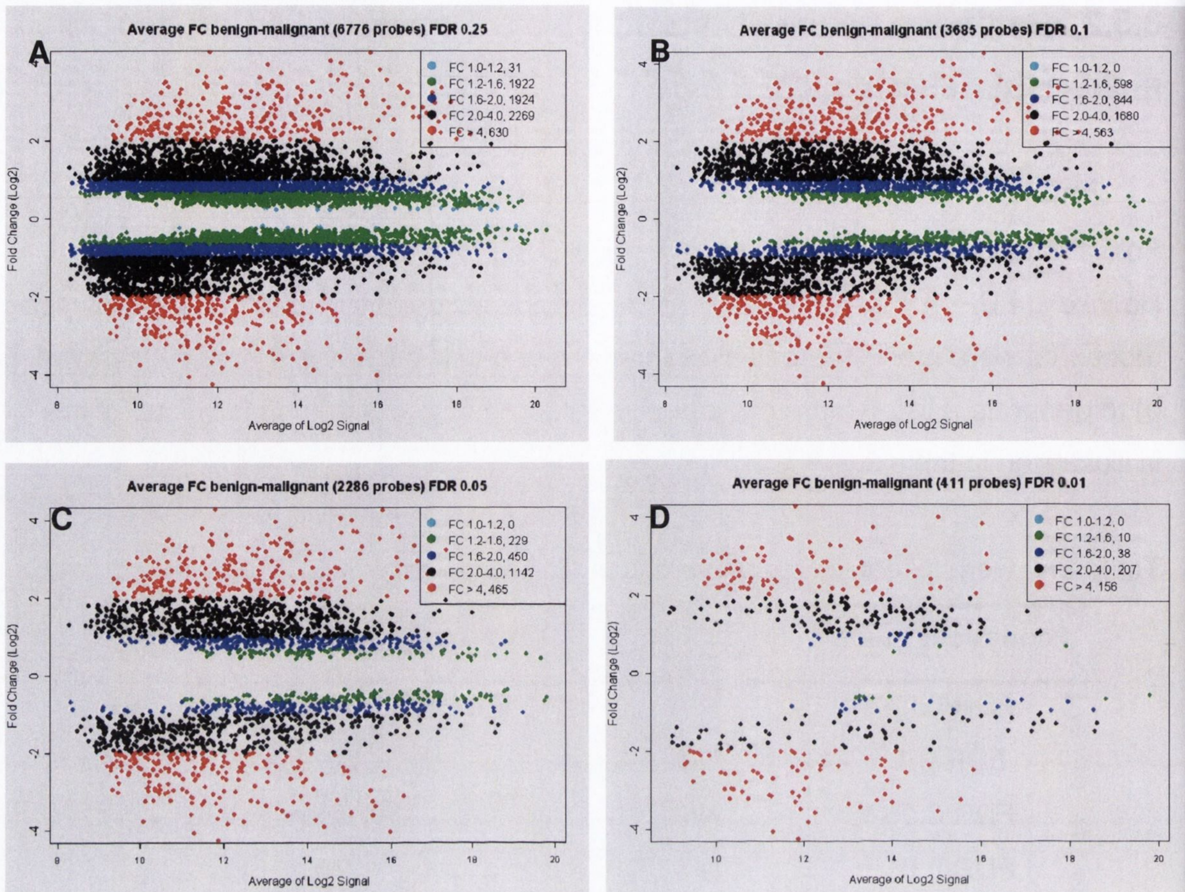


Figure 3.xv: Step-wise filtering of genes differentially expressed between benign and malignant oesophageal tissue

Hierarchical Clustering

Unsupervised hierarchical clustering appropriately separated benign and malignant tissue in to the two respective groups with one exception; one of the benign samples did not seem to have features that related it to either benign or malignant tissue. This was excluded from further analysis. Having obtained differentially expressed gene lists (13 tumour, 11 normal) supervised hierarchical clustering was performed (Figure 3.xvi).

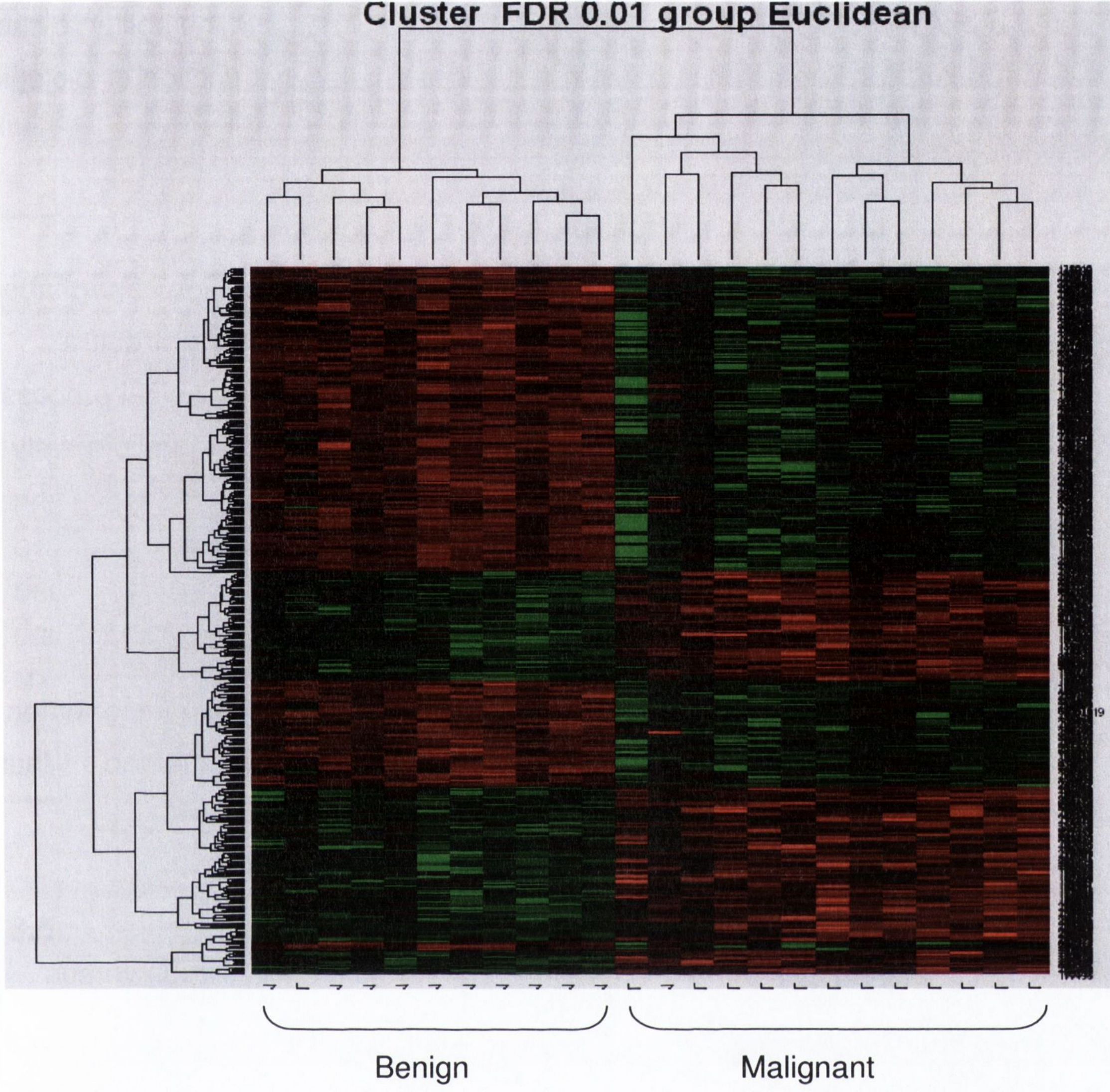


Figure 3.xvi: Hierarchical clustering for benign versus malignant oesophageal specimens based on gene lists obtained from comparison analyses

Secondary Analyses

Of the 411 differentially expressed probes (FDR 0.01) 168 corresponded to fully annotated up-regulated genes and 211 to fully annotated down-regulated genes in benign versus malignant oesophageal samples.

PANTHER

The gene lists were uploaded and analysed in the online database – Panther[127]. Its functions include the ability to merge and differentiate genes within lists, the ability to

graphically breakdown the protein families of the selected genes and the ability to check for over- or under-representation of genes in a particular molecular function or biological process or pathway.

Compared to the reference human genome up-regulated genes in benign oesophageal tissue were involved in the following biological processes: cell adhesion, structure, motility and communication and signal transduction ($p<0.001$). The biological process most significantly represented by down-regulated genes was that involving lipid, fatty acid and steroid metabolism ($p<0.0001$).

Further Analysis

Narrowing the group further 22 probes were differentially expressed by a factor of ten or more. These corresponded to 11 genes that up-regulated in benign tissue and 11 that were down-regulated in malignant tissue (Tables).

Table 3.vi: Genes with assigned names (and corresponding AB 1700 Probe identification number) that were 10-fold (or more) down-regulated in benign versus malignant oesophageal tissue (FDR0.01)

Symbol	**Gene Name**	**Fold Change**	**Adjusted p-value 0.01**	**Probe ID**
unassigned	unassigned	41.3	0,0079	103778
COL1A2	collagen, type I, alpha 2	11.7	0,0065	105493
PXDN	peroxidasin homolog	10.3	0,0048	110561
COL5A1	collagen, type V, alpha 1	12.0	0,0060	110570
COL5A2	collagen, type V, alpha 2	10.8	0,0054	114784
FKBP10	FK506 binding protein 10, 65 kDa	16.9	0,0025	116397
G0S2	G0/G1switch 2	11.3	0,0046	162119
SPP1	secreted phosphoprotein 1	23.5	0,0065	186825
POSTN	periostin, osteoblast specific factor	19.3	0,0087	188528
HTRA3	HtrA serine peptidase 3	14.2	0,0037	208657
MMP1	matrix metallopeptidase 1	41.8	0,0065	215808

Table 3.vii: Genes with assigned names (and corresponding AB 1700 Probe identification number) that were 10-fold (or more) up-regulated in benign versus malignant oesophageal tissue (FDR0.01)

Symbol	Gene Name	Fold Change	Adjusted p-value 0.01	Probe ID
PADI1	peptidyl arginine deaminase, type	10.9	0.0035	135075
CRISP3	cysteine-rich secretory protein 3	40.3	0.0047	145236
ANXA9	annexin A9	10.3	0.0064	145458
SPINK5	serine peptidase inhibitor, Kazal type 5	14.0	0.0087	153856
PRSS27	protease, serine 27	13.1	0.0099	162550
C18orf25	chromosome 18 open reading frame 25	10.2	0.0064	183473
DSG1	desmoglein 1	10.8	0.0046	188189
LOC342897	similar to F-box only protein 2	10.8	0.0097	201940
MXD1	MAX dimerization protein 1	11.0	0.0094	202348
ECM1	extracellular matrix protein 1	15.8	0.0075	209238
unassigned	unassigned	19.3	0.0076	231528

3.5.3 To identify genes that predicted the response to chemoradiation

It was not possible to apply the False Discovery Rate to these samples.

There were 492 probes at the p<0.05 level that differentiated good from poor responders to neo-adjuvant chemoradiation and 103 probes at the p<0.01 level (Figure 3.xvii and Appendix).

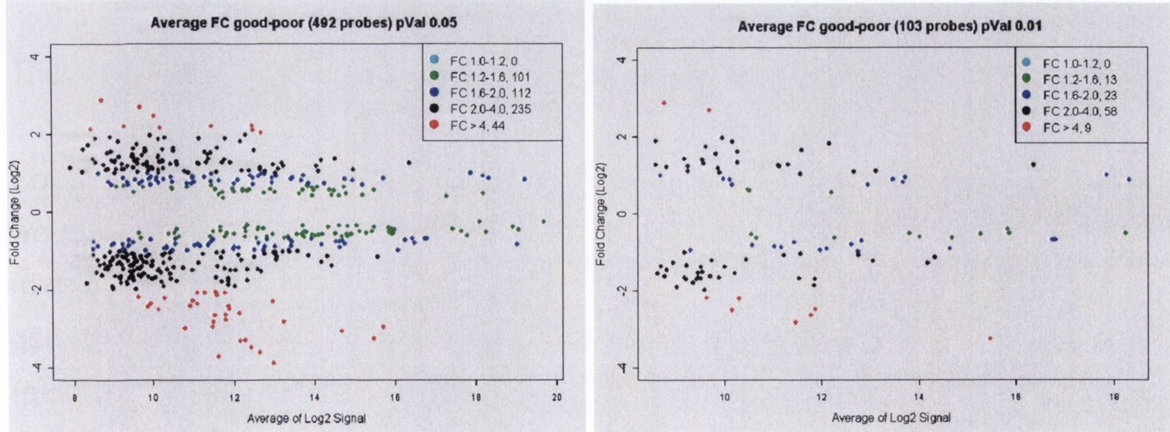


Figure 3.xvii: Step-wise filtering of genes differentially expressed between good and poor responders to chemoradiation

Table 3.viii: Gene Filtering: Good versus poor responders

Statistical Value	Fold Change	Differentially Expressed Probes
P<0.05	N/A	492
P<0.01	N/A	103
P<0.005	N/A	44
P<0.001	N/A	8
P<0.01	>2	67
P<0.01	>4	9

Of the 492 differentially expressed probes (p0.05) 127 corresponded to fully annotated up-regulated genes and 234 to fully annotated down-regulated genes in good versus poor responders to chemoradiation.

Hierarchical Clustering

Unsupervised hierarchical clustering separated the tumour samples into three clusters; one sample (TRG 3) bore no obvious resemblance to either type but was grouped, appropriately, with the poor responders. After obtaining differentially expressed gene lists, supervised hierarchical clustering was performed as demonstrated in figure 3.xviii. The 13 samples separated in to their respective groups; responders and non-responders to chemoradiation.

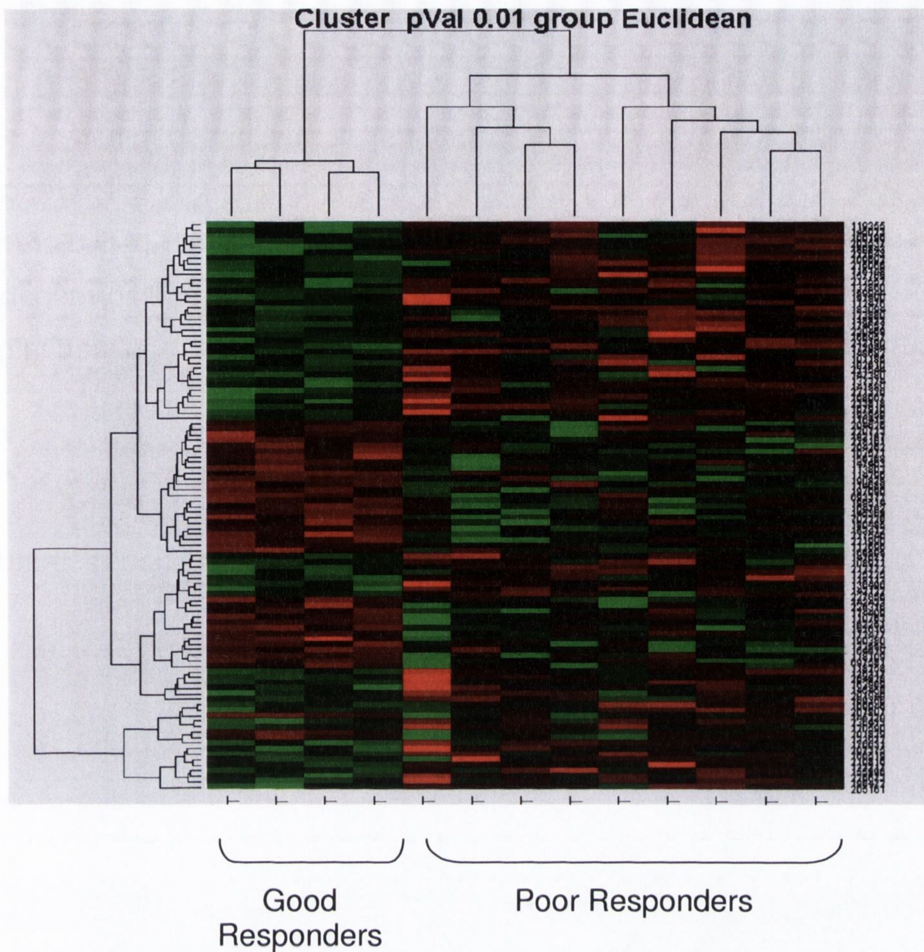


Figure 3.xviii: Hierarchical Clustering Heat map of responders and non-responders to neo-adjuvant chemoradiation based on the gene lists obtained from comparison analysis

PANTHER

The gene lists were uploaded and analysed in the online database – Panther[127].

Compared to the reference human genome up-regulated genes in good responders were involved in cell communication, protein metabolism and signal transduction ($p<0.01$). The biological processes most significantly represented by down-regulated genes were those involved in developmental processes and amino acid metabolism ($p<0.01$).

Further Analysis

Of the 103 differentially expressed probes (p<0.01) 57 corresponded to fully annotated/assigned down-regulated genes and 40 to up-regulated genes in responders versus non-responders. Of these 39 were down-regulated by a factor of two or more and 25 up-regulated. Tables 3.ix and x demonstrate those genes (and corresponding AB 1700 probe identification numbers) that were either down or up regulated in good responders versus poor responders (p<0.01).

Table 3.ix: Genes with assigned names (and corresponding AB 1700 Probe identification number) that were 2-fold (or more) down-regulated in good responders versus poor responders (p<0.01)

Probe ID	Symbol	Gene Name	Fold Change
101185	ABHD3	abhydrolase domain containing 3	2.1
145458	ANXA9	annexin A9	5.6
166957	B3GALT5	betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 5	3.8
205161	C15orf48	chromosome 15 open reading frame 48	9.5
125725	C5orf15	chromosome 5 open reading frame 15	2.3
162989	CCL28	chemokine (C-C motif) ligand 28	3.6
166519	CKMT1A	creatine kinase, mitochondrial 1A	2.5
166519	CKMT1B	creatine kinase, mitochondrial 1B	2.5
126317	CYP2C18	cytochrome P450, family 2, subfamily C, polypeptide 18	3.3
207375	DHRS1	dehydrogenase/reductase (SDR family) member 1	2.2
204928	EPB41L3	erythrocyte membrane protein band 4.1-like 3	3.2
118833	FLJ37440	hypothetical protein	2.3
118713	HSD17B4	hydroxysteroid (17-beta) dehydrogenase 4	2.1
221320	IL7	interleukin 7	2.6
141589	IRF8	interferon regulatory factor 8	2.3
115148	KCNJ2	potassium inwardly-rectifying channel, subfamily J, member 2	3.5
212772	KLHL2	kelch-like 2, Mayven (Drosophila)	2.0
109356	KRT23	keratin 23 (histone deacetylase inducible)	7.1
192990	LOC147645	hypothetical protein	3.2
201940	LOC342897	similar to F-box only protein 2	6.2
145220	LOC392437	similar to hypothetical protein	2.4
230469	LOC400705	similar to polycythemia rubra vera 1	3.9
166196	LOC441027	similar to hypothetical protein	2.6
212190	LPHN2	latrophilin 2	2.1
167910	MUC15	mucin 15	3.2
205161	NMES1	normal mucosa of esophagus specific 1	9.5
183094	NOV	nephroblastoma overexpressed gene	3.2
222543	PCDHA13	protocadherin alpha 13	3.1
168667	PEX6	peroxisomal biogenesis factor 6	3.1
110415	PON3	paraoxonase 3	5.7
157769	POU5F1	POU domain, class 5, transcription factor 1	2.9
109692	RARB	retinoic acid receptor, beta	2.6
180843	RGL1	ral guanine nucleotide dissociation stimulator-like 1	3.2
215065	RNPC1	RNA-binding region (RNP1, RRM) containing 1	2.9
112476	RRAGD	Ras-related GTP binding D	3.2
123880	STAT5B	signal transducer and activator of transcription 5B	2.0
193950	TCP11L2	t-complex 11 (mouse) like 2	4.6
174652	VNN2	vanin 2	4.5
			2.7
212552	ZNF546	zinc finger protein 546	

Probe ID	Symbol	Gene Name	Fold Change
190825	CD3EAP	CD3E antigen, epsilon polypeptide associated protein	3.3
118109	Cep290	centrosome protein	2.4
112696	DPF1	D4, zinc and double PHD fingers family 1	2.4
221990	FLJ43987	similar to RIKEN cDNA 4930433I11 gene	3.7
118258	FLRT3	fibronectin leucine rich transmembrane protein 3	3.5
114296	HNT	neurotrimin	2.9
197090	HOXC8	homeo box C8	3.1
132554	INSIG2	insulin induced gene 2	2.0
172477	KIAA1731	KIAA1731	2.1
104762	LENG1	leukocyte receptor cluster (LRC) member 1	2.7
180485	LIN28B	lin-28 homolog B (C. elegans)	2.3
229316	LOC442053	similar to Methionine aminopeptidase 2	2.1
153472	MYBPH	myosin binding protein H	2.3
199514	NAG8	nasopharyngeal carcinoma associated gene protein-8	2.2
156268	PCDHB16	protocadherin beta 16	3.7
114689	PPP2R3A	protein phosphatase 2 (formerly 2A), regulatory subunit B", alpha	3.9
164929	RTKN	rhotekin	2.4
702228	SAMD7	sterile alpha motif domain containing 7	2.5
190380	SEC61A2	Sec61 alpha 2 subunit (S. cerevisiae)	3.1
166505	SPAG17	sperm associated antigen 17	7.3
145309	ZFYVE9	zinc finger, FYVE domain containing 9	2.6
113803	ZNF177	zinc finger protein 177	2.5
147403	ZNF583	zinc finger protein 583	2.1
697581	ZNF658	zinc finger protein 658	2.3
697581	ZNF658B	zinc finger protein 658B	2.3

Table 3.x: Genes with assigned names (and corresponding AB 1700 Probe identification number) that were 2-fold (or more) up-regulated in good responders versus poor responders ($p<0.01$)

3.5: DISCUSSION

3.5.1: Benign versus Malignant Oesophageal Tissue

A number of the most differentially expressed genes have previously been reported in oesophageal cancer, some have been described in malignant disease but not of the oesophagus and some have not been documented in cancer.

3.5.1.1: Genes Down-regulated (>10 fold) in Benign versus Malignant Tissue

COL1A2 (collagen, type I, alpha 2) was down-regulated in benign tissue by a factor of 11.7 ((p=0.0065). It has previously been reported in gastric cancer endothelium and, along with COL1A1, thought to play an important role in angiogenesis and desmoplasia formation[128]. Oue et al performed serial analysis gene expression (SAGE) on 4 gastric cancers and 1 lymph node metastasis[129]. This allowed them to identify several genes and tags that, with reference to their gastric cancer library, might be involved in invasion, metastasis, and carcinogenesis. From these, they selected 27 genes and measured mRNA expression levels in an additional 46 gastric cancer samples by quantitative reverse transcription-PCR. They found that COL1A2 was over-expressed by a factor >2 in tumour:normal in 58.7% of cases and that the gene was significantly associated with tumour stage (p=0.0156).

POSTN (periostin) is a disulfide linked 90 kDa protein secreted by osteoblasts and osteoblast-like cell lines. It is thought to function as a cell adhesion molecule for pre-osteoblasts and to participate in osteoblast recruitment, attachment and migration[130]. In this study it was down-regulated by a factor of 19.3 (p=0.0087) and this adds to the growing body of data in other cancers, including head and neck[131], breast[132], colon[133] and lung[134]. From gene expression and RT-PCR data in oral cancer the gene would appear to be involved in invasion and metastatic progression[131]. More recently it has been reported in both Barrett's oesophagus and oesophageal adenocarcinoma, along with another stromal gene that we found to be markedly down-regulated in benign tissue (COL5A2)[135]. In

light of its finding in Barrett's Hao et al postulate that such findings imply that the stromal and extracellular matrix genes associated with tumour growth are expressed prior to pathological evidence of dysplasia.

MMP1 (matrix metalloproteinase 1) is one member of a well-described family of zinc dependant proteolytic enzymes that are responsible for the breakdown of connective tissue proteins. The enzymes play an important role in the normal processes of growth, differentiation and repair. MMP1 in this study was markedly down-regulated in benign specimens (41.8 fold, p=0.0065). There is now considerable evidence that aberrant matrix metalloproteinase expression contributes to the invasive local growth and spread in different tumour types, including oesophageal cancer[136,137]. Interestingly Yamashita et al found that the mean tumour:normal (T:N) ratio amongst 51 paired oesophageal samples was of a similar magnitude to this study (42.5 fold)[137]. They went on to demonstrate that tumours with a T:N ≥ 10 were more likely to involve the muscularis propria, whilst Murray et al found that MMP1 expression was associated with a worse prognosis[138]. In this study, however, the gene was not found to be significantly up-regulated in poor responders to CRT.

The wealth of information on matrix metalloproteinases has resulted in the generation of clinically-utilised enzyme inhibitors (MMPI) tested at phase III level[139]. Patients with advanced gastric or gastro-oesophageal cancer received either placebo or marimastat (an MMPI). Three hundred and sixty nine patients were recruited and a 2 year survival advantage of 9% vs 2% was demonstrated (hazard ratio 1.23, p=0.024) for the latter. The findings were even more significant when the analysis was limited to those patients that had previously received chemotherapy.

SPP1 encodes an extracellular glycosylated bone phosphoprotein, osteopontin, widely investigated in malignant disease, where it may play a role in tumourigenesis and metastasis[140]. It has been described in a number of cancers including lung and colon[141,142] and furthermore is secreted into urine, milk and blood. In this regard it has been studied as a plasma marker of response in the irradiation of head and neck cancers[143]. It appears to be most abundant in cancers of gastrointestinal, genitourinary and gynaecological origin where it is

strongly correlated with tumour progression[144]. In this study was down-regulated by a factor of 19.3 in benign specimens (p=0.0087) and clearly warrants further investigation.

3.5.1.2: Genes Up-regulated (>10 fold) in Benign versus Malignant Tissue

ANXA9

The annexins are a family of closely related calcium- and membrane-binding proteins expressed in most eukaryotic cell types. Members of the family are frequently reported to be differentially regulated in cancer[145]. Annexins A1, A2, A4, A6, and A11, for example, have all been shown to be down-regulated in hormone refractory prostate cancer[146]. In oesophageal tumours annexin A1 seems to be down-regulated in oesophageal SCC (*cf* normal)[147] whilst A1 and A8 were down-regulated in poor responders to chemoradiation[96]. Annexin A9 (ANXA9) has not been reported in oesophageal cancer and may be an atypical member of its family; it is not capable of binding acidic phospholipids in the presence of sub-millimolar Ca^{2+} concentrations[148]. It was 10 fold up-regulated in benign tissue (p=0.0064) but amongst tumour samples it was 5.6 times down-regulated in good responders (p=0.0082).

CRISP3

Members of the cysteine-rich secretory protein (CRISP) family are expressed predominantly in the male reproductive tract and are implicated in spermatogenesis and post-testicular sperm maturation. CRISP-3 appears to be up-regulated in chronic inflammation of the salivary glands, prostate and pancreas and has also been implicated in prostate cancer[149,150]. By searching publicly available expressed sequence tag sequences for prostate genes, Asman et al found 9 genes that showed statistically significant differences in their EST counts between cancer and normal prostate[150]. One of these genes, CRISP 3, was further validated using RT-PCR and found to be up-regulated >50 fold in malignant tissue. It is thought to be associated with cell to cell adhesion. It has only been reported in other type of cancer – oesophageal. Luthra et al found that it was significantly down-regulated in poor responders to CRT (p<0.0001)[96]. That it was 40 fold up-regulated in this study in normal oesophageal samples (p=0.0047) warrants, at least, RT-PCR validation. The normal samples in this study were taken at a

distance of >5cm from the macroscopic primary tumour. Perhaps CRISP3 expression is a marker of peri-tumoural inflammation/oesophagitis or even of oesophageal cancer susceptibility. It is worth noting that amongst tumour samples CRISP3 was more abundant in poor responders by a factor of 7.7 (p=0.03). The normal samples in this study were taken from the patients who also had cancer. It would probably be prudent to confirm the findings with samples from entirely normal organs.

SPINK5 encodes LEKTI, a 15-domain serine protease inhibitor expressed in epithelial and mucosal surfaces and also in the thymus. It is located on the short arm of chromosome 5. It is well documented in various dermatological conditions and is involved in extra-cellular matrix remodelling. A mutation of the gene appears to be the principal abnormality in patients with Netherton syndrome, a rare skin disease characterised by ichthyosiform dermatosis, hair shaft abnormalities and an atopic diathesis[151]. It was up-regulated in benign tissue 14 fold in this study (p=0.0087), but not significantly associated with response. Interestingly it has only previously been reported in cancer once before; Luthra et al found it to be down-regulated in the poorer responding (to CRT) group of oesophageal tumours (p<0.0001)[96]. Whilst the gene clearly needs to be validated perhaps SPINK5 up-regulation, as CRISP3, represents a malignant precursor/association not dissimilar to the relationship between tylosis (palmoplantar keratoderma) and squamous cell oesophageal cancer.

The extracellular matrix protein 1 (**ECM1**) is a secreted protein that has been implicated with cell proliferation, angiogenesis and differentiation[152]. In their immuno-histochemical study Wang et all found that the protein was expressed to a greater degree in epithelial malignancies (including oesophageal cancer) as opposed to sarcomas or normal epithelium. Microarray data in thyroid cancer confirm similar findings[153]. In this study ECM1 was, however, more abundant in normal tissue (fold change 15.8, p=0.0075). This, of course, needs to be validated with RT-PCR because it is not clear why there should be such discordance.

The study has identified a number of highly differentially expressed genes which, in general, are supported by the available literature in the field. Fascinatingly two

of these genes - SPINK5 and CRISP3 – had, until recently, not been reported in cancer let alone oesophageal tumours. The MD Anderson study found that both were significantly down-regulated in poor responders to CRT and with this data would seem to support their importance in the disease[96]. Validation of this study is clearly essential and this should ideally be performed using a different set of oesophageal cancer samples as well as tissue from entirely normal organs.

3.5.2: Good versus Poor Responders to Neo-adjuvant CRT

Whilst not without controversy, patients with localised, operable oesophageal cancer are frequently treated with neo-adjuvant chemotherapy or CRT. Following such regimes survival figures are most favourable in those that achieve a complete or near-complete pathological response to treatment[20]. However it is currently not possible to predict those patients who are likely to respond well and consequently many patients experience significant treatment-related toxicity with no therapeutic benefit.

In a preliminary attempt to identify a panel of biomarkers associated with a good response to neo-adjuvant CRT gene expression array analysis was performed on 13 diagnostic tumour biopsies. Four (31%) achieved a good response (TRG1-2) and nine a poor response (TRG3-5)[41]. With a few exceptions a number of novel genes, not previously reported in oesophageal cancer, have been identified. The data has been validated by selecting 12 candidate genes and comparing their expression values using RQ-PCR. To date, median time to local or metastatic progression has not been reached.

When compared to the analysis of benign versus malignant samples there were far less statistically significant differences between good and poor responders. The same is true for biological pathways, molecular functions and processes. These features are most likely to be as a consequence of the smaller sample number, although gene expression differences amongst tumour samples, regardless of chemoradiation sensitivity, may not be as great as those between benign and malignant tissue. This issue will become more apparent as the study continues to accrue more patients.

From the most differentially expressed genes identified in our study (tables 2 and 3) only two have previously been reported in oesophageal cancer. Normal mucosa of esophagus specific 1 gene (**NMES1**) was down-regulated by a factor of 9.5 (p=0.008) in those that responded well to CRT. The gene was first described in 2002[154]. Suppression subtractive hybridisation and reverse Northern blot analysis of 36 squamous cell oesophageal tumours (and adjacent normal tissue) found it to be consistently expressed in the latter, but markedly down-regulated or absent in malignant tissue. This led to the hypothesis that the gene may play a role in suppressing oesophageal carcinogenesis. Our data suggest an additional role; its expression may allow for more efficient DNA damage repair following the insult of ionising radiation and cytotoxic therapy leading to relative resistance to the neo-adjuvant protocol.

Vanin2 (**VNN2**) is a gene that encodes proteins associated with adhesion and migration of inflammatory and cancer cells. We found it to be down-regulated by a factor of 4.5 (p=0.003) in good versus poor responders. Kawamata et al studied the gene expression profiles of two oesophageal squamous cell carcinoma cell lines; one metastasizing and the other non-metastasizing[155]. Thirty four genes showed a more than 3-fold differential expression. VNN2 was up-regulated in the metastasizing line. Whilst this characteristic does not necessarily imply responsiveness to CRT, it would not be surprising if tumours with a greater propensity to spread were more resistant to treatment.

The majority of the genes we have identified have not previously been associated with oesophageal cancer. Rhotekin (**RTKN**) encodes a target molecule for the small GTPase, Rho, and is a member of the Ras superfamily[156]. It was moderately up-regulated (p0.006) in those tumours that responded well to treatment. Liu et al demonstrated that the gene is frequently over-expressed in gastric adenocarcinoma and that sustained activation of RTKN confers increased survival signal on the cell[157]. They showed that RTKN activation links the Rho signal to nuclear factor-κβ (NF κβ) activation, leading to increased cell survival by trans-activating anti-apoptotic genes downstream of NFkB. By blocking NF- κβ activity the resistance to cytotoxic drugs was markedly attenuated. They postulated that

over-activation of the Rho signalling pathway might be a common feature in the initiation and progression of the cancer.
Most of our patients had lower oesophageal or oesophago-gastric junction adenocarcinomas and so the genetic phenotype is unlikely to be that different from gastric tumours. However, our data would seem to suggest the converse; that over-expression of RTKN is associated with enhanced sensitivity and the gene warrants further investigation.

Nephroblastoma over-expressed gene (**NOV**) is a matri-cellular protein of the CCN family and may be involved in regulating cell growth and adhesion as well as promoting survival. Altered expression of the gene has been observed in a variety of tumours, including hepatocellular carcinomas, Wilm's tumors, Ewing's sarcomas, gliomas, rhabdomyosarcomas, and adrenocortical carcinomas[158]. Lin et al have demonstrated that it acts directly upon endothelial cells to stimulate pro-angiogenic activities, and induces angiogenesis in vivo. Several studies suggest that enhanced angiogenesis is associated with a poor outcome after radiotherapy[159,160]. That NOV is relatively up-regulated in the poor responding tumours in this study would certainly support this observation.

There have been only two groups that have used gene expression analysis to identify oesophageal cancers with different outcomes and, prior to the inception of this study, none that had looked at response markers to CRT[95,96]. The Japanese adjuvant chemotherapy study identified 52 genes that they deemed biologically significant in distinguishing different survival patterns. None of these genes were identified in this study. However the study groups were quite different; in the Japanese study all the tumours were squamous cell, none of the patients had radiotherapy and the results were being equated with survival not pathological response. In the MD Anderson study unsupervised hierarchical clustering separated the 19 patients in to two molecular subtypes. Most cancers (five of six) that achieved a pCR following neo-adjuvant CRT clustered in molecular subtype I. Subtype II, with one exception, consisted of cancers that achieved less than a pCR. Genes differentially expressed between the two sub-types were then analysed and three selected for RT-PCR validation. Our study, however, included within the good responders patients who, following CRT, still had “rare residual tumour cells”. These samples would, in the Luthra study, have fallen into the less

than pCR group. Following CRT in our study only two (15%) achieved a pCR – probably far too small a number to glean any meaningful statistical information from. Their paper only documents those genes that were down-regulated in molecular subtype II (essentially poor responders). Whilst none of these genes were identified in our response grouping analysis several identical (CRISP3, SPINK5) or very similar (annexins, desmoglein) were found when comparing benign and malignant samples. This would seem to lend weight to their importance as genes somehow involved in oesophageal cancer.

3.5.2.1: RNA Integrity

One of the significant difficulties of this study was obtaining adequate RNA from very small tissue fragments. A number of steps were taken (see chapter 2) to optimize the process but many samples still had to be excluded. One of the contributing factors was, without doubt, sample size. As opposed to other tumour sites (rectum, breast, thyroid) obtaining large quantities of oesophageal tissue is not without risk to the patient; oesophageal perforation, aspiration, haemorrhage and length of time under sedation. Furthermore at any one endoscopic procedure biopsy samples were also being taken for routine histopathology and at least two other research studies. Secondly, the biochemical milieu in which such tumours arise may well contribute to RNA degradation. This could occur immediately following resection and whilst it was being withdrawn by the endoscope, prior to insertion in RNA*later*. Every effort was taken to minimise the thawing and re-freezing of samples but RNA integrity will have certainly been compromised during these necessary steps.

3.5.2.2: Validity of Sampling

It is possible that each sample contained components of non-malignant tissue which may have impacted on the genetic signature. Laser capture micro-dissection would overcome this but arguably the inclusion of such cells probably provides a more representative picture of the tumour microenvironment. This is perhaps particularly of value, when the aim is to associate molecular signatures with pathological response. Conceivably, the genes associated with response

between oesophageal adenocarcinoma and squamous cell carcinoma may differ and ideally the two sub-types should be analysed separately. Equally a far larger cohort of patients with stringent validation is necessary before even considering clinical application. However, as with the Luthra dataset, our study indicates that the analysis of combinations of genes, readily analysed using PCR or tissue microarrays, may be ideal for identifying oesophageal tumours that will and will not respond to chemoradiation.

CHAPTER 4

Validating the Gene Expression Array Data using Real Time Quantitative PCR

4.1: SUMMARY

Validation of the microarray data was performed using Real Time Quantitative PCR.

In order to select the most appropriate endogenous control for oesophageal samples the Applied Biosystems TaqMan® Human Endogenous Control Plate was used.

12 targets were then chosen from the microarray data. This was done by selecting some of those genes that were most differentially expressed between responders and non-responders at the $p<0.01$ level.

Sufficient RNA remained from 10 of the initial 13 tumour samples. RNA was reverse-transcribed using a Superscript III kit from Invitrogen, UK. Primers and probes were obtained by using Applied Biosystems' pre-designed TaqMan® Gene Expression Assays. PCR was carried out using an ABI PRISM 7900 Sequence Detection System (Applied Biosystems, CA, USA). Analysis of relative gene expression data was performed using the $\Delta\Delta C_T$ method[161] with 18s ribosomal RNA as an endogenous control/reference assay.

4.2: INTRODUCTION

In the world of genomic research, the process of experimentally validating microarray data is often performed as a side issue in many microarray studies. Indeed, unlike many early papers in the field that typically included a series of validation experiments[162], there are now an increasing number of microarray-related papers in the literature that have actually forgone performing such validations altogether. This might suggest that the microarray platform has successfully evolved to a level of accepted technological maturity with regards to reliability and consistency. There is some truth to this, particularly with the increasing availability of inexpensive commercially-fabricated microarrays, which

are typically of high quality and display minimal chip to chip variance. However, experimental validations are still probably necessary, because depending on the particular platform used, microarray data is inherently noisy. The large numbers of gene expression measurements obtained in a typical microarray experiment, by virtue of their sheer numbers, can often yield significant numbers of false positive and negative results. Such artefacts can arise for numerous experimental and technical reasons, including (but not limited to) : spurious signals caused by microarray probes cross-hybridizing to related transcripts of similar sequence similarity, artefacts induced by the sample preparation technique (e.g. degradation of RNA during the extraction process), and artefacts caused by the hybridisation procedure itself (e.g. biased hybridisations due to the Cy3 or Cy5 flourophore used) and mistakes in the assignment of probe identities. The latter is of particular concern when dealing with cDNA microarrays, since many cDNA clone libraries invariably contain a certain degree of mistaken assignments. These examples imply that microarray technology is still very much an evolving field, and consequently, it is essential to have the resources to perform at least a measure of independent validation of the microarray result. In addition, it is also worth noting that in certain cases, the validation exercise may lead to unexpected novel biological findings.

4.2.1: Validation Methods

There are two approaches to independently confirming microarray data: *in silico* analysis and laboratory-based analysis.

4.2.1.1: *In silico* Technique

The *in silico* method compares array results with information available from the literature and in public or private expression databases. It allows data validation without performing further experiments. For example, in a meta-analysis of prostate cancer gene data sets, several differentially expressed genes were found to be common to the majority of the studies[163]. Moreover, some of these genes, such as glutathione-*S*-transferase, have previously been identified as aberrantly expressed in prostate cancer in studies using methods other than microarrays[164]. The similarity between array results from different groups coupled with known expression information in the literature, tends to validate the general performance

of a system. It serves to provide confidence in the overall data, including the unique and novel discoveries made in a study. It is probable that this *in silico* approach to validation will become more useful now that standardized methods for reporting array data, such as the MIAME format (minimal information about a microarray experiment) are being increasingly utilised.

4.2.1.2: Laboratory-Based Validation

Laboratory-based validation of data provides independent, experimental verification of gene-expression levels, and typically begins with the same samples that were studied in the initial array experiment(s). The methodology used varies depending on the scientific question, but commonly used techniques include semi-quantitative reverse transcription PCR (RT–PCR), real-time RT–PCR, northern blot, ribonuclease protection assay, and *in situ* hybridization or immunohistochemistry using tissue microarrays. Real-time RT–PCR is the optimum choice in many publications since it is rapid, relatively inexpensive and requires a minimal starting template[165,166]. However, it requires a significant up-front effort to optimize amplification conditions, and the method has potential pitfalls that must be carefully monitored. Rajeevan et al, for example, found that whilst the majority of array results were qualitatively accurate consistent validation was not achieved for those genes where there was a less than four-fold difference on the array165. Additionally, for many of the genes examined, there were significant quantitative differences between array and RT-PCR-based data.
In view of the paucity of oesophageal microarray literature, particularly with respect to response prediction, it was elected to validate the array data by means of Real-Time Quantitative PCR.

4.2.2: Selecting Targets to Validate

Assuming a laboratory validation technique is decided upon it is then necessary to select an appropriate gene set for follow-up analysis. In general, the choice depends on the aim(s) of the study, but is also influenced by factors such as the relative difference in expression among the samples, biological function, abundance levels, and availability of appropriate reagents (probes and antibodies).

Investigators often choose the genes with the highest differential expression ratios, as such differences are more likely to be valid. There is concern, however, that this approach might overlook genes of significant interest; quantitative information from arrays may be imprecise for transcripts showing small differences in expression[167]. In the near term, genes on an array that show significant differences in expression may serve as important clues and point investigators towards biochemical pathways whose members should be studied in detail using more quantitative methods.
It is not clear, from the literature, the ideal or most appropriate number of targets with which to validate array data, but it is presumed that the greater the number of consistencies the more robust the data-set.

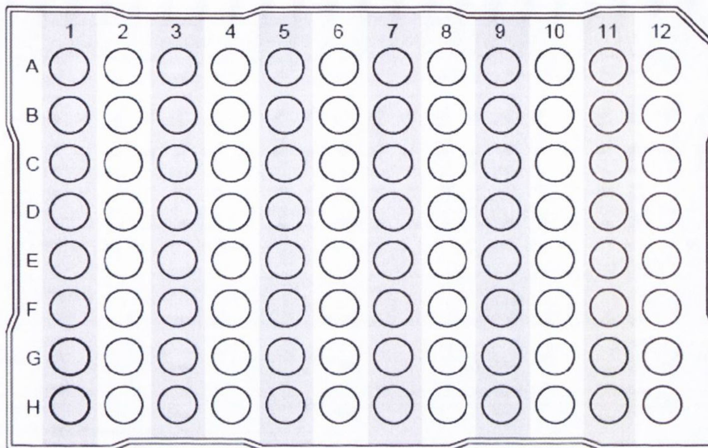
In addition to validating array results at the mRNA level, it is equally important to evaluate expression levels of the corresponding protein products, but that is beyond the scope of this study.

4.2.3: Selecting an Endogenous Control

Recent oesophageal cancer studies have employed various endogenous control genes, such as GAPDH[168], 18s-rRNA[169] and ß-glucuronidase[170] for RT-PCR. However, no endogenous control gene has been validated in oesophageal cancer and normal oesophageal tissues. Therefore, prior to performing validation of the gene arrays a small study was performed to identify an endogenous control gene suited for investigating experimental designs covering benign and malignant oesophageal tissue.

The TaqMan® Human Endogenous Control Plate (Applied Biosystems) was used. This is a MicroAmp® Optical 96-Well Reaction Plate divided into 12 columns, one for every control assay. Each column consists of eight identical wells containing TaqMan primers and probes for the detection of one target gene. (Figures 4.i: A and B). In this way 11 endogenous controls could be analysed simultaneously.

A



B

Column	Control Assay	Abbreviation
1	Internal Positive Control	IPC
2	18S rRNA	18S
3	Acidic ribosomal protein	huPO
4	Beta-actin	huβA
5	Cyclophilin	huCYC
6	Glyceraldehyde-3-phosphate dehydrogenase	huGAPDH
7	Phosphoglycerokinase	huPGK
8	β_2-Microglobulin	huβ2m
9	β-Glucronidase	huGUS
10	Hypoxanthine ribosyl transferase	huHPRT
11	Transcription factor IID, TATA binding protein	huTBP
12	Transferrin receptor	huTfR

Figure 4.i: TaqMan® Human Endogenous Control Plate (A) showing the location of the 11 endogenous controls to be tested (B)

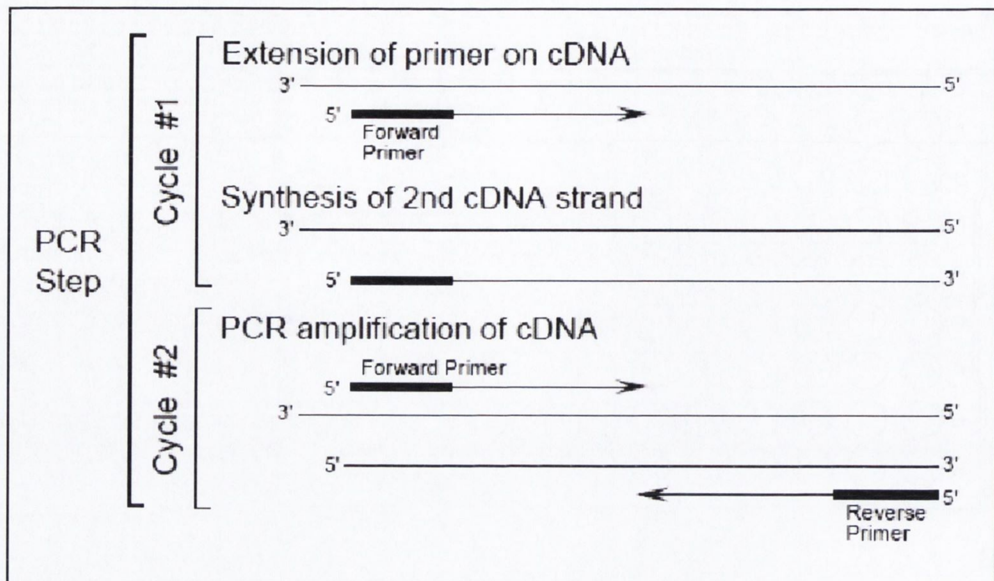


Figure 4.ii: The PCR reaction.

The PCR reaction exploits the 5´ nuclease activity of AmpliTaq Gold® DNA Polymerase to cleave a TaqMan® probe during PCR (Figure 4.ii). The TaqMan probe incorporates a VIC reporter dye at the 5´ end of the probe and a quencher dye at the 3´ end of the probe.

During the reaction, cleavage of the probe separates the VIC reporter dye and the quencher dye, which results in increased fluorescence of the reporter.

Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye as shown in figure 4.iii.

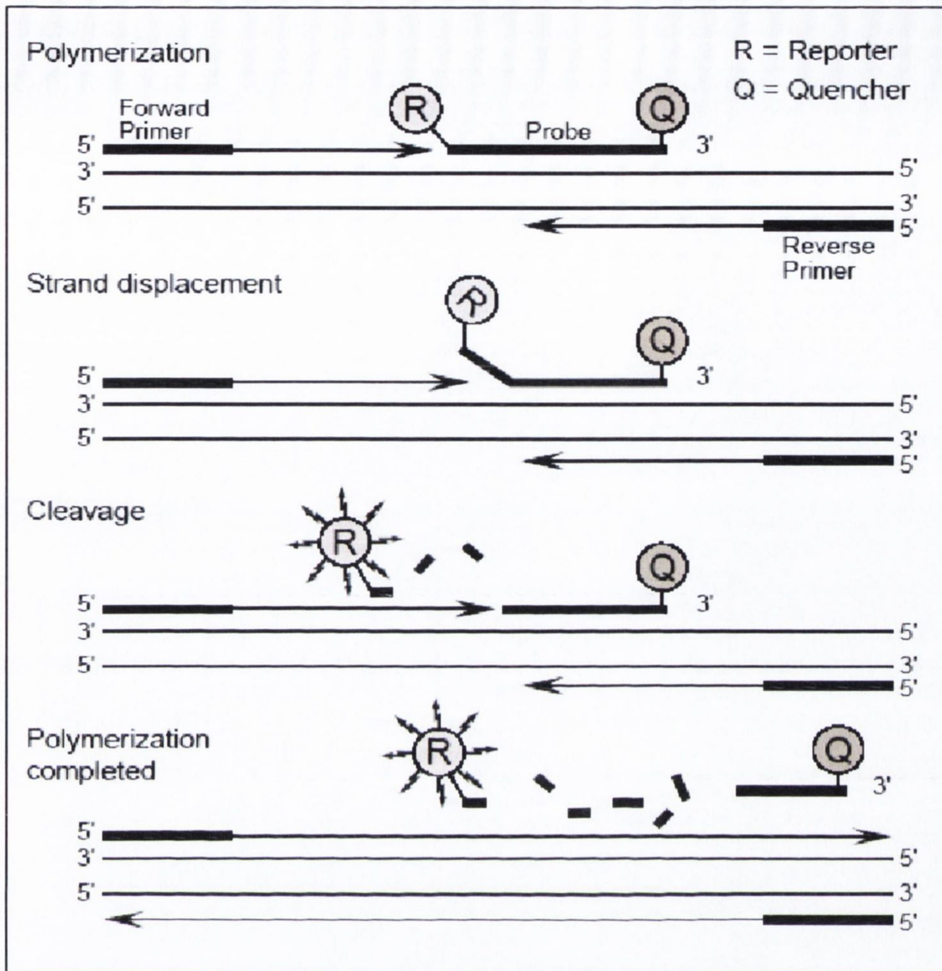


Figure 4.iii: Detecting accumulation of PCR products

When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence. During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites.

The 5′→3′ nucleolytic activity of the AmpliTaq Gold® DNA Polymerase cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3′ end of the probe is blocked to prevent extension of the probe during PCR.
This process occurs in every cycle and does not interfere with the exponential accumulation of product.

The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, any nonspecific amplification is not detected.

4.3: AIMS

To identify the most appropriate endogenous control to use with oesophageal samples.

To study the expression of a subset of genes identified in the microarray study that differentiated responders from non-responders, using Real Time Quantitative PCR.

To correlate the expression levels between the two experiments.

4.4: MATERIALS and METHODS

4.4.1: Generation of cDNA

4.4.1.1: Principle

The Invitrogen protocol version 3.0 was used (Invitrogen, UK.). First strand cDNA is generated from 1μg total RNA by reverse transcription. This process uses random hexamers to prime the RNA and M-MLV reverse transcriptase to convert single stranded RNA into a double stranded RNA / cDNA hybrid. RNA degrades very easily with handling and as such, it should be handled to a minimum, and kept on ice as much as possible. Samples are processed in batches of 10.

4.4.1.2: Preparation

- cDNA is prepared in the class II safety cabinet. Before starting, the cabinet should be wiped clean with ethanol and irradiated with *UV* light for 20 minutes.
- Remove the following kit reagents from a -20°C freezer: RT 5X Buffer, dNTP mixture (10mM), Random hexamer primers (3µg/µl), 0.1M DTT, nuclease-free water, and allow to thaw in the safety cabinet.
- Calculate the volume of RNA required to obtain 1µg as follows:

  1µg = 1/1000 x RNA concentration (ng/µl)
- The above volumes of RNA should be ≥1µl and ≤11.7µl. If the RNA concentration exceeds 900ng/µl, a 1/10 dilution of the neat RNA should be made by adding 9µl of RNase-free water to 1µl of RNA. The diluted sample should then be used to calculate the volume of 1µg.
- Calculate how much RNase free water needs to be added to each sample to make a total volume of 11.67µl (i.e. 11.67 minus volume of 1µg of RNA).
- If the volume of 1µg of RNA exceeds 11.67µl, the sample concentration is below 1µg in 11.67µl the maximum volume of RNA is used (11.67µl). The corresponding amount or RNA in ng is noted. Alternatively the RNA sample can be concentrated.
- Remove the RNA samples from the –80°C freezer and allow them to thaw slowly on ice in a class II safety cabinet.
- Into the bottom of each 0.2µl tube place the amount of nuclease-free water calculated in the previous step. Add the calculated amounts of 1µg of each RNA corresponding tube.
- Make an 11X (for 10 samples) premix of the following reagents in a 0.2ml tube:

	1X	11X
Random Primers (3µg/µl)	0.33µl	3.63µl
dNTP mix (10mM)	1µl	11µl

- Add 1.33µl of the above mixture to tubes 1-10. Incubate at 65°C for 5 minutes.

- Make an 11X premix of the following reagents in a 0.5ml microcentrifuge tube:

	1X	11X
5X RT Buffer	4μl	44μl
DTT (0.1M)	1μl	11μl
RnaseOut (40U/μl)	1μl	11μl
SuperScript III (200U/μl)	1μl	11μl

  Mix all of the reagents by gentle pipetting and place the mixture on ice.
- Add 7μl of the above mixture to tubes 1-10, opening only one lid at a time and mix by pipetting. Place the samples in the 2720 thermal cycler and run at: 25°C for 5mins, 50°C for 60 minutes, 70°C for 15 minutes followed by indefinite 4°C soak.
  Note: Change tips between each sample.
- When the reaction is completed, remove the tubes from the thermal cycler and spin down any condensation.
- To each tube add a further 30μl of RNase-free water to make up volume to a total: 50μl
- Store samples until required in a –20°C RNA freezer.

Appendices

Requisition of SuperScript III Reagents

Invitrogen Cat No 18080-093 2,000 units

Invitrogen Cat No 18080-044 10,000 units

Invitrogen Cat No 18080-085 4 x 10,000 units

(Includes 5X buffer and 0.1M DTT)

Requisition of RNAseOut (40U/ml)

Invitrogen Cat No 10777-019 5,000 units

Random Primers 3mg/ml

Invitrogen Cat# 48190-011

4.4.2: Real Time Quantitative PCR

4.4.2.1: Selection of Endogenous Control

Having obtained informed consent biopsy material was taken from three different oesophageal tumours and one benign specimen. RNA was extracted according to the protocol (section 3.4.7)

- Having created cDNA (1µg/20µl) as described in section 4.4.1.2 the products are synthesized from these samples using the TaqMan Universal PCR Master Mix (No UNG).
- Pipette 650µl of TaqMan Universal PCR Master Mix (No UNG) into each of 4 microcentrifuge tubes (3 test tumour samples and 1 benign calibrator sample).
- Dilute the 3 test samples and the calibrator sample from the cDNA step to a volume of 650µl with RNase-free water in separate microcentrifuge tubes.
- Combine the 2 tubes and mix by gentle inversion.
- Centrifuge the tubes to spin down the contents and eliminate air bubbles from the solution
- Transfer by pipette 50µl aliquots to the wells of the endogenous control plate (figure 4.iv)

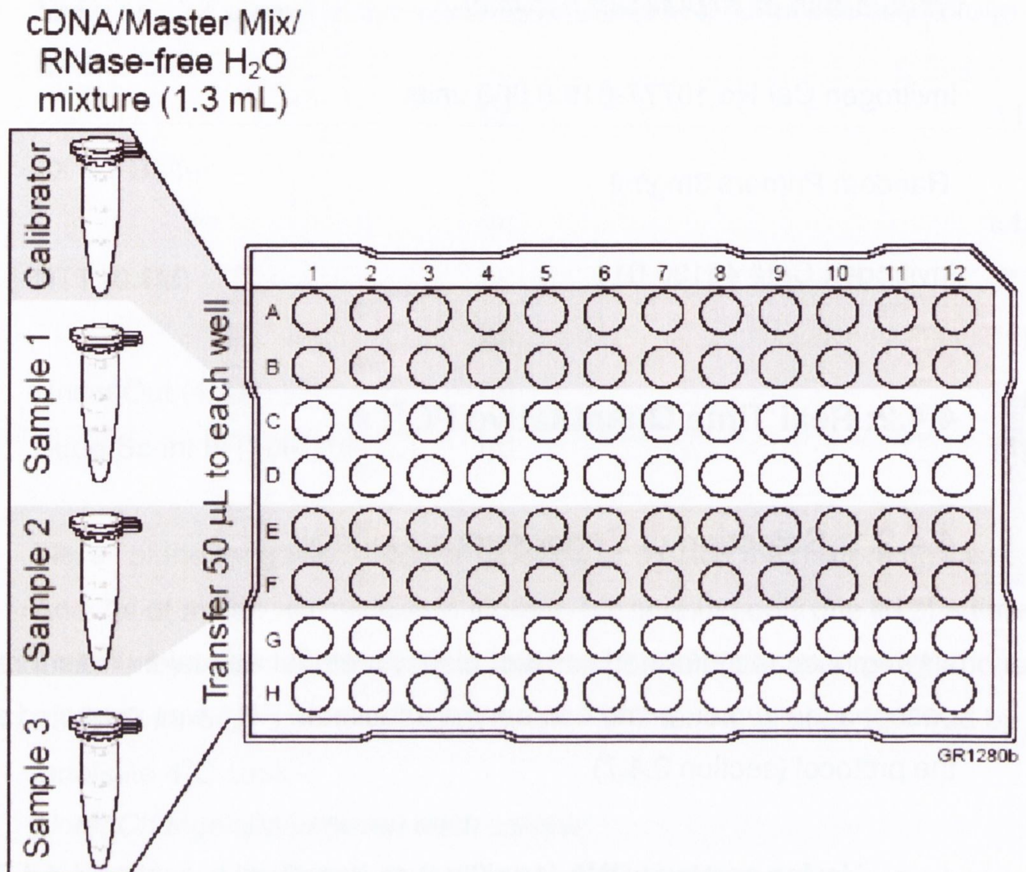


Figure 4.iv: Loading the endogenous control plate. Calibrator = Normal oesophageal tissue, Samples 1, 2 and 3 = three different oesophageal tumour specimens.

- Seal the wells with optical caps
- Briefly centrifuge the 96-well plate to eliminate air bubbles
- Load the plate into ABI Prism 7700 Sequence Detection system and run the programme (95°C for 10mins and 40 cycles of 95°C for 15 seconds/60°C for 1 minute).

Relative expression of the marker genes was calculated using the $2^{-\Delta\Delta C_T}$ method previously described[161].

4.4.2.2: Selecting Targets to Validate

A number of steps were taken before deciding the final targets with which to validate.

- Genes that were not differentially expressed between responders and non-responders at the $p<0.01$ level were excluded
- From this list genes that were either less than two-fold up or down regulated were then excluded
- Any gene that did not have a designated name or symbol assigned was excluded

These steps left a final list of 64 genes (see Microarray Results 3.5.4.2)

- Any gene whose biological function or process or biological pathway was significantly up or down regulated ($p<0.05$) in responders versus non-responders was included
- A PubMed[171] search was performed to identify which, if any, of these genes were reportedly involved in either gastro-intestinal malignancies or in responsiveness to chemotherapy and/or radiotherapy.
- The remainder of the targets were chosen if they were highly significantly under or over expressed ($p<0.005$)

In total 12 targets were selected to validate the array data.

4.4.2.3: PCR

4.4.2.3.1: Preparation

Order primers and probes from Applied Biosystems' pre-designed TaqMan® Gene Expression Assays.

Generate worksheets (Figure 4.v) to correspond with TaqMan® PCR validation ABI PRISM 7900 Sequence Detection System (Applied Biosystems, CA, USA)

- The targets (in triplicate) and the endogenous control (in duplicate) are represented vertically
- Each tumour sample is allocated a row
- One row is reserved for the cDNA of a normal specimen. This acts as a calibrator.
- One row will have no cDNA in its wells. This acts as a control

4.4.2.3.2: The Process

- PCR is carried out in a class II safety cabinet. Before starting, the cabinet should be wiped clean with ethanol and irradiated with *UV* light for 20 minutes.
- Remove the following kit reagents from a -20°C freezer: nuclease-free water, pre-prepared cDNA, primers and probes and allow to thaw in the safety cabinet.
- In a 500µl tube create a pre-mix of 5µl TaqMan Universal PCR Master Mix (No UNG), 0.5µl of the target and 3.5µl nuclease-free water sufficient to fill two columns of a 384-well plate
- Mix by gentle inversion and briefly centrifuge
- Carefully pipette 9µl of the pre-mix into the corresponding well on a 384 well plate (Figure 4.v)
- Repeat the same step for each target
- Once all appropriate wells are filled pipette 1µl of cDNA into each well of the row pertaining to that sample
- Seal the plate with clear adhesive film ensuring no gaps
- Briefly centrifuge
- Load into ABI PRISM 7900 Sequence Detection System (Applied Biosystems, CA, USA)
- Perform PCR: 40 cycles of 15 seconds melt at 95°C and 60 seconds anneal/extend at 60°C

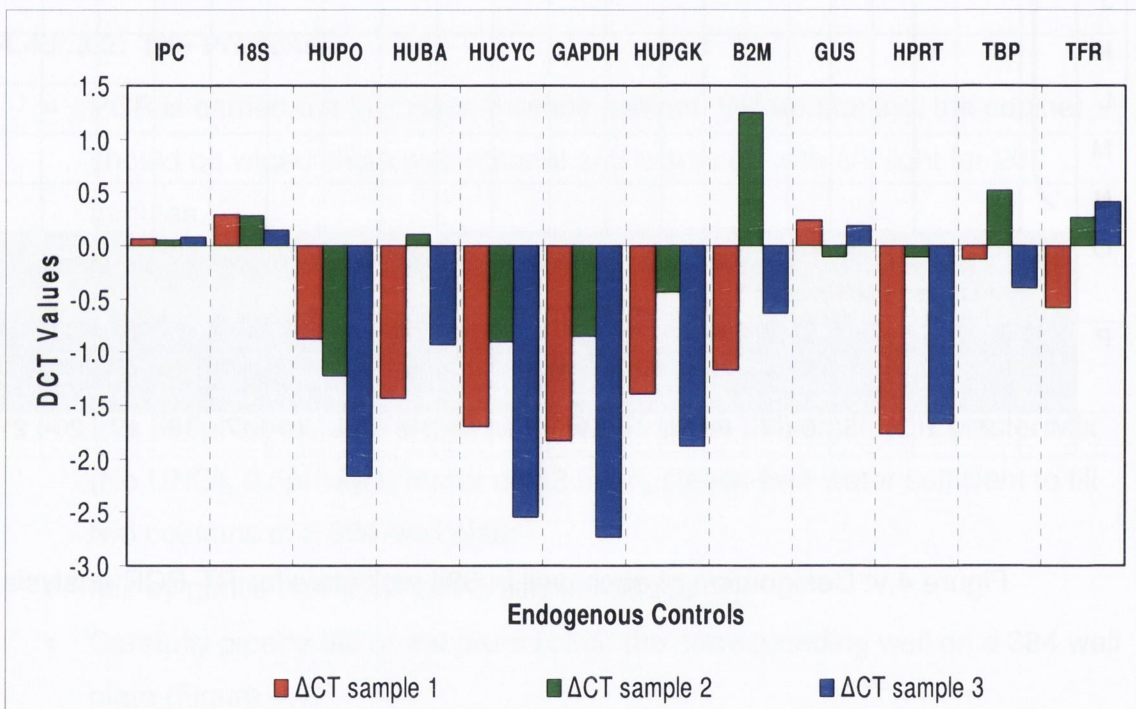
	Targets																						18s	
A																								
B																								
C																								
D																								
E																								
F																								
G	Tumour cDNA samples																							
H																								
I																								
J																								
K																								
L																								
M																								
N																								
O	cDNA from normal specimen - calibrator																							
P	No cDNA - control																							
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24

Figure 4.v: Designation of each well in 384 well plate for RT-PCR analysis.

4.5: RESULTS

4.5.1: Endogenous Control Plate

The results of the endogenous control plate are expressed in ΔC_T greater than or less than the calibrator (normal oesophageal tissue). Thus, the calibrator serves as a baseline for the assay and is shown as zero on graph 4.i.



Key: IPC – internal positive control, 18s – 18s ribosomal RNA, HUPO – acidic ribosomal protein, HUBA – beta actin, HUCYC - cyclophilin, GAPDH – glyceraldehyde-3-phosphate dehydrogenase, HUPGK - phosphoglycerokinase, B2M – β2-microglobulin, GUS – β-glucuronidase, HPRT – hypoxanthine ribosyl transferase, TBP – transcription factor IID-TATA binding protein, TFR – transferring factor.

Graph 4.i: Samples 1-3 represent different oesophageal tumour sample CT values minus benign oesophageal calibrator sample CT value. Samples with positive values have initial template concentrations higher than that of the calibrator sample, whilst those with negative values have lower concentrations.

The expression of 18S ribosomal RNA and GUS remained relatively constant across the test (mean (SD): 0.24 (±0.08) and 0.10 (±0.18) respectively). Both assays produced ΔC_T values that deviated little from zero, indicating a fairly stable level of gene expression relative to the other candidate controls. In contrast B2M and GAPDH (-0.19 (±1.27) and -1.80 (±0.95) respectively) varied widely. Since 18S ribosomal RNA varied the least it was chosen as the endogenous control for the subsequent validation PCR experiments.

4.5.2: Validation Targets

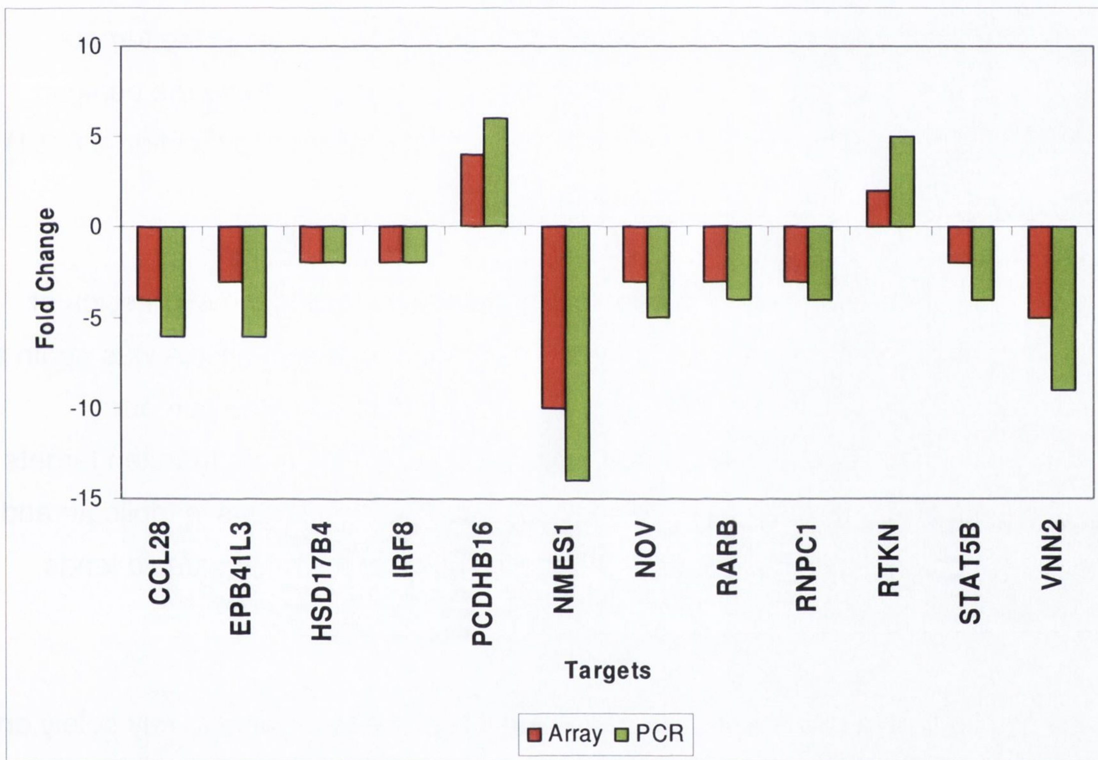
Twelve targets were selected following the methods described in section 4.4.2.2. Ten were down-regulated (2.0-9.5 fold) in good responders and two were up-regulated (2.4-3.7 fold). All of them were highly significantly differentially expressed ($p \leq 0.008$) and most had not previously been reported in oesophageal cancer. Table 4.i lists these genes and, separate from the significance level and fold change, for what other reasons they were chosen.

Table 4: Depicting the 12 targets that were chosen for validation.

Applied Biosystems Probe ID	p value	Gene Name	Symbol	Why chosen
162989	0.0020	chemokine (C-C motif) ligand 28	CCL28	p<0.005 and identified in colorectal cancer[172]
204928	0.0016	erythrocyte membrane protein band 4.1-like 3	EPB41L3	p<0.005. Methylation of gene associated with poor prognosis in non-small cell lung cancer[173]
118713	0.0007	hydroxysteroid (17-beta) dehydrogenase 4	HSD17B4	p<0.001 and molecular function significantly involved at p<0.05
141589	0.0012	interferon regulatory factor 8	IRF8	p<0.005 and molecular function significantly involved at p<0.05
156268	0.0026	protocadherin beta 16	PCDHB16	p<0.005
205161	0.0081	normal mucosa of esophagus specific 1	NMES1	Previously described in oesophageal SCC[154]
183094	0.0025	nephroblastoma overexpressed gene	NOV	p<0.005. Low levels associated with poor prognosis in breast cancer[174]
109692	0.0049	retinoic acid receptor, beta	RARB	p<0.005. expression progressively lost during oesophageal carcinogenesis[175]
215065	0.0008	RNA-binding region (RNP1, RRM) containing 1	RNPC1	p<0.001 and molecular function significantly involved at p<0.05
164929	0.0063	rhotekin	RTKN	confers survival in gastric adenocarcinoma[176]
123880	0.0040	signal transducer and activator of transcription 5B	STAT5B	p<0.005 and molecular function significantly involved at p<0.05. May promote survival in melanoma[177]
174652	0.0032	vanin 2	VNN2	p<0.005. Up-regulated in human metastasizing oesophageal squamous cell cancer cell line[178]

4.5.3: PCR

Sufficient RNA with which to perform RT-PCR remained from 10 of the initial 13 samples. The microarray results were validated using a reverse transcription reaction followed by TaqMan® PCR for 12 gene targets. The $2^{-\Delta\Delta CT}$ method was used to analyse relative gene expression data. 18s was used as an endogenous control and a benign oesophageal specimen was chosen as an arbitrary calibrator sample. Gene expression profiles for TaqMan® PCR were plotted in conjunction with those for microarray results in Graph 4.ii. The Pearson co-efficient comparing data from microarray analysis and TaqMan® RT-PCR was 0.788.



Graph 4.ii: TaqMan® PCR validation of microarray experiments.

Profile bar charts of gene expression levels comparing results obtained by microarray analysis (n=13) to TaqMan® PCR analysis (n=10).

4.6: DISCUSSION

By selecting 12 of the genes that were most differentially regulated between good and poor responders to CRT and performing RT-PCR on the original RNA we have identified reassuring consistency between the two laboratory techniques. Additionally this step was preceded by a careful assessment of the most appropriate endogenous control. It is accepted that the data would have, perhaps, carried more significance had the relative expression been confirmed on RNA taken from different oesophageal tumour samples. However the paucity of fresh frozen diagnostic biopsies precluded such an analysis. It is unfortunate that there was insufficient material to perform RT-PCR on all the tumour samples (10 out of 13), which meant that we were also unable to validate the benign versus malignant array data. The problem lies, as previously alluded to (section 3.6.2.1) in obtaining adequate quantities of good quality biopsy material.

The data would have presumably also been strengthened by testing the expression of more (than 12) differentially expressed targets. The amount of residual RNA was again a limiting factor, but there would seem to be no agreed standard as to the number of targets with which to validate. Many publications report using little more than ten targets and some use as few as three[96]. That we performed our PCR on targets in triplicate and endogenous control in duplicate, coupled with the inclusion of a benign sample lends additional credence to our findings.

As already highlighted in this chapters' introduction it is probably unwise to rely solely on the genes generated by individual microarray platforms and some form of validation is essential before the information can translate into clinical practice. Additionally there remains on-going controversy about the reliability of the data generated not only between different platforms but also between different laboratories[179,180]. Millenaar et al evaluated gene expression with six different platforms using the same data. They found that different genes were differentially regulated depending on the method used to such an extent that there was only a 27-36% overlap. Similar findings have been identified by other groups, whilst conversely others have found encouraging consistency[181]. It is unlikely that these issues are going to be readily resolved and consequently the use of arrays should probably best be seen as a stepping stone to aid in the search for critical

genes and pathways involved in a particular disease entity. Thereafter a panel of genes, following international and inter-laboratory collaboration ideally, should be formally tested under the auspices of randomised phase III clinical trials such as is occurring in breast cancer following van't Veer's seminal work[182,183].

CHAPTER 5

Correlation of Early Quantitative changes in FDG-PET Uptake with Tumour Regression grade in patients with Localised Oesophageal cancer undergoing Neo-adjuvant Chemoradiation.

5.1: SUMMARY

In an attempt to identify the clinical utility of ^{18}FDG PET as a predictive tool in the management of localised oesophageal cancer scans were performed at diagnosis and following the first week of neoadjuvant chemoradiation. The initial value of two parameters; the mean maximum standardised uptake value (SUV) and the volume of metabolically active tissue were recorded. The change between these and those taken following one week of pre-operative therapy was compared with the final tumour regression grade (TRG). Pathological responders to treatment were deemed those who achieved a TRG of 1-2, whilst non-responders were those with a score of 3-5.
Neither the diagnostic values nor the change following treatment significantly predicted the response of the tumour to chemoradiation.

5.2: INTRODUCTION

The traditional method of assessing cancer response is by measuring changes in lesion size, but unless the target lesions disappear, there are numerous difficulties. First, a "lesion" seen on an image may not be entirely composed of malignant cells. Consequently a change in size does not necessarily mean regression or progression of disease. In a series of pulmonary neoplasms, a reactive zone ranging from 2% to 48% of the lesion diameter was documented[184]. Secondly, a tumour may respond to treatment without an obvious change in its size if the cancer is replaced by fibrosis or necrosis. Finally the measurement of change may not always be reliable. Viability after treatment is a metabolic, not a geometric, characteristic of a lesion. As imaging becomes more able to depict biochemical and molecular biology features, the options for cancer diagnosis and monitoring are expanding rapidly; particularly as knowledge accumulates about the ways in which malignant and non-malignant tissue differ chemically.

The first major success in metabolic imaging dates back to the discovery, by the biological chemist Otto Warburg in the 1920's, that cancer cells generate most of their energy by glycolytic metabolism of glucose, whereas benign cells employ aerobic metabolism. Glycolysis is inefficient: cancer cells need to increase their

metabolic rate dramatically to obtain enough energy to support themselves and their rapid replication.

Like glucose, FDG is taken up in abundance by most cancer cells and phosphorylated to FDG-6-phosphate. Unlike glucose-6-phosphate, however, FDG-6-phosphate cannot be processed further by glycolysis, nor can cancer cells de-phosphorylate it quickly, so it is trapped in the cells for detection by PET.

The utility of PET in detecting and staging many types of cancer, including oesophageal, is well established[185,186] and increasingly it is being used to monitor treatment. In 22 patients with advanced breast cancer, FDG uptake by drug-responsive tumours had fallen below 55% of the baseline value as early as the first course of chemotherapy[187]. The eventual histopathologic response of a given cancer could be predicted with an accuracy of 88% after the first course of drugs and 91% after the second course. In another series of 30 women with large or locally advanced breast cancers, the mean pre-treatment dose uptake ratio (DUR) was significantly higher in the tumours that responded completely to chemotherapy, and there was a correlation between the amount of decline in the DUR and the extent of the tumour response. In this series, PET scans after the first course were able to predict the eventual response with a sensitivity of 90% and a specificity of 74%[188].

Similar studies have been performed in oesophageal cancer (table 1.i). In the main, the second PET scan has been performed *after* the completion of the neoadjuvant phase of therapy[109,111,112,113,114,116,117,119]. It would be more clinically useful if a prediction of response could be identified at an earlier time-point. This would spare those who were not going to respond from the inherent toxicities of chemoradiation and allow their treatment to be tailored more individually. Only four published studies to date have performed the second scan during pre-operative treatment; three where the patients (all adenocarcinoma) received chemotherapy only[108,110,115] and the other where the patients (all squamous cell carcinoma) received chemoradiation[118]. In this study patients with oesophageal cancer (adenocarcinoma or squamous cell carcinoma) scheduled to receive neoadjuvant chemoradiation had PET scans performed at diagnosis and following the first week of treatment.

5.3: AIMS

To determine whether the initial ^{18}FDG-PET scan in patients scheduled to receive neoadjuvant chemoradiation for localised oesophageal cancer could predict the final tumour regression grade identified following surgery.

To determine whether the changes seen on ^{18}FDG-PET images between diagnosis and following the first week of chemoradiation could predict those patients that ultimately respond well to neoadjuvant therapy and those that respond poorly.

5.4: METHODS

5.4.1: Patients and Treatment

Between January 2003 and October 2005, 41 consecutive patients with histologically confirmed oesophageal carcinoma scheduled to undergo multimodal therapy had a PET scan at diagnosis and following the first week of CRT. Pre-treatment investigations also included computerized tomography of the neck, thorax and abdomen, and oesophago-gastroscopy. The criteria for inclusion in the multimodal protocol was as previously described (Chapter 3, Section 4.1)

The neo-adjuvant protocol and surgical procedure was as previously described (Chapter 3, Section 4.2 and Figure 3.ix).

5.4.2: PET Imaging

PET scans were performed on all patients as part of their staging and in the and in the week following completion of the induction combination chemotherapy and radiation therapy. The PET images were acquired on a high-resolution dedicated

PET scanner 47-78 minutes after intravenous injection of 340–450 MBq of fluorine-18-flurodeoxyglucose (^{18}F-FDG). In so far as was possible, the scanning conditions were kept constant facilitating comparison of the pre-treatment and intra-treatment scans i.e. same acquisition protocol, reconstruction algorithm and uptake time (mean $\Delta t = 3.9$ min). Patients fasted for 6 hours prior to imaging to ensure that serum glucose and endogenous serum insulin levels were low at the time of FDG administration. Blood glucose levels were measured before each PET scan. Whole body scans extending from base of skull to mid thigh were obtained in 2-D mode on either the GE supplied *PET Advance* scanner or *Discovery-ST* PET/CT scanner. The images were reconstructed using ordered subsets expectation maximum (OSEM) iterative reconstruction.

Semi-quantitative measurements of metabolic uptake in FDG-avid tumours following pre-treatment and intra-treatment scans were compared and evaluated for their potential to predict histopathological response to CRT. The tumour FDG uptake was measured using a region of interest (ROI) method employed by Stahl et al[189]. Essentially a cylindrical ROI with a diameter of 1.5 cm was manually placed over the tumour site on the hottest trans-axial slice, avoiding the edges of the tumour. The mean activity concentration within the ROI was determined and expressed as the SUV, where SUV is the ratio of the activity in the tissue to the decay-corrected activity injected into the patient. This technique combines the advantages of little interference by statistical count rate fluctuations and little influence by non-viable tumour zones or partial volumes effects. The other parameter we studied was the volume of metabolically active disease. This was selected by choosing a threshold SUV value, in which only voxels with SUV values greater than or equal to the selected threshold were included in the volume. All SUV measurements were normalised for patient body weight (SUV). The relative changes in tumour SUVs between baseline and follow-up were calculated and correlated with subsequent histopathological tumour response to therapy.

The percentage change (Δ) in each of the parameters (P) between diagnosis (pre) and during treatment (intra) was calculated using the following formula:

$\Delta P = \{[P_{intra} - P_{pre}]/P_{pre}\} \times 100$

A negative value indicated a reduction in that parameter following therapy and a positive value indicated an increase.

5.4.3: Histology

All the surgical specimens were classified by one experienced pathologist who was unaware of the clinical and PET data and who graded and staged the specimens in accordance with the criteria of the International Union Against Cancer and the American Joint Committee on Cancer Staging[125]. Tumour response to treatment was classified according to the criteria described by Mandard et al[41]. The patients were then subdivided into groups for analysis: responders (TRG 1-2) versus non-responders (TRG3-5).

5.4.4: Statistical Analysis

A statistical analysis was performed using commercial software SPSS for Windows (version 12.0). Intra-individual comparisons were performed using the Wilcoxon signed-rank test and inter-individual comparisons using Wilcoxon/Kruskal-Wallis.

5.5: RESULTS

The basic demographics are shown in Table 5.i. The median age was 59, there was a male preponderance, and most patients had adenocarcinoma. Most patients had clinical Stage 2 disease.

Table 5.i: Patient Characteristics

Male/female		34/7
Mean Age (range)		59 (37-75)
Pathology	Squamous cell	7
	Adenocarcinoma	34
Grade	Well Differentiated	2
	Moderately differentiated	8
	Poorly differentiated	19
	Not evaluable	2
Primary Site	Middle	4
	Lower	22
	OG Junction	15
Clinical Stage	II	35
	III	6
Radiotherapy (RT) Dose	44Gy in 22 fractions	12
	40.05Gy in 15 fractions	29
Fractions of RT completed before 2nd PET	Mean (range)	7 (5-10)
*Days following CRT before surgery	Mean (range)	55 (31-76)

*excluding 4 who did not proceed to surgery

Thirty seven of 41 patients proceeded to surgery. Of the four who did not it was due to disease progression in two and deterioration in performance status in the other two. We analysed the data in two ways; first designating a TRG of 1-2 as responders with the rest non-responders and second designating TRG of 1-3 as responders in keeping with the original Mandard paper[41].

5.5.1: TRG 1-2 versus 3-5 (Table 5.ii and Figures 5.iv and 5.v)

Of the 37 patients who proceeded to surgery, 9 (24%) achieved a total or near total response (TRG 1 or 2) and 26 (74%) had less or no response (TRG 3, 4 or 5). In one of the patients the tumour size was below the spatial resolution of PET both before and during treatment, and this data was excluded. In three patients a marked inflammatory response made analysis of the second PET scan unreliable. In the fifth patient whose data had to be excluded, the initial injection extravasated and made interpretation of the images unreliable. The one-year survival in the TRG1-2 group was 87% compared with 67% in TRG 3-5 (p=0.091).

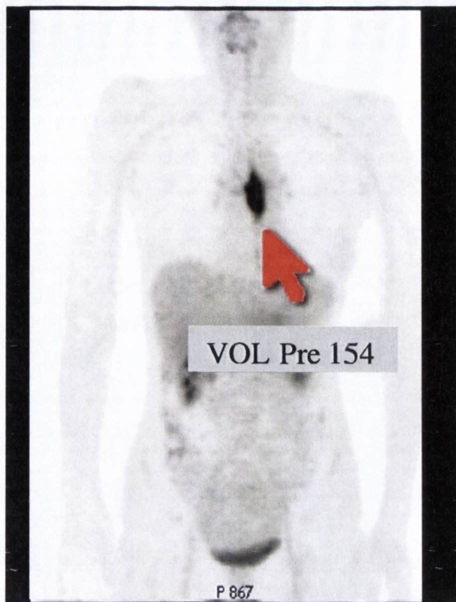
In the evaluable responder group (9/32) the mean (standard deviation) maximum SUV fell from 12.6 (6.3) pre-treatment to 8.1 (2.9) following one week of chemoradiation (p = 0.070). In the evaluable non-responders (23/32) the results were 9.7 (5.4) and 7.1 (3.8) respectively (p=0.003). The mean volume in good responders fell from 36.6cm^3 (22.7) to 22.3cm^3 (10.4) during treatment (p=0.180), whilst in poor responders this fell from 35.9cm^3 (36.7) to 31.9cm^3 (52.7) (p=0.405). The change in mean maximum SUV pre treatment and after induction CRT was not significantly different between responders and non-responders (p = 0.645). Similarly the change in metabolically active tumour volume pre-treatment and after induction CRT was not significantly different between responders and non-responders (p = 0.305). There was a mean reduction of mean maximum SUV in good and poor responders of 25.2% and of 22.3% respectively (p=0.902). There was a mean reduction in volume in good and poor responders of 30.4% and of 15.1% respectively (p=0.621).

In an attempt to identify a threshold above or below which response could be more accurately predicted a reduction of more than 20% in each of the parameters was used as a criterion. The positive predictive values were 27% and 35% for changes in mean maximum SUV and volume respectively. The negative predictive values were 71%, and 80% respectively for the same parameters. No other cut-off value was found to differentiate responding from non-responding tumours better. If we analysed the initial (pre-treatment) scores it was clear that neither parameter significantly predicted the ultimate pathological response.

5.5.2: TRG1-3 versus TRG4-5 (Table 5.ii and Figure 5.iv and 5.v)

Analysing the data in this way there were 27 evaluable responders and 5 non-responders. In the responder group the mean (standard deviation) maximum SUV fell from 10.6 (5.7) pre-treatment to 7.5 (3.5) following one week (p = 0.002). In non-responders the results were 10.1 (6.3) and 6.7 (4.2) respectively (p=0.125). The mean volume in good responders fell from 32.7cm^3 (26.8) to 22.3cm^3 (24.3) during treatment (p = 0.124), whilst in poor responders this fell from 54.5cm^3 (57.2) to 66.0cm^3 (99.1) (p=0.893). The change in mean maximum SUV pre treatment and after induction CRT was not significantly different between responders and non-responders (p = 0.640). Similarly the change in metabolically active tumour volume pre-treatment and after induction CRT was not significantly different between responders and non-responders (p = 0.517). There was a mean reduction of mean maximum SUV in good responders of 21.6% and of 31.4% in poor responders (p=0.479). There was a mean reduction in volume in good responders of 21.6% and of 7.8% in poor responders (p=0.841).

In an attempt to identify a threshold above or below which response could be more accurately predicted a reduction of more than 20% in each of the parameters was used as a criterion. The positive predictive values were 80% and 88% for changes in mean maximum SUV and volume respectively. The negative predictive values were 12%, and 20% respectively for the same parameters. No other cut-off value was found to differentiate responding from non-responding tumours better. If we analysed the initial (pre-treatment) scores it was clear that neither parameter significantly predicted the ultimate pathological response.



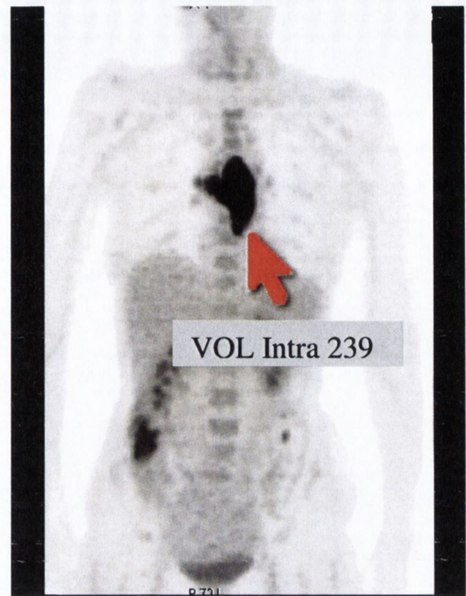
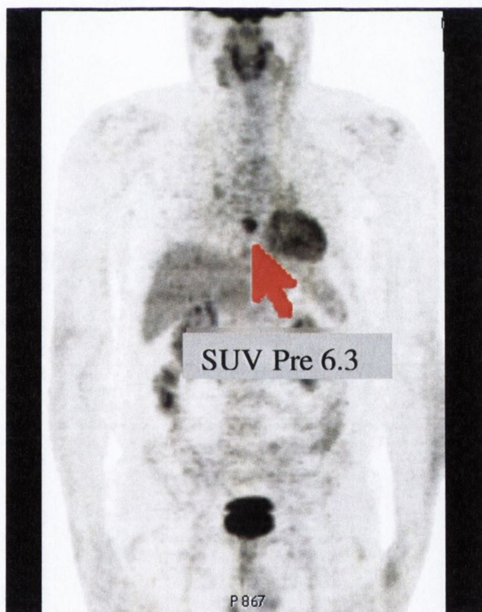


Figure 5.ii: Non-responder (patient 29) demonstrating increase in volume (VOL) from before (PRE) to during (Intra) chemoradiation



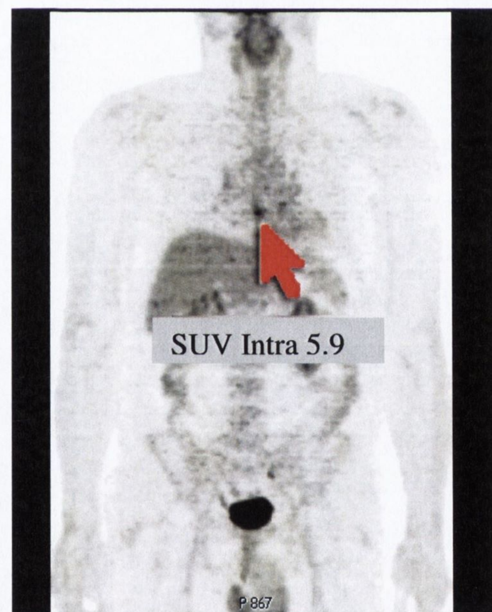


Figure 5.iii: Responder (patient 1) demonstrating reduction in SUV from before (PRE) to during (Intra) chemoradiation

Table 5.ii: Evaluable patients – clinical stage, pathological response and FDG uptake

Patient	Pre-CRT Clinical Stage	TRG	Stage	SUV Pre	SUV Intra	Reduction in SUV (%)	VOL Pre	VOL Intra	Reduction in Volume (%)
1	T3N0M0	1	ypT0N0	6.3	5.9	6.4	24.6	19.2	21.9
2	T3N0M0	1	ypTxN0	6.1	3.5	42.6	13.4	3.7	72.1
3	T3N1M0	1	ypT0N0	12.8	11.5	10.4	35.6	34.5	3.2
4	T3N0M0	1	ypT0N0	12.8	12.8	0.0	35.3	22.4	36.5
5	T3N0M0	1	ypT0N0	9.8	9.5	3.0	19.6	20.6	-5.0
6	T3N0M1a	2	ypT3N0	5.1	5.9	-15.7	13.2	16.2	-23.2
7	T3N0M0	2	ypT1N0	19.2	8.9	53.4	80.0	33.0	58.8
8	T3N0M0	2	ypT3N0	21.4	8.3	61.2	61.6	35.3	42.7
9	T3N0M0	2	ypT1N0	19.6	6.8	65.3	46.0	15.5	66.7
10	T3N0M0	3	ypT3N1	19.7	8.6	56.4	48.5	13.7	71.7
11	T3N1M0	3	ypT3N1	10.0	4.8	52.0	25.4	4.7	82.0
12	T3N0M0	3	ypT3N0	9.5	7.0	26.3	25.4	10.1	60.1
13	T3N0M0	3	ypT3N1	20.9	18.3	12.4	95.9	87.6	8.7
14	T3N1M0	3	ypT2N1	12.4	4.7	62.1	22.3	5.5	75.4
15	T3N1M0	3	ypT2N0	12.6	11.7	7.0	4.6	5.8	-25.3
16	T3N0M0	3	ypT3N1	6.4	5.9	7.8	10.2	5.8	43.0
17	T3N0M0	3	ypT3N0	6.0	7.3	-21.7	17.7	13.3	24.9
18	T3N0M0	3	ypT3N0	5.7	4.5	21.1	22.9	28.3	-23.4
19	T2N1M0	3	ypT3N1	9.3	4.7	49.5	46.6	15.9	66.0
20	T3N0M0	3	ypT3N1	4.3	3.8	11.6	7.9	7.1	10.8
21	T3N0M0	3	ypT2N1	5.6	5.7	-1.8	11.6	23.6	-102.5
22	T3N0M0	3	ypT3N0	8.2	8.5	-3.7	21.3	22.6	-6.2
23	T3N0M0	3	ypT3N1	4.4	3.2	27.3	26.0	12.4	52.3
24	T3N0M0	3	ypT3N0	19.1	13.0	31.9	53.3	19.6	63.2
25	T3N0M0	3	ypT3N1	6.2	5.1	17.7	7.6	10.9	-43.3
26	T3N0M0	3	ypT2N1	4.3	4.3	0.0	3.5	4.9	-41.2
27	T3N0M0	3	ypT3N1	8.7	8.7	0.0	103.7	111.3	-7.3
28	T3N0M0	4	ypT3N0	6.2	3.5	43.6	16.9	0.0	100.0
29	T3N0M0	4	ypT4N1	20.8	11.9	42.8	154.0	239.0	-55.2
30	T3N0M0	4	ypT3N1	10.5	10.5	0.0	32.4	58.2	-79.6
31	T3N1M0	4	ypT3N0	7.8	2.9	62.8	49.8	13.9	72.1
32	T3N0M0	4	ypT3N1	5.2	4.8	7.7	19.2	18.9	1.7

Key: FDG – fluorodeoxyglucose, CRT – chemoradiation, TRG – tumour regression grade, SUV – mean maximum standardised uptake value, Pre – before CRT, Intra – during chemoradiation, VOL – metabolically active volume

Figure 5.iii: Percentage change in mean maximum SUV (A) and metabolically active volume (B) from pre- to during CRT relative to differing pathological response groups. TRG, tumour regression grade

Figure 5.iv.A:

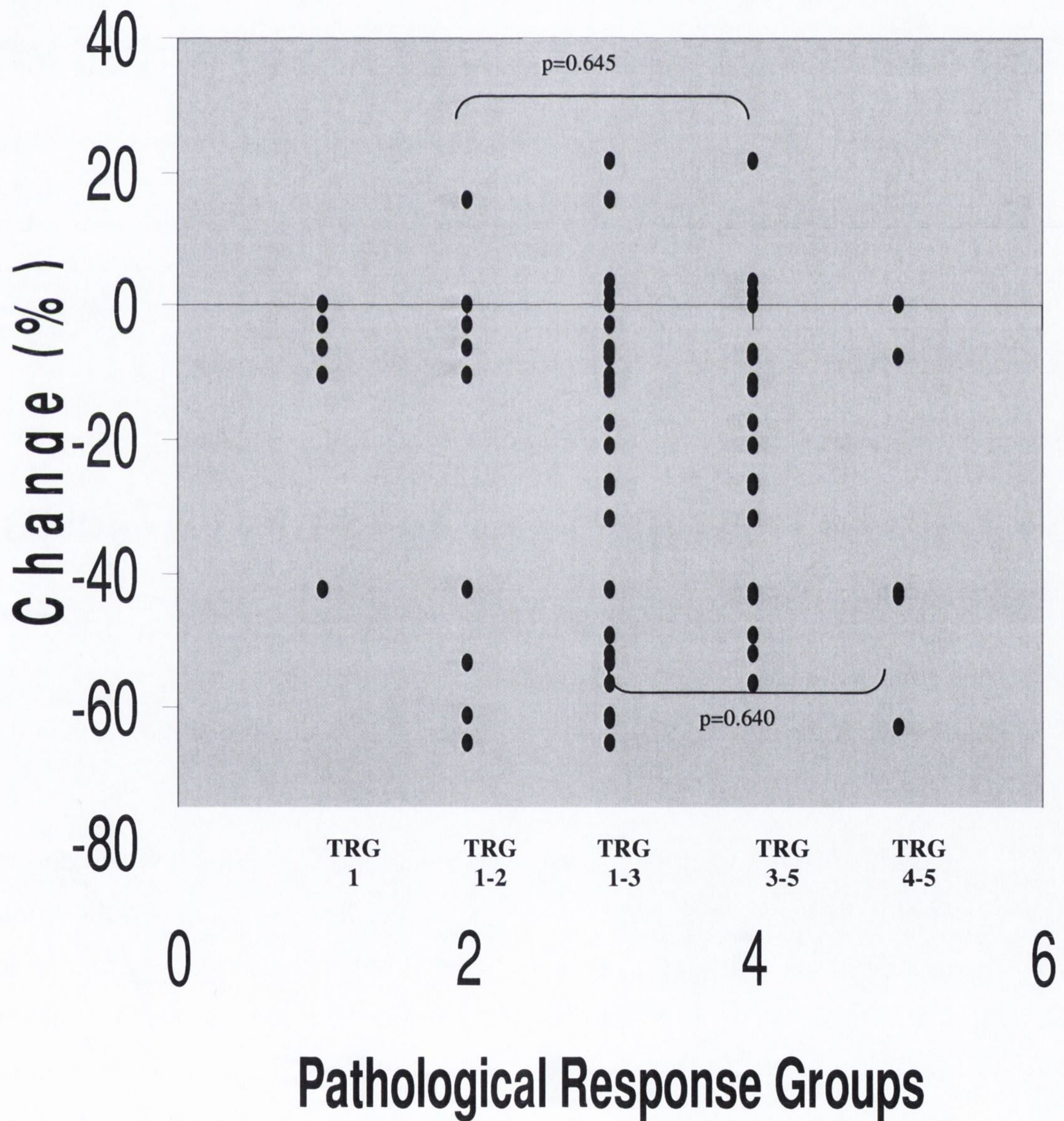
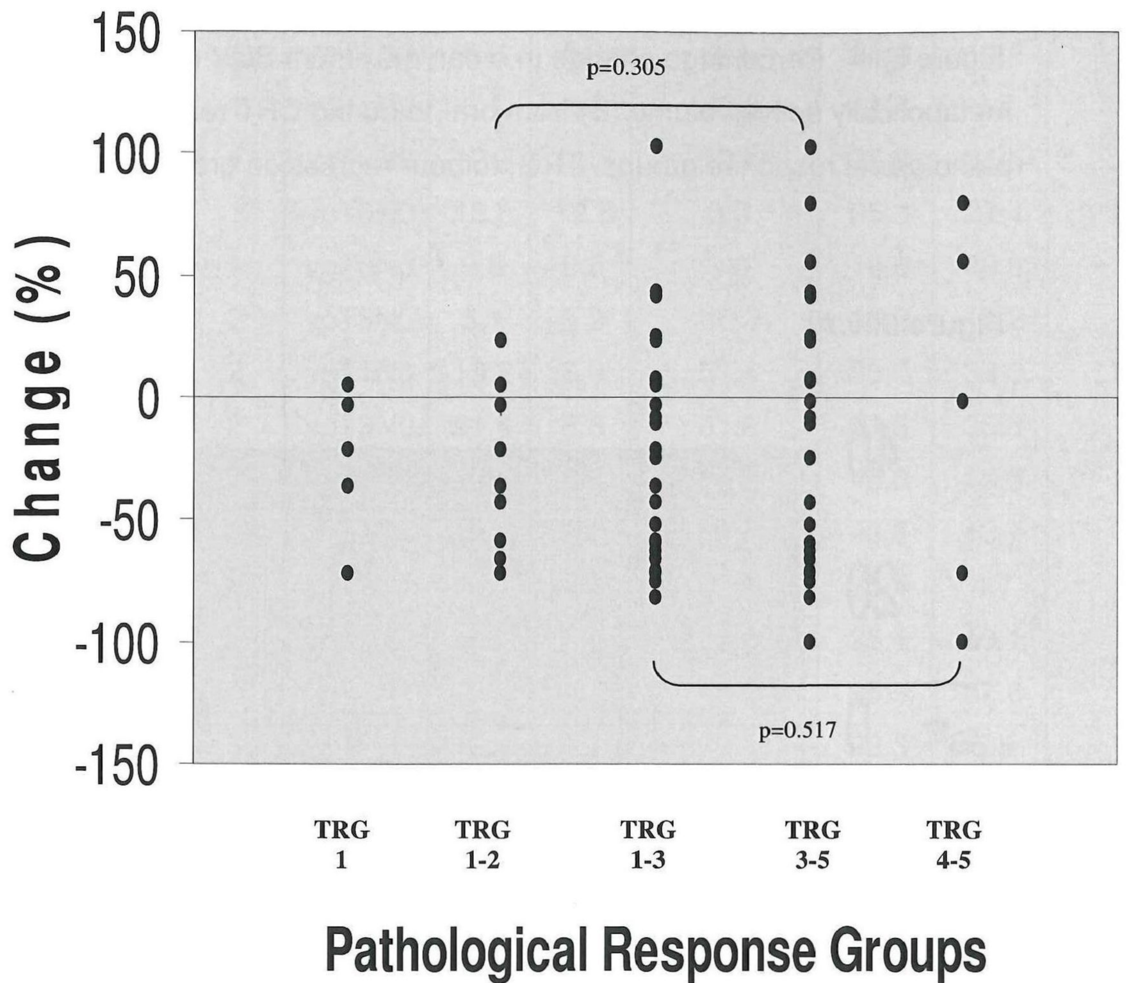


Figure 5.v.B:



5.6: DISCUSSION

In contrast to endoscopic ultrasound (EUS) and CT imaging which cannot differentiate fibrous tissue from viable tumour tissue in patients undergoing neoadjuvant treatment regimens, PET scanning holds greater promise as it monitors glucose turnover in glucose-avid tumour cells, and, intuitively, diminished metabolism should be anticipated to correlate with a tumour response. Moreover, the metabolic changes may precede structural changes, and this has been confirmed for some solid tumours[190].

In studies in oesophageal cancer, the PET scan has predominantly been performed following completion of the neoadjuvant protocol, as distinct from early in the treatment. The evidence from these studies suggests that PET scans following treatment significantly correlates with pathologic response and survival. Brücher et al[109] prospectively evaluated 37 patients following chemoradiation for T2-4 squamous cell tumours, and 24 proceeded to surgery. In those that responded, determined as less that 10% viable tumour cells and correlating closely with TRG1 and 2 in this study, FDG uptake decreased by 72 ± 11%, while in non-responders the decrease was significantly ($p=0.002$) less at 42 ± 22%, and this was associated with worse overall survival. Brink et al[116] prospectively studied 20 consecutive patients who underwent surgery following chemoradiation. Although the SUV decreased ($p<0.01$) in all patients following the neo-adjuvant component, the percentage change did not differ significantly between TRG groups.

An early marker of response offers the greatest potential clinical advantage, particularly if those not benefiting from treatment could be identified and offered alternative approaches. Weber at al[110] performed the second PET scan in 40 consecutive patients just prior to the second of two cycles of neo-adjuvant chemotherapy. No radiation therapy was included in their treatment protocol. A significant difference in tumour FDG uptake between responders and non-responders was observed and, applying a cut-off value of 35% reduction of initial FDG uptake as a criterion for metabolic response, they were able to predict clinical response with 93% sensitivity and 95% specificity. The most comparable study is from Wieder et al[118] from Munich who performed the second PET scan following two weeks of neo-adjuvant chemoradiation in 27 patients with oesophageal squamous cell cancer. Mean tumour SUV was 9.3 ± 2.8 before therapy and decreased to 5.7 + 1.9 (-38%±18%; $p<0.001$) after two weeks. In histopathologic responders (less than 10% viable tumour cells in the resected specimen), the decrease in SUV from baseline to day 14 was 44% ± 15%, whereas it was only 21% ± 14% in non-responders ($p=0.005$). In this series, in contrast, although both maximum SUV and metabolically active tumour volume decreased more in responders compared with non-responders, this has not approached statistical significance. There are some differences in the studies: the report by the Munich group was in squamous cell cancer, whereas this study was

predominantly in patients with adenocarcinoma; and the second week of induction CRT was completed in the Munich study before the second PET scan was performed, in contrast to this study where the second scan was performed after one week of induction therapy. There are several other factors that might explain why our study failed to demonstrate significant differences between responders and non-responders. First, it may be difficult for PET to differentiate an inflammatory response, which will be induced by ionising radiation, from active malignant tissue[191]. Such an effect would be expected in both responders and non-responders and might mask a reduction in SUV. Second, there was variation in the date of surgery after completion of chemoradiotherapy, in large part due to variation in the recovery of neutrophil counts and overall performance indices, and it is possible that the TRG achieved by therapy may be different at say four compared with eight weeks following completion of therapy. Third, it is possible that metabolic changes will not be observed until beyond the time chosen in this study to repeat the PET scan, and the premise that PET is an early predictor of response or resistance is incorrect. Finally, the size of the study group as in all reports is small and changes may be masked at this stage, and the trend for change between responders and non-responders in maximum SUV and metabolically active volume would support this possibility.

In conclusion, analysis of 32 patients in whom 9 achieved a good histologic response failed to show significant differences between responders and non-responders. The hypothesis that early tumour responses after induction chemoradiotherapy may be evident in PET scans has not been proven. Larger studies are required, but the inflammatory response to radiation may be a true confounding variable making metabolic imaging easier to evaluate in trials of induction chemotherapy alone prior to the administration of radiation therapy.

General Discussion

The worldwide incidence of oesophageal cancer is increasing, essentially as a consequence of a rise in adenocarcinoma of the lower oesophagus and gastro-oesophageal junction. Very early stage disease is associated with a favourable long-term outcome following surgery alone. However, most patients present when the disease is more advanced and therapeutic success is hampered both by recurrence (local and distant) and treatment-related toxicity. In recent years there have been a number of strategies employed with the aim of improving the outcome. If the disease is deemed potentially operable then a multi-modal approach is generally adopted. This utilises various combinations of chemotherapy and/or radiotherapy given prior to definitive surgery. Unlike in the management of other solid malignancies the use of adjuvant therapy has not been widely investigated and is therefore infrequently used. It is evident that those patients whose tumours respond well or completely to neo-adjuvant therapy experience the most favourable outcomes. However the reverse is also true; not only is the survival of non-responders poor but they also endure treatment-related toxicity with minimal gain. If the pathological response could be predicted early on therapy could be planned on a much more individualised basis.

Many studies exist that have tried to identify markers of response for oesophageal cancer pre-operatively. These include p53, survivin, p21, VEGF and Bax amongst others. Although many have proved promising, most have not proved specific enough for use as biomarkers and a single gene has yet to be translated into clinical practice.

This study has shown that molecular analysis reveals genes that can distinguish between benign and malignant oesophageal tissue, as well as between good and poor responders to chemoradiation. Larger sample numbers are clearly required and this study is on-going. Furthermore the data obtained will have to be carefully and precisely validated before it can become incorporated into routine clinical practice. There seems little doubt that the integration of genome-wide techniques into the clinical arena will increase in the years that follow.

A further advance has been seen with the development of metabolic imaging. Our PET study was not able to predict the final histological response. This is perhaps because of the effect ionising radiation has on the oesophageal mucosa or because the second PET scan was being performed before the treatment had had a significant cytotoxic effect. The study is continuing albeit with the second PET being performed a week later. Other studies have shown that following completion of the neo-adjuvant protocol PET is much more effective at predicting the response and this raises the question of the necessity of surgery. If a pathological complete response could be accurately predicted either before or after the start of chemoradiation the surgery could possibly be avoided. Non-surgical, definitive chemoradiation trials have a substantial number of long-time survivors. Although a pCR has not been confirmed in these patients the figures appear to equate to the pCR rates and survival reported in phase III trials of neo-adjuvant chemoradiation followed by surgery[31,32,33].

Closing Comments

The ultimate aims of predicting response to cancer therapy, to which this body of work has contributed, is to improve the prognosis and minimise the toxicity for those afflicted with the disease. Advances in translational medicine and enhanced imaging modalities hold great promise. The way in which cancer is treated is undergoing rapid change and the focus is increasingly on the patient rather than the pathology.

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