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The Epidermal Growth Factor (EGFR) in Non-small Cell Lung Cancer (NSCLC): Biomarker and Therapeutic Target.

Thesis submitted for the degree of Doctor of Medicine at the University of Dublin, Trinity College.

Linda Emma Coate
MB BCh BAO, MRCPI

June 2010
Thoracic Oncology Research Group, St. James’ Hospital and Trinity College, Dublin.
DECLARATION

I hereby declare that this thesis is my own work. It is a record of work planned and carried out by myself excepting those instances specifically referred to and gratefully acknowledged. All previous work has been fully accredited and referenced. This work was carried in Dublin between 2004 and 2008, with further analysis completed with the resulting data in Toronto from 2008 to 2010.

This work has not been submitted as an exercise for a degree at this or any other University. I also grant permission for the library to lend or copy the thesis upon request.

There are no financial conflicts of interests relating to this work

Linda Coate
Toronto
April 2010
ACKNOWLEDGEMENTS

Ken O’Byrne
Kathy Gately
Martin Barr
Helen
Christine
Tom
Emily
Steven
Graham
Dermot
Sinead
Mary-Clare
Anne-Marie

My thanks to the Thoracic Oncology Research Group, based at St. James’ Hospital in Dublin.

This work is dedicated to R
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CHAPTER ONE

INTRODUCTION
1.1 Non-Small-Cell Lung Cancer (NSCLC)

Lung cancer is the leading cause of cancer death in industrialized nations (Parkin, Bray et al. 2005). With approximately 1.35 million people diagnosed with the disease worldwide each year, and the incidence rising, lung cancer is set to become a global epidemic (Youlden, Cramb et al. 2008). From 1985 to 2005, the number of cases diagnosed annually increased by more than 50% globally. There are nearly 1.2 million lung cancer related deaths every year, accounting for 29% of total cancer-related deaths (Parkin, Bray et al. 2005). In the US and parts of Europe, lung cancer is responsible for as many deaths in men as deaths from prostate, colorectal and stomach cancers combined (Jemal, Siegel et al. 2006). Although incidence rates in women throughout the world are lower, lung is now the third most common cancer site after breast and cervix. In certain regions lung cancer has overtaken breast cancer as the most common cause of cancer-associated mortality among women (Patel, Bach et al. 2004).

Lung cancer can occur at any age but the median age of diagnosis is 70. There is significant geographical variation in the incidence and mortality rates of the disease, with highest rates reported in industrialized nations. Whereas a peak in incidence appears to have been observed in Western and Northern Europe, rates continue to increase in Southern and Eastern Europe, with overall age-adjusted incidence in males of more than 100/100,000 cases per year in some countries (Janssen-Heijnen and Coebergh 2003; Ferlay, Autier et al. 2007).
1.1.1 Staging of Lung Cancer

At the time of this work, patients with NSCLC were staged according to the sixth edition of the international TNM classification system (Table 1.1) on the basis of primary tumour characteristics (T), the presence or absence of regional lymph node involvement (N) and the presence or absence of distant metastases (M). The TNM system is a powerful prognostic tool that correlates strongly with outcome, and is used to determine the most appropriate therapeutic strategy. This classification was published in 1997 by Clifton Mountain, a surgeon in Houston Texas, and is based on data from a relatively small US database of 5,319 surgical patients (Mountain 1997). It is both the relative small size of the sample used to derive the 1997 TNM staging system and the fact that it was incompletely validated, amongst other factors, that led to the International Association for the Study of Lung Cancer (IASLC) to commence an initiative to refine the staging system used in lung cancer. This was published in 2008, and most institutions have started using it at the beginning of this year. In this work, as it was performed pre-2008, the older 1997 staging system is used.

1.1.2 New Lung Cancer Staging System

The TNM staging guidelines for lung cancer changed in 2008, since the 1997 edition was based on a selected patient population treated as a single institution. The various descriptors had not undergone rigorous validation, and the patients’ studies all emanated from a single geographic region. As a result, the IASLC established a committee in 1998 with the specific aim of
updating the TNM classification of lung cancer. This objective was to be accomplished by the collection of data worldwide from lung cancer patients. Information from nearly 68,000 NSCLC patients who fulfilled inclusion criteria was studied, and cases of NSCLC treated with all modalities of care between 1990 and 2000 were entered into a single database. Following analysis and validation of the database, several recommendations were made by the revision committee (Goldstraw, Crowley et al. 2007).

Tumour size was confirmed as a crucial determinant of outcome, and the IASLC committee proposed subclassification of T1 tumours into T1a (T1a ≤2 cm) and T1b (2 < T1b ≤3 cm), and T2 tumours into T2a (>3 T2a ≤5 cm) and T2b (>5 T2b ≤7 cm). It was also suggested that T2 tumours larger than 7 cm be reclassified as T3, and that T4 disease due to malignant pleural effusion be moved to M1 classification. An additional recommendation was that satellite lesions in the same lobe as the primary tumour, previously designated as T4 (stage IIIB), be changed to T3, reflecting the improved prognosis of this population compared to stage IIIB patients overall. Nodule(s) in a different lobe in the ipsilateral lung would become T4 instead of M1. These various designations were validated for each of the histopathological subtypes (Rami-Porta, Ball et al. 2007).
Primary Tumour (T)

TX The primary tumour cannot be assessed, or there are malignant cells in the sputum or bronchoalveolar lavage but not seen on imaging or bronchoscopy.

Tis Carcinoma \textit{in situ}

T0 No evidence of primary tumour

T1 Tumour that is 3 cm or less in its greatest dimension, does not invade the visceral pleura, and is without bronchoscopic evidence of invasion more proximal than a lobar bronchus

T2 Tumour that has any of the following features:
- Size more than 3 cm in its greatest dimension
- Involvement of a mainstem bronchus, with a proximal extent at least 2 cm away from the carina
- Invasion of the visceral pleura
- Association with atelectasis or obstructive pneumonitis that extends to the hilar region, but does not involve the entire lung

T3 Tumour of any size with any of the following features:
- Invasion of the chest wall (including superior sulcus tumours), diaphragm, mediastinal pleura, or parietal pericardium
- Involvement of a mainstem bronchus within 2 cm of the carina, but without invasion of the carina
- Association with atelectasis or obstructive pneumonitis of the entire lung

T4 Tumour of any size with any of the following features:
- Invasion of the mediastinum, heart, great vessels, trachea, oesophagus, vertebral body, or carina
- Association with a malignant pleural or pericardial effusion
- Satellite tumour nodule(s) within the same lobe of lung that contains the primary tumour

Table 1.1 Staging of non-small cell lung cancer (TNM classification).
(From: Mountain, Chest, 1997;111:1710-17.)
**Lymph Nodes (N)**

- **N0** No regional lymph node involvement
- **N1** Involvement of ipsilateral intrapulmonary, peribronchial, or hilar lymph nodes
- **N2** Involvement of ipsilateral mediastinal or subcarinal lymph nodes
- **N3** Involvement of contralateral mediastinal or hilar lymph nodes. Alternatively, involvement of either ipsilateral or contralateral scalene or supraclavicular lymph nodes

**Distant Metastasis (M)**

- **M0** No distant metastasis
- **M1** Distant metastasis or pulmonary nodules in separate lobe from primary

**OVERALL STAGE**

<table>
<thead>
<tr>
<th>Grouping</th>
<th>TNM Staging</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occult carcinoma</td>
<td>TX N0 M0</td>
</tr>
<tr>
<td>Stage 0</td>
<td>Tis N0 M0</td>
</tr>
<tr>
<td>Stage IA</td>
<td>T1 N0 M0</td>
</tr>
<tr>
<td>Stage IB</td>
<td>T2 N0 M0</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T1 N1 M0</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>T2 N1 M0</td>
</tr>
<tr>
<td></td>
<td>T3 N0 M0</td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>T1 N2 M0</td>
</tr>
<tr>
<td></td>
<td>T2 N2 M0</td>
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<tr>
<td></td>
<td>T3 N1 M0</td>
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<tr>
<td></td>
<td>T3 N2 M0</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>Any T N3 M0</td>
</tr>
<tr>
<td></td>
<td>T4 Any N M0</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any T Any N M1</td>
</tr>
</tbody>
</table>

Table 1.1 (contd.) Staging of non-small cell lung cancer (TNM classification)  
(From: Mountain, *Chest*, 1997;111:1710-17.)
As the current staging system for lymph node involvement was validated from the IASLC database, it was recommended that the N descriptors be maintained as they currently exist. The staging committee however recommended changing the current M staging system into M1a and M1b subgroups, with M1a reflecting patients with metastatic disease confined to nodules in the contralateral lung, and M1b indicating extrapulmonary tumour spread because of the differing underlying behaviours of these tumours.

1.1.3 Current Treatment Paradigms in NSCLC

The treatment of the patient with NSCLC is dependent on stage and clinical performance indicators. For stage I and II of the disease, surgical resection with systematic mediastinal lymph node dissection at time of surgery is the treatment of choice (Scott, Howington et al. 2007). It has been consistently shown that patients operated on in centres specializing in the care of patients with lung tumours have better outcomes as the multidisciplinary teams involved derive great expertise in dealing with high volumes of these patients.

In those patients assessed to have excessive surgical risk, Stereotactic Body Radiotherapy (SBRT) may be a potential option for therapy. For SBRT, tumour position, size and in particular tumour position relative to vital organs determine the suitability of this modality.
In locally advanced or stage III disease involving mediastinal lymph nodes, the treatment of choice is combined modality therapy with chemotherapy and high-dose radiation, either delivered concurrently or sequentially (Robinson, Ruckdeschel et al. 2007). For patients not fit to receive combined modality therapy, there is a role for high-dose radiation, which has a small but real survival advantage and good rates of local control.

Routine adjuvant chemotherapy has been recommended after complete resection of stage IIIA lung cancer encountered unexpectedly at surgery by virtue of the discovery of micrometastatic ipsilateral nodal disease.

The optimal treatment for pre-operatively confirmed stage IIIA disease and minimal N2 involvement remains controversial, though neoadjuvant chemoradiation followed by surgery may provide a survival advantage in those patients who will not ultimately need a pneumonectomy, although another large European trial showed no survival benefit from the addition of surgery (van Meerbeeck, Kramer et al. 2007).

As a result of four seminal clinical trials, adjuvant chemotherapy is now the standard of care for resected stage II and III disease. (Arriagada, Bergman et al. 2004; Winton, Livingston et al. 2005). These studies have shown both overall and disease-free survival advantages using a platinum-based combination of agents in this setting (Douillard, Rosell et al. 2006; Strauss, Herndon et al. 2008). However, the benefit of chemotherapy for
fully-resected stage I patients remains unproven, and the treatment may even be detrimental.

For those patients with distant metastasis and adequate performance status, systemic chemotherapy may be appropriate (Azzoli, Giaccone et al.; Jett, Schild et al. 2007; Socinski, Crowell et al. 2007). Treatment with palliative chemotherapy using a platinum-based doublet regimen is superior to best supportive care in those without significant weight-loss or comorbidity (Stinchcombe and Socinski 2009). Treatment with chemotherapy significantly reduces symptoms. The response rate lies at about 30%, with modest overall survival benefit.

Novel targeted therapies have been the focus of intense research in NSCLC for a decade. The epidermal growth factor receptor is discussed in detail below. Multiple ongoing studies are currently investigating other drug targets.
1.2 The Epidermal Growth Factor Receptor (EGFR)

The epidermal growth factor receptor (EGFR), also known as HER1 and ErbB1, is a transmembrane cell surface receptor that belongs to the ErbB superfamily of receptor Tyrosine Kinases (TKs), and plays a significant and complex role in numerous malignancies, including NSCLC (Yarden and Sliwkowski 2001; Herbst and Bunn 2003). As EGFR is abnormally activated in many epithelial tumours leading to enhanced proliferation, invasion, metastasis and a poor prognosis is expressed in ~ 40-85% of primary NSCLC. The dimerization of EGFR in response to ligand binding activates the TK domain, leading to phosphorylation of proteins, which induces cellular proliferation, cellular migration and angiogenesis (Mosesson and Yarden 2004). The activation of EGFR TK activity also inhibits malignant cell apoptosis and contributes to tumour progression (Woodburn 1999). EGFR plays a role in the cell-cell adhesion required for tumour cell invasion of the surrounding tissues via interaction with the integrin pathway (Hazan and Norton 1998; Herbst and Bunn 2003). In addition, EGFR activates matrix metalloproteinases, thereby stimulating tumour cell motility and facilitating metastasis (Ellerbroek, Halbleib et al. 2001). Gain-of-function or activating mutations occur within the $EGFR$ gene in some NSCLCs, resulting in constitutive TK activity and enhanced sensitivity to EGFR inhibitors (Pao, Miller et al. 2004; Riely, Politi et al. 2006; Sharma, Bell et al. 2007).
1.2.1 EGFR as a Target for Therapy

EGFR is expressed in many malignancies, including 50% to 90% of NSCLCs. The EGFR can be targeted in a number of ways. Monoclonal antibodies directed at the extracellular domain of EGFR, and small molecules that inhibit the Intracellular TK domain (TKIs), are two different strategies for blocking the downstream, ligand-induced signalling of EGFR. (Figure 1.1)

![Diagram of EGFR targeting](image)

**Figure 1.1** Targeting the EGFR and downstream effects.
Gefitinib was the first reversible EGFR TKI to demonstrate anti-tumour activity in NSCLC in two randomized phase II trials (Fukuoka, Yano et al. 2003; Kris, Natale et al. 2003), although larger placebo-controlled trials failed to demonstrate a survival benefit in patients who received gefitinib rather than placebo or docetaxel (Thatcher, Chang et al. 2005); (Niho, Ichinose et al. 2007). It is worthwhile to note that although these trials did not reach their primary end points they all identified demographic factors which were predictive of benefit: female sex, adenocarcinoma and never smokers. Conversely, the double-blind, placebo-controlled, randomized phase III BR.21 study showed that another reversible EGFR TKI, erlotinib, administered to chemotherapy pre-treated patients with NSCLC, demonstrated an increased median survival of approximately two months with minimal toxicity (Shepherd, Rodrigues Pereira et al. 2005), as well as (many clinicians would regard more importantly) a significant improvement in symptoms and in quality of life (Bezjak, Tu et al. 2006).

Not all patients benefit from TKI treatment, and several investigators have sought to identify a priori markers that will predict response to, and benefit from, treatment. A number of different potential candidate molecular predictive markers of anti-EGFR therapy have been investigated, utilizing different methodologies to assess them.

The EGFR status of a tumour can be examined at the protein level by immunohistochemistry (IHC). Gene copy number can be evaluated by several methods including Fluorescent In Situ Hybridization (FISH) and Real Time quantitative Reverse Transcription PCR (qRT-PCR). Finally,
EGFR tyrosine kinase domain gene mutations/deletions can be detected by direct sequencing.
1.2.2 EGFR Biomarkers

1.2.2.1 EGFR protein

IHC has the advantage of being a technique that most pathology laboratories are capable of performing. However, it requires careful standardisation, and discordance between antibodies has been reported (Buckley and Kakar 2007; Hirsch, Dziadziuszko et al. 2008). EGFR over-expression by IHC occurs in 40-80% of NSCLC (Rusch, Klimstra et al. 1997; Fontanini, De Laurentiis et al. 1998; Hsieh, Shepherd et al. 2000; Hirsch, Varella-Garcia et al. 2003).

In both BR.21 and ISEL (the two largest phase III trials investigating erlotinib and gefitinib respectively), patients with EGFR IHC+ tumours treated with the active drug demonstrated significantly higher response rates than did those with IHC- tumours (7.5% v. 3.75%, p=0.03 in BR.21, 8.2% v. 1.4%, p value not reported in ISEL) (Tsao, Sakurada et al. 2005; Hirsch, Varella-Garcia et al. 2006). In both studies, a survival benefit was seen in IHC+ patients treated with TKI compared to those given a placebo, whereas no significant survival benefit was seen in IHC- patients (Table 1.2). The interaction in ISEL was significant (p=0.049) but not in BR.21 (p=0.25). Although EGFR IHC may be weakly prognostic and likely weakly predictive, it cannot currently be recommended as a test to exclude patients from treatment with a TKI.
1.2.2.2 EGFR gene copy or dosage

The *EGFR* gene is located on the short arm of chromosome 7 (7p21), which is commonly overrepresented or amplified in NSCLC. There are several methods for detecting and determining EGFR gene copy number and dosage, including fluorescence in situ hybridization (FISH), Chromogenic In Situ Hybridization and real-time quantitative polymerase chain reaction (qPCR).

1.2.2.3 EGFR copy number

In BR.21 and ISEL, a high copy number was prognostic and associated with poorer survival in patients treated with the placebo. The association between high EGFR copy, as measured by FISH (FISH+), and higher response to EGFR TKIs has been demonstrated in a number of studies. Of perhaps greater importance, in both BR.21 and ISEL, a significant association between high copy number and greater survival benefit compared to placebo for both gefitinib and erlotinib was reported (Tsao, Sakurada et al. 2005; Hirsch, Varella-Garcia et al. 2006). (Table 1.2)

A recent study also demonstrated that FISH+ tumours treated with chemotherapy and cetuximab had longer survival than FISH- patients (Hirsch, Herbst et al. 2008). All patients received cetuximab, and so this study does not demonstrate interaction between copy number and cetuximab treatment.
Although both BR.21 and ISEL support FISH as a predictive test, the INTEREST trial that compared gefitinib to docetaxel did not find FISH-positivity to be predictive of response or survival benefit to gefitinib. (Kim, Hirsh et al. 2008). In the INVITE study, which compared vinorelbine to gefitinib in elderly patients, patients with FISH+ tumours benefited more from treatment with vinorelbine than gefitinib (Crino, Cappuzzo et al. 2008). This suggests that predictive markers that are identified in trials comparing active treatment to placebo or a “no-treatment” control arm may not be predictive when two active treatments are compared to each other. Although the treatments may have very different modes of action, they may exert similar effects in the molecular subsets under study. Hopefully, molecular analyses from prospective marker validation studies such as the MARVEL trial, which compares erlotinib to pemetrexed in the second-line setting, and for which EGFR FISH is mandatory, will define the validity of EGFR copy number.

1.2.2.4 CISH

CISH is an alternative assay to FISH for determining gene copy number and amplification in tissue sections. The major difference between the two methods is that FISH requires an epifluorescent system, while CISH can be evaluated by the pathologist using bright-field microscopy, and correlated directly with histopathology. Furthermore, as with IHC, CISH-stained slides can be archived and kept permanently.
The DNA probes are labelled with digoxigenin or biotin (Gallegos Ruiz, Floor et al. 2007; Sholl, John Iafrate et al. 2007; Chang, Liu et al. 2008; Li, Chitale et al. 2008), and then detected using a CISH Detection Kit (Zymed Laboratories, South San Francisco, CA). The gene/chromosome probes are visualized as dark brown dots, which are counted in parallel sections using standard light microscopy, and converted to the number of nuclear signals per cell. CISH may be useful in samples that are difficult to interpret by fluorescence microscopy due to sample heterogeneity, high autofluorescent background signal, or both (Daniele, Macri et al. 2007). The main potential drawback of CISH compared with FISH is that most CISH studies have been performed on frozen sections, and only a single colour can be developed per section, meaning that one is not able to evaluate a control probe for aneusomy. Therefore, the complex scoring system proposed for FISH (Cappuzzo, Hirsch et al. 2005) may not be extrapolated automatically to CISH. Nevertheless, with a scoring system that depends only on EGFR gene signal, CISH is an alternative method to assess EGFR gene copy status.

A study comparing the performance of FISH and CISH for the detection of increased EGFR copy number in 77 Taiwanese patients with NSCLC treated by surgery alone, a significant correlation was found between the number of signals detected by both methods (Spearman r = 0.81; P <0.0001) (Sholl, John Iafrate et al. 2007). Discrepancies between the two tests were seen in only 7% of cases. At a signal of 4.5, CISH had very high sensitivity (89%) and specificity (89%) for the discrimination of low and high EGFR polysomy (P <0.0001). At a signal of 7.1, CISH was also effective at distinguishing between high polysomy and amplification (P = 0.0003). Another study comparing CISH and FISH in 58 NSCLC samples
demonstrated similar results with a high concordance between copy number determined by the two tests (Gallegos Ruiz, Floor et al. 2007). Overall, these data support the feasibility of using CISH to evaluate the role of EGFR gene copy as a predictive marker for EGFR TKI therapy, although further evaluation and validation in clinical trial samples is needed before this technique can be adopted clinically.

1.2.2.5 qPCR

Several different methods of performing qPCR measurements can be used to detect small amounts of RNA or DNA (Bustin 2002). Using probes that are labelled with both a fluorophor and fluorescence-quenching molecule, amplicon generation can be detected and quantified in real time during a PCR reaction. An alternative and less expensive method uses the double-stranded DNA intercalating agent SYBR® GreenER™ as the signal emitter. Using multiple primers labelled with different fluorophors, current qPCR technology can quantitate multiple genes, including control genes, simultaneously in one reaction.

The greatest pitfall for using qPCR to measure gene dosage is the inherent inability to control for contamination from normal host cells that result in the dilution of tumour gene copy. Performing laser-capture microdissection can reduce this problem, but this is laborious and clinically impractical. The other major issue with qPCR-based gene dosage estimation is normalization. Normalization using another region of the same chromosome as the studied gene, such as centromeric sequences, will detect
true amplification, but will not detect increased gene copy number due to polysomy. Normalization using centromeric sequences of multiple chromosomes may allow assessment of the degree of aneusomy.

There have been two studies on EGFR gene dosage by qPCR to predict treatment benefit in patients with NSCLC receiving TKI therapy. Both studies failed to demonstrate its clinical applicability (Bell, Lynch et al. 2005; Dziadziuszko, Witta et al. 2006). EGFR qPCR was not predictive of response to treatment, disease control, progression-free survival, or overall survival, nor did it correlate with FISH, IHC or mRNA expression (Bell, Lynch et al. 2005; Takano, Ohe et al. 2005; Dziadziuszko, Witta et al. 2006; Dziadziuszko, Holm et al. 2007).

1.2.2.6 EGFR mutations

In 2004, two independent research groups simultaneously reported the discovery of somatic mutations in the TK domain of the EGFR gene (Riely, Politi et al. 2006; Sakurada, Shepherd et al. 2006). Classical EGFR activating mutations have since been defined as deletions in exon 19 and a specific point mutation in exon 21. Clinicopathological features that correlate with these mutations include East-Asian ethnicity, adenocarcinoma histology, female sex and never-smoker. The groups of patients harbouring activating mutations are similar to those found to correlate with benefit in the randomised trials versus placebo in the second-line setting. The prevalence of EGFR mutations varies by ethnicity from \(~10\%\) among Caucasians to \(~40\%\) in Asian populations. There is differential efficacy
observed between patients with the two different "classical" sensitizing mutations, with patients harbouring the exon 19 mutation showing preferential responses to treatment with the EGFR TKI’s.

Several studies have reported that untreated NSCLC patients with EGFR mutations have a more favourable prognosis compared to EGFR wild-type patients. In BR.21, untreated patients with EGFR mutant tumours achieved a median survival of 8.3 months compared with 3.3 months for EGFR wild-type. However a recent poster presentation at the American Society of Clinical Oncology 2010 Annual meeting showed a large surgical series where there was no significant impact of mutational status on overall survival, merely a trend (D'Angelo, Janjigian et al.).

Several early phase II studies have now demonstrated overall response rates as high as 80% in patients harbouring mutations compared to 10% or less in wild-type patients. In BR.21 and ISEL, patients whose tumours harboured mutations achieved significantly higher response rates (27% v. 7%, p=0.035 in BR.21, 37.5% v. 2.6%, p not reported in ISEL). Also in BR.21, greater overall survival benefit from erlotinib compared to placebo was seen in patients with mutations (HR 0.55 p=0.12), although it should be highlighted that patients with wild-type EGFR also derived survival benefit from treatment (HR 0.74 p=0.09) (Zhu, da Cunha Santos et al. 2008). However, the interaction p value was not significant, perhaps due to the small number of patients with mutations. Survival data has never been reported for mutation patients in ISEL.
In the recently reported phase III Iressa Pan-Asia Study (IPASS), patients were randomised to receive first-line treatment with gefitinib or chemotherapy consisting of carboplatin and paclitaxel (Mok, Wu et al. 2008). The overall response rate in the gefitinib arm was significantly higher (43% v 32.2%, p=0.0001) and PFS was also significantly improved with gefitinib (HR 0.74 p<0.0001). In patients with mutations, first line gefitinib significantly improved PFS (HR 0.48 p<0.0001) and the interaction p value was also highly significant at p= <0.0001. Interestingly in mutation negative patients, gefitinib was significantly inferior to chemotherapy, suggesting that mutation status in this population predicts not only response to gefitinib but potentially also harm from gefitinib in those patients who are mutation negative. As the IPASS trial is negative for survival, this means that the sequence of EGFR inhibitor followed by chemotherapy is not inferior to chemotherapy followed by EGFR inhibitor.

1.2.2.7 Genetic polymorphisms and EGFR targeted drugs

The *EGFR* gene contains numerous genetic polymorphic variants. The evaluation of germline genetic variants uses DNA extracted from any non-tumour source, including leukocytes from whole blood. Several genetic variants are associated with alterations in mRNA gene expression (Liu, Innocenti et al. 2005). Both the *EGFR* −216G/T and −191C/A polymorphisms are located in the transcriptional start site region of the promoter where multiple nuclear regulatory affinity sites are located (Liu, Innocenti et al. 2005). Two studies in white patients reported that the −216G/T variant, alone, or in combination with −191C/A, was associated with improved outcome, greater toxicity to gefitinib, or both (Cusatis, Gregorc et
al. 2006; Liu, Gurubhagavatula et al. 2008), while a third found no association (Gregorc, Hidalgo et al. 2008). Among Asians these variants are rare (Ichihara, Toyooka et al. 2007). Most of these studies were performed using the candidate gene approach, which has lately fallen out of favour.

One of the enhancer elements for EGFR is located in intron 1 (Maekawa, Imamoto et al. 1989; Haley and Waterfield 1991). Shorter alleles of the dinucleotide CA repeat sequence polymorphism in intron 1 of EGFR are associated with greater EGFR expression than are the longer repeats (Gebhardt, Zanker et al. 1999; Etienne-Grimaldi, Pereira et al. 2005). In Asian patients, shorter CA repeat length was associated with greater EGFR expression and copy number (Zhou, Cheung et al. 2006). Asian patients tend to have longer CA repeat lengths. Two Asian (Han, Jeon et al. 2007; Nie, Wang et al. 2007) and one American study (Liu, Gurubhagavatula et al. 2008) found associations with lung cancer survival or toxicity with gefitinib, whilst others found no significant association (Ichihara, Toyooka et al. 2007; Gregorc, Hidalgo et al. 2008). Shorter CA repeat lengths were associated with poorer survival in the absence of therapy with an EGFR TKI, which is a reversal of expectations in TKI-treated patients (Dubey, Stephenson et al. 2006).

The multidrug transporter ABCG2 has been shown to be active in removing gefitinib from cells (Elkind, Szentpetery et al. 2005). In published reports, ABCG2 polymorphisms have been identified as being associated with increased EGFR concentrations, toxicity, or both, in patients treated with gefitinib and erlotinib (Cusatis, Gregorc et al. 2006; Li, Cusatis et al. 2007; Rudin, Liu et al. 2008).
In summary, genetic polymorphisms may be important contributors to the variability in response to EGFR TKI therapy, but the evidence is thus far contradictory. Several reasons exist to explain the inconsistent data: (1) Studies have been underpowered with sample sizes generally lower than 200 patients; (2) Different definitions are used for key variables, such as “short” and “long” intron 1 CA repeat lengths and “clinical toxicity”; (3) Confounders such as race, polymorphism allele frequencies, mutation rates, copy number changes, and other clinical and molecular prognostic and predictive factors have rarely been accounted for in these polymorphism analyses. Comprehensive analyses are needed to determine whether genetic polymorphisms are independently predictive with EGFR TKI therapy, are simply correlated with other molecular or clinical prognostic factors, or are prognostic but not predictive.
<table>
<thead>
<tr>
<th>Study</th>
<th>Arms</th>
<th>HR for high expression, gene copy number or presence of mutation*</th>
<th>P-value</th>
<th>HR for low expression, gene copy number or absence of mutation*</th>
<th>P-value</th>
<th>Interact P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EGFR Protein Expression</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR. 21 (Shepherd, Rodrigues Pereira et al. 2005; Tsao, Sakurada et al. 2005)</td>
<td>Erlotinib versus Placebo</td>
<td>0.68</td>
<td>0.02</td>
<td>0.93</td>
<td>0.70</td>
<td>0.10</td>
</tr>
<tr>
<td>ISEL (Thatcher, Chang et al. 2005; Hirsch, Varella-Garcia et al. 2006)</td>
<td>Gefitinib versus Placebo</td>
<td>0.77</td>
<td>0.13</td>
<td>1.57</td>
<td>0.14</td>
<td>0.05</td>
</tr>
<tr>
<td>BMS099 (S. Khambata-Ford and Dakhil 2008; T. J. Lynch and Hermann 2008)</td>
<td>Carboplatin/paclitaxel/cetuximab versus Carboplatin/paclitaxel</td>
<td>1.02</td>
<td>0.91</td>
<td>1.86</td>
<td>0.31</td>
<td>NR</td>
</tr>
<tr>
<td><strong>EGFR Copy Number</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR.21 (Shepherd, Rodrigues Pereira et al. 2005; Zhu, da Cunha Santos et al. 2008)</td>
<td>Erlotinib versus Placebo</td>
<td>0.43</td>
<td>0.004</td>
<td>0.80</td>
<td>0.35</td>
<td>0.12</td>
</tr>
<tr>
<td>ISEL (Thatcher, Chang et al. 2005; Zhu, da Cunha Santos et al. 2008)</td>
<td>Gefitinib versus Placebo</td>
<td>0.61</td>
<td>0.07</td>
<td>1.16</td>
<td>0.42</td>
<td>0.05</td>
</tr>
<tr>
<td>INTEREST (Douillard, Rosell et al. 2006; J. Douillard 2008)</td>
<td>Gefitinib versus Docetaxel</td>
<td>1.09</td>
<td>0.62</td>
<td>NR</td>
<td>NR</td>
<td>0.52</td>
</tr>
<tr>
<td>BMS099 (S. Khambata-Ford and Dakhil 2008; T. J. Lynch and Hermann 2008)</td>
<td>Carboplatin/paclitaxel/cetuximab versus Carboplatin/paclitaxel</td>
<td>1.92 (PFS)</td>
<td>0.03</td>
<td>0.84 (PFS)</td>
<td>0.57</td>
<td>NR</td>
</tr>
<tr>
<td>INVITE (Crino, Cappuzzo et al. 2008)</td>
<td>Gefitinib versus Vinorelbine</td>
<td>3.13 (PFS)</td>
<td>NR</td>
<td>0.93 (PFS)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>INSTEP (Goss, Ferry et al. 2009)</td>
<td>Gefitinib versus Placebo</td>
<td>0.29 (PFS)</td>
<td>NR</td>
<td>0.74 (PFS)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td><strong>EGFR Mutation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR.21 (Shepherd, Rodrigues Pereira et al. 2005; Zhu, da Cunha Santos et al. 2008)</td>
<td>Erlotinib versus Placebo</td>
<td>0.55</td>
<td>0.12</td>
<td>0.74</td>
<td>0.09</td>
<td>0.47</td>
</tr>
<tr>
<td>IPASS (Mok, Wu et al. 2008)</td>
<td>Gefitinib versus Cisplatin/paclitaxel</td>
<td>0.48</td>
<td>&lt;0.0001</td>
<td>2.85</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMS099 (S. Khambata-Ford and Dakhil 2008; T. J. Lynch and Hermann 2008)</td>
<td>Carboplatin/paclitaxel/cetuximab versus Carboplatin/paclitaxel</td>
<td>1.62 (PFS)</td>
<td>0.38</td>
<td>0.91 (PFS)</td>
<td>0.61</td>
<td>NR</td>
</tr>
</tbody>
</table>
NR: Not reported; *HR: hazard ratio. HR is for OS unless otherwise stated.

Table 1.2 Summary of EGFR biomarker analyses from randomised trials.

1.2.2.8 KRAS gene mutation

RAS is considered a “master switch” signal transduction protein downstream from multiple types of cell surface receptors, including tyrosine kinase and G-protein-coupled receptors (Aviel-Ronen, Blackhall et al. 2006; Molina and Adjei 2006). A point mutation on codons 12, 13 and 61 of all RAS family genes will lead to the inability of the RAS-Guanosine Triphosphatase (RAS-GTPase) activating protein to hydrolyze bound GTP to Guanosine Diphosphate (GDP), thus resulting in constitutive activation of the RAS protein. In NSCLC, >90% of RAS mutations involve the KRAS gene, with most occurring in codon 12. The most direct way to detect KRAS codon 12 and 13 mutations is by PCR amplification of exon 2, and direct sequencing of the amplicon. However, all the caveats for the detection of EGFR mutations using this technique on FFPE samples apply, especially the need to enrich samples to at least ≥50% tumour cellularity, and not use samples with necrotic tissue. The need for repeat PCR to confirm mutations is less of a concern, as any mutations detected outside codons 12, 13 and 61 can be ignored.
Pao et al. suggested first that patients with NSCLC whose tumours harbour mutations are resistant to EGFR TKI (Pao, Wang et al. 2005). To date, among 75 NSCLC patients harbouring KRAS mutations who were treated with TKIs (Pao, Wang et al. 2005; Hirsch, Varella-Garcia et al. 2006; Massarelli, Varella-Garcia et al. 2007; Miller, Riely et al. 2008; Zhu, da Cunha Santos et al. 2008), only one patient has been reported to respond (Zhu, da Cunha Santos et al. 2008). Interestingly, this patient also had \textit{EGFR} gene amplification. Based on TKI response rates of up to 10\% in patients with wild-type EGFR, it is expected that at least seven to eight patients should have responded, suggesting that mutated \textit{KRAS} results in TKI resistance. In the Tarceva responses in conjunction with paclitaxel and carboplatin (TRIBUTE) study, patients with \textit{KRAS} mutant tumours, who were treated with a combination of erlotinib and chemotherapy, demonstrated poorer survival than did patients treated with chemotherapy alone, or patients with wild-type \textit{KRAS}, regardless of treatment type (Eberhard, Johnson et al. 2005). In the BR.21 study, \textit{KRAS} mutations were found in 30 (15\%) of 206 patients tested. The hazard ratio (HR) of erlotinib to placebo treatment in 30 patients with a \textit{KRAS} mutation was 1.67 (95\% CI, 0.62 to 4.50; \(P = 0.31\)), while that for 176 patients with wild-type \textit{KRAS} was 0.69 (95\% CI, 0.49 to 0.97; \(P = 0.03\)), with an insignificant interaction \(P\) value of 0.09. In a multivariate analysis, \textit{KRAS} mutation was neither prognostic for poorer survival in untreated patients nor predictive of differential treatment effect (Zhu, da Cunha Santos et al. 2008). Overall, these results suggest that patients with NSCLC who have \textit{KRAS} mutations are unlikely to derive benefit from current TKI therapy, although the number of reported cases remains limited.
<table>
<thead>
<tr>
<th>Study</th>
<th>Patient Population</th>
<th>Arms</th>
<th>Method</th>
<th>Marker High or Present HR (95%CI)*</th>
<th>Marker Low or Absent HR (95%CI)*</th>
<th>Interaction P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>JBR.10 (Winton, Livingston et al. 2005; Tsao, Aviel-Ronen et al. 2007)</td>
<td>Adjuvant</td>
<td>Cisplatin/ vinorelbine versus Observation</td>
<td></td>
<td>0.95 0.87 0.69 0.03 0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BR.21 (Shepherd, Rodrigues Pereira et al. 2005; Tsao, Sakurada et al. 2005)</td>
<td>2nd-3rd-line</td>
<td>Erlotinib versus Placebo</td>
<td>Mutation Analysis</td>
<td>1.67 0.30 0.69 0.03 0.09</td>
<td></td>
<td>0.09</td>
</tr>
<tr>
<td>INTEREST (J. Douillard 2008)</td>
<td>2nd-line</td>
<td>Gefitinib versus Docetaxel</td>
<td>(PFS) 0.16 0.68 1.23 (PFS) 0.19</td>
<td>0.52 (OS)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.81 (OS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMS099 (S. Khambata-Ford and Dakhil 2008; T. J. Lynch and Hermann 2008)</td>
<td>1st-line</td>
<td>Carboplatin/ paclitaxel versus Carboplatin/paclitaxel/cetuximab</td>
<td></td>
<td>0.69 0.68 NR 0.69</td>
<td>0.68</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.3 Response to treatment of patients with *KRAS* mutations
1.3 Neoadjuvant Chemotherapy in NSCLC

As a result of three pivotal phase III trials published in recent years, the administration of adjuvant chemotherapy for resectable lung cancer has become a standard of care for patients with stage II and III tumours (Arriagada, Bergman et al. 2004; Winton, Livingston et al. 2005; Le Chevalier 2008). Patients with stage IB tumours were included as they were included in other neoadjuvant trials during the timeframe of this trial’s inception (Felip, Rosell et al. 2007).

Because of the morbidity and mortality associated with resection of lung cancer, some patients are not fit for adjuvant chemotherapy. Furthermore, several recent trials have demonstrated a similar survival advantage with the administration of neoadjuvant chemotherapy (Gilligan, Nicolson et al. 2007).

The epidermal growth factor receptor (EGFR) is overexpressed in 80% of NSCLC, making it an attractive target for therapy in this malignancy. EGFR may be targeted by inhibition of the intracellular tyrosine kinase domain by such agents as erlotinib and gefitinib, or by inhibition extracellularly by a monoclonal antibody.

Cetuximab is a monoclonal antibody directed against EGFR. Its mode of action is competitive ligand binding. There is preclinical evidence demonstrating antibody dependent cytotoxicity (Kurai, Chikumi et al. 2007), which may be an additional mode of action in the clinical model. Cetuximab is licensed for use in irinotecan refractory metastatic colon cancer.
Cetuximab has been studied with chemotherapy in the treatment of advanced NSCLC. In two published phase II studies, the response rate was significantly increased by the addition of cetuximab to standard platinum doublet chemotherapy (Butts, Bodkin et al. 2007; Rosell, Robinet et al. 2008).

This finding was replicated in the ensuing phase III studies (T. J. Lynch and Weber 2007; Pirker, Szczesna et al. 2008). The response rate in the BMS 099 study was 17% in the chemotherapy arm compared to 26% in the cetuximab and chemotherapy arm. The larger FLEX study, which included 1,025 untreated patients with metastatic disease, also showed a significant increase in response rate with the addition of cetuximab. Unlike the BMS 099, there was also a short but statistically significant improvement in overall survival. It should be noted that patients were selected for entry into the FLEX study (but not the BMS 099) based on EGFR expression as detected by immunohistochemistry. Before FLEX, agents targeting EGFR, administered in conjunction with cytotoxic chemotherapy, have failed to demonstrate a significant survival advantage when translated to the clinic.

A large body of translational research exists examining potential biomarkers of response to the tyrosine kinase inhibitors of EGFR. Multiple papers have demonstrated the predictive significance of mutations in exons 19-22 of the \textit{EGFR} gene in the treatment of NSCLC with small molecule tyrosine kinase inhibitors (TKI's) directed against the EGFR (Lynch, Bell et al. 2004; Tsao, Sakurada et al. 2005). Mutations in these exons of the \textit{EGFR} gene do not seem to confer tumour sensitivity to treatment with cetuximab. There is limited evidence investigating biomarkers of response to cetuximab.
in all solid tumours for which it is used. Expression of EGFR detected by IHC has been used to select patients for entry into studies examining the effect of cetuximab. In colon cancer, however, even patients whose tumours are negative for EGFR by IHC (Chung, Shia et al. 2005) have demonstrated response to treatment with cetuximab. To date, there is no reliable predictive marker that selects patients who might benefit from treatment with cetuximab in NSCLC.
CHAPTER TWO

MATERIALS AND METHODS
2.1 Materials

2.1.1 General Chemicals and Reagents

All the general chemicals and reagents were purchased from Sigma-Aldrich Company Ltd (Poole, UK) unless otherwise stated.

2.1.2 Primary Antibodies

- EGFR (Novocastra Laboratories Ltd)
- Phosphorylated EGFR (Calbiochem Laboratories Ltd.)

2.1.3 Secondary Antibodies

- Biotinylated rabbit anti-mouse whole immunoglobulins (DAKO)
- Biotinylated donkey anti-goat

2.1.4 Kits

- Streptavidin-biotin peroxidase complex (ABC) (DAKO, UK)
- Diaminobenzidine tetrahydrochloride (DAB) (Sigma, UK)
- Trilogy Immunohistochemistry (IHC) Reagent X10 solution (Cell Marque, UK)

2.1.5 Buffers

Lysis buffer:

10Mm Tris-HCl Ph 8.0 10 ml from a 1M stock
320 Mm Sucrose 109.5 g
5 Mm MgCl₂ 5ml from a 1M stock (or 0.45g MgCl₂.6H₂O)
1% Triton X-100 10 ml

Ph adjusted to 8.0 using 10N NaOH.

2.1.6 Blood Sample Preparation for Blood Samples Drawn at Various Time Points from Clinical Study Patients

Lysis buffer:

10Mm Tris-HCl Ph 8.0 10 ml from a 1M stock
320 Mm Sucrose 109.5 g
5 Mm MgCl₂ 5ml from a 1M stock (or 0.45g MgCl₂.6H₂O)
1% Triton X-100 10 ml

Ph adjusted to 8.0 using 10N NaOH.
General reagents and chemicals

Unless otherwise specified all reagents and chemicals were purchased from Sigma Aldrich Company Ltd

2.1.7 Immunohistochemistry

Primary antibodies

- AntiEGFR purified mouse monoclonal antibody (Novocastra Laboratories Ltd). Clone EGFR.113 (Isotype IgG2a).

Secondary antibodies

- Biotinylated rabbit anti-mouse whole immunoglobulins (DAKO)

Other reagents

- Streptavidin-biotin peroxidase complex (ABC) (DAKO, UK)
- Diaminobenzidine tetrahydrochloride (DAB) (Sigma, UK)
- Trilogy Immunohistochemistry Reagent X10 solution (Cell Marque, UK)

2.1.8 Sequencing Exons 19 and 21 of the EGFR

- Cutting sections
- Microtome
- Microtome blades
- Clean 1.5ml tube (eppendorf)

- Xylene 100%
- Ethanol 100%
- Heated shaking block
- Pipette with clean pipette tips
- Clean 0.5ml tubes (eppendorf)

- Qiagen DNeasy kit (QIAGEN)
- Amplitaq (Applied Biosystems, US)

<table>
<thead>
<tr>
<th>Direction</th>
<th>Primer Sequence <strong>EGFR</strong> Exon 19</th>
<th>Primer sequence <strong>EGFR</strong> Exon 21</th>
<th>Primer sequence <strong>KRAS</strong> exon 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5'-tccaaatgagctggcaagtg</td>
<td>5'-gtcagagcctggcatgaa</td>
<td>5'-ttatgtgtgacatgttctaat</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-ccacacagcaaagcagaaactcac</td>
<td>5'-cat cctcctgcgttgt</td>
<td>5'-agaatggctctgcaccagtaa</td>
</tr>
</tbody>
</table>

**Table 2.1** Primer sequence for PCR

- QIAquick PCR purification kit (QIAGEN)
- MJ Research PIC-200 Thermal cycler
- BigDye® (ABI Biosystems, US)
- GenAmp PCR 2400 Thermal Cycler
- PE 3100 Sequencing Instrument (AB Biosystems, US)
2.1.9 Quantification of EGFR Gene Expression Using Real-time Quantitative PCR

- Genomic DNA as above
- Genomic DNA from buffy coat of pooled normal volunteers
- TaqMan MasterMix ®
- ABI 7000 Sequence detection system (*Applied Biosystems, Foster City, CA*)

- ABI SDS software
- Excel software

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>CAATTGCCAGTTAACGTCTT</td>
<td>TTTCTCACCTTCAGTGATC</td>
<td>TCTCTCTGTCATAC</td>
</tr>
<tr>
<td></td>
<td>CCTT</td>
<td>CA</td>
<td>AC tamra</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GGGGAGCCAAAAGGGTCAT</td>
<td>GACGCCTGCTTCACCACC</td>
<td>CAAGCTTCCCCGTTG</td>
</tr>
<tr>
<td></td>
<td>CATCT</td>
<td>TTCTTG</td>
<td>AGCCtamra?</td>
</tr>
</tbody>
</table>

Table 2.2 Primer and probe sequences for qPCR.

2.1.10 Phase II Clinical Trial

All patients referred to St. James’ Hospital with operable stage IB to IIIA NSCLC were screened for entry into the clinical study.
2.2 Methods

2.2.1 Immunohistochemistry

2.2.1.1 Paraffin-embedded formalin fixed tissue

Resection tissue was routinely processed, being formalin fixed for 24 to 72 hours prior to selection. Standard automated processing was performed at 53 - 56° C with tissue blocks embedded in paraffin wax. Blocks were kept in the dark at 20 °C prior to sectioning. Only blocks containing > 60% tumour were evaluated.

2.2.1.2 Tissue sectioning

Tissue sections of 4μm thickness were cut onto Vectabond™-treated glass slides. Sections on slides were stored in the dark at 4 °C until evaluation. Freshly cut sections were used for the phospho-EGFR staining as phosphorylated antigens are very labile with disappearance of expression occurring very soon after the sections are cut.
2.2.1.3 EGFR and p-EGFR immunohistochemistry

Slides were dewaxed, rehydrated and antigen retrieval was achieved by treating according to the manufacturer’s instructions with TRILOGY reagent. Endogenous peroxidises were blocked with the application of a few drops of 3% hydrogen peroxide to the sections for 5 minutes. Primary antibody (mouseEGFR 1:100 dilution) was applied and sections were left at room temperature for an hour. Following three PBS washes, secondary antibody was applied and the slides were incubated at room temperature for 1 hour prior to three more washes in PBS. Avidin/Biotin Complex-Horseradish peroxidase (ABC-HRP) solution was then applied to each slide and the slides incubated for a further 30 minutes at room temperature. 3-diaminobenzidine tetrahydrochloride (DAB) was applied to each slide for 5 mins. Slides were then rinsed in tap water for 5 minutes, counterstained in haematoxylin (20 sec), and dehydrated through graded alcohols. The slides were transferred to fresh xylene and mounted with DPX.

Negative controls were performed to assess non-specific antibody staining. Primary antibody was omitted and replaced by 100 µl of normal serum. All other steps were as outlined above.

Table 2.3 shows the grading system devised by the pathologist who scored the slides. Both the percentage of staining and the intensity of staining was determined by both the author and the scoring pathologist.
<table>
<thead>
<tr>
<th>Grade</th>
<th>0</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staining pattern</td>
<td>No staining</td>
<td>Weak cytoplasmic staining in less than 10% of cells</td>
<td>Weak cytoplasmic staining in greater than 10% of cells</td>
<td>Strong cytoplasmic staining in greater than 10% of cells</td>
</tr>
</tbody>
</table>

Table 2.3 Immunohistochemistry grading system
2.2.2 Sequencing exons 19 and 21 of the *EGFR* gene

2.2.2.1 DNA extraction from paraffin-embedded tissues blocks.

2.2.2.1.1 Cutting sections for DNA extraction.

The microtome was cleaned with 100% ethanol ensuring any residual sections had been removed. A clean blade was placed on the microtome before cutting each sample. 3 x 10\(\mu\)m sections from the paraffin-embedded block were cut and placed in a cryovial tube. The tubes were centrifuged at 14,000rpm for 1 minute to bring down paraffin sections to the bottom of the tubes.

2.2.2.1.2 De-paraffinisation and rehydration of samples.

600\(\mu\)l of xylene was added to each sample. The samples were then incubated at room temperature for 5 minutes on the shaking heating block. The samples were centrifuged for 3 minutes at 13,500rpm to pellet the tissue. The xylene was removed into a xylene waste container. A total of three xylene washes were performed.

600\(\mu\)l of 100% ethanol was added (to remove the xylene). This was then incubated for 5 minutes on shaking heating block. The sample was then
centrifuged for 3 minutes at 13,500rpm. This was repeated for a total of two ethanol washes.

### 2.2.2.1.3 Cell lysis and DNA extraction using QIAamp DNA mini kit.

180μl of Buffer ATL was added to each sample. 20μl of Proteinase K was then added and mixed by vortexing. The samples were then incubated at 56°C shaking for 48 to 72 hours. The samples were then briefly centrifuged to remove drops from the lids of the tubes. 200μl Buffer AL was added, mixed by vortexing for 15 second, then incubated at 70 °C for 10 min. They were then pulse centrifuged. 200μl ethanol was added and mixed by pulse vortexing for 15 seconds. The samples were then pulse centrifuged, then added to a QIAamp spin column and centrifuged at 8,000rpm for 1 minute. The columns were then placed into a new 2ml collection tube, and the first collection tube was discarded (containing filtrate).

500μl of Buffer AW1 was then added and the columns centrifuged at 8,000rpm for 1 minute. The columns were then placed in a clean 2 ml collection tube and the filtrate discarded. Each column was opened and 500μl of Buffer AW2 was added. The caps were closed and the columns centrifuged at 14,000rpm for 3 minutes. The columns were placed in a new 2ml collection tube and centrifuged for 14,000rpm for 1 minute. The sample was then placed in a clean 1.5ml tube labelled with the sample name and date. 100μl of Buffer AE was added and incubated at RT for 1 minute. The tube was centrifuged at 8,000rpm for 1 minute to elute the DNAs. DNA
concentrations were determined using the Nanodrop spectrophotometer. 200ng of DNA in 10μl was needed for the nested-PCR reaction.

2.2.2.1.4 Amplification of Exons 19 & 21 from genomic DNA using nested-PCR

2.2.2.1.4.1 PCR 1 of nested PCR

The reagents were removed from -20°C freezer and allowed to thaw on ice. 0.5ml tubes were labelled with the sample number and “PCR1”.

Using a 1.5ml tube a “Master Mix” was set up for 20 reactions according to the table below. The Amplitaq was added last and kept in the freezer until just before its addition to the mix. 40μl of this Master Mix was added to each of the DNAs. 200ng (in 10μl) of DNA was added to each tube. All the tubes were centrifuged briefly at 8,000rpm. The tubes were put in the thermocycler using the PCR program below.
<table>
<thead>
<tr>
<th>Reagents</th>
<th>x1</th>
<th>x</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x Buffer</td>
<td>5μl</td>
<td>μl</td>
</tr>
<tr>
<td>10mM MgCl₂</td>
<td>3μl</td>
<td>μl</td>
</tr>
<tr>
<td>Forward Primer (20pmol)</td>
<td>1μl</td>
<td>μl</td>
</tr>
<tr>
<td>Reverse Primer (20pmol)</td>
<td>1μl</td>
<td>μl</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>1μl</td>
<td>μl</td>
</tr>
<tr>
<td>Amplitaq (5U/μl)</td>
<td>0.2μl</td>
<td>μl</td>
</tr>
<tr>
<td>Template DNA (200ng)</td>
<td>10μl</td>
<td>-</td>
</tr>
<tr>
<td>dH₂O</td>
<td>28.8μl</td>
<td>μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>50μl</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.4 PCR1**

**2.2.2.1.4.2 PCR program**

STEP 1 - 96°C for 2.00
STEP 2 - 94°C for 0.30
STEP 3 - 58°C for 1.00
STEP 4 - 72°C for 1.00
STEP 5 - Goto 2, 30 times
STEP 6 - 72°C for 10.00
STEP 7 - 4°C forever
STEP 8 - end
2.2.2.1.4.3 PCR 2 of nested PCR

After PCR1 was complete, tubes were removed from the thermocycler and placed on ice. The 0.5ml tubes were labelled with sample number and “PCR2”.

Using a 1.5ml tube a “Master Mix” (for 20 reactions) was set up according to the table below (Table 2.5) 48μl of this Master Mix was added to each of the labelled tubes. 2μl of the reaction from PCR1 was added to the corresponding tube labelled PCR2. All the tubes were centrifuged briefly at 8,000rpm. The tubes were placed in the thermocycler using the PCR program below.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>x1</th>
<th>x</th>
</tr>
</thead>
<tbody>
<tr>
<td>10xBuffer</td>
<td>5μl</td>
<td>μl</td>
</tr>
<tr>
<td>10mM MgCl₂</td>
<td>3μl</td>
<td>μl</td>
</tr>
<tr>
<td>Forward Primer (20pmol)</td>
<td>1μl</td>
<td>μl</td>
</tr>
<tr>
<td>Reverse Primer (20pmol)</td>
<td>1μl</td>
<td>μl</td>
</tr>
<tr>
<td>10mM dNTPs</td>
<td>1μl</td>
<td>μl</td>
</tr>
<tr>
<td>Amplitaq (5U/μl)</td>
<td>0.2μl</td>
<td>μl</td>
</tr>
<tr>
<td>Template DNA (from PCR1)</td>
<td>2μl</td>
<td>-</td>
</tr>
<tr>
<td>dH₂O</td>
<td>36.8μl</td>
<td>μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>50μl</td>
<td></td>
</tr>
</tbody>
</table>

*Table 2.5 PCR2*
2.2.2.1.4.4 PCR program

STEP 1 – 96°C for 2.00
STEP 2 – 94°C for 0.30
STEP 3 – 58°C for 1.00
STEP 4 – 72°C for 1.00
STEP 5 – Goto 2, 30 times
STEP 6 – 72°C for 10.00
STEP 7 – 4°C forever
STEP 8 – end

2.2.2.1.4.5 QIAquick PCR purification kit

Five volumes of Buffer PB were added to 1 volume of the PCR sample and mixed.

QIAquick spin columns were placed in a 2ml collection tubes. The sample was added to the QIAquick columns and centrifuged for 30 to 60 sec at 13,000rpm.

The flow-through was discarded. The QIAquick column was placed back into the same tube. 0.75ml Buffer PE was added to the QIAquick column and centrifuged for 30 to 60 sec at 13,000rpm.
The flow-through was discarded and the QIAquick column was placed back in the same tube. The column was centrifuged for an additional 1 minute at 13,000rpm.

The QIAquick column was placed in a clean 1.5ml tube. To elute DNA, 30µl Buffer EB was added to the centre of the membrane in the column. This was allowed to stand for 1 minute and then centrifuged at 13,000rpm for 1 minute.

2.2.2.1.5 Cycle sequencing using BigDye Terminator v3.1

The sequencing reaction took place in 0.2ml thin wall tubes. Each tube was labelled with sample number (on the lid and side of tube). The Master Mix was made up as per table below. 18µl of Master Mix was added to each 0.2ml tube. 2µl of each nested-PCR reaction from PCR 1 was added to the corresponding tube.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>x1</th>
<th>x</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template (20/50ng)</td>
<td>(20/50ng)</td>
<td>-</td>
<td>1µl pGem</td>
</tr>
<tr>
<td>Primer (1/50 diln of 100pmoles)</td>
<td>2pmoles</td>
<td>µl</td>
<td>2µl M13 primer</td>
</tr>
<tr>
<td>BigDye Terminator Mix v3.1</td>
<td>4µl</td>
<td>µl</td>
<td>4µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>µl</td>
<td></td>
<td>14µl</td>
</tr>
<tr>
<td><strong>Total Volume</strong></td>
<td>20µl</td>
<td></td>
<td>20µl</td>
</tr>
</tbody>
</table>

*Table 2.6 Cycle sequencing using BigDye Terminator v3.1*
The reactions were placed on the GeneAmp 2400 thermal cycler using the following program:

STEP 1: 96°C for 1 minute
STEP 2: 96°C for 10 seconds
STEP 3: 50°C for 5 seconds
STEP 4: 60°C for 4 minutes
STEP 5: Go to STEP 2, 25 times
STEP 6: 4°C forever
STEP 7: end

2.2.2.1.6 Clean up of sequencing products with Qiagen DyeEx spin columns

The DyeEx column was vortexed to resuspend the resin and loosen the cap of the column. The bottom of the column was snapped off and placed in a 2ml collection tube. The column was spun at 3000rpm for 3 minutes. The waste was emptied from the collection tube and 300µl of dH₂O was placed on the resin bed. The tubes were spun at 3000rpm for 3 minutes. The collection tube was removed and placed in the column in a 1.5ml microfuge tube. 20µl of the sequencing reaction was placed in the middle of the surface of the resin bed and spun at 3,000rpm for 3 minutes. The eluted DNA was then dried in a Speedyvac at medium heat for 20 mins.
2.2.2.1.7 Sequence detection

The pellet was resuspended in 10μl of HiDi formamide and loaded into a 96 well optical plate for sequencing on the PE 3100 sequencing instrument (ABI Foster City CA).

2.2.2.1.8 Sequence analysis

The sequence trace files were then analysed using DNASTAR software and were also manually read by eye and compared against the wild-type sequence of exons 19 and 21.

2.2.3 TaqMan Real Time Quantitative PCR

2.2.3.1 DNA extraction

DNA was extracted from paraffin embedded tumours as described previously.

2.2.3.1.1 Set up of real-time PCR

100ng of DNA was found to be the optimal amount of template DNA. This amount was confirmed by measuring DNA concentration and purity of stored DNA on the Nanodrop spectrophotometer (ND-1000, Thermo Fischer
Scientific, Wilmington, DE) before analysis. The following probe/primer concentrations were found to be the optimal conditions (Table 2.7, Table 2.8).

<table>
<thead>
<tr>
<th></th>
<th>X1</th>
<th>X2</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR Master Mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200nm probe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>300nm primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taqman Master Mix</td>
<td>12.5μL</td>
<td>25μL</td>
</tr>
<tr>
<td>Primer f</td>
<td>1μL</td>
<td>2μL</td>
</tr>
<tr>
<td>Primer r</td>
<td>1μL</td>
<td>2μL</td>
</tr>
<tr>
<td>Probe</td>
<td>1μL</td>
<td>2μL</td>
</tr>
<tr>
<td>H20μL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.7 Optimized qPCR EGFR

<table>
<thead>
<tr>
<th></th>
<th>X1</th>
<th>X2</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH Master Mix</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150nm probe</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150nm primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taqman Master Mix</td>
<td>12.5μL</td>
<td>25μL</td>
</tr>
<tr>
<td>Primer f</td>
<td>1μL</td>
<td>2μL</td>
</tr>
<tr>
<td>Primer r</td>
<td>1μL</td>
<td>2μL</td>
</tr>
<tr>
<td>Probe</td>
<td>1.5μL</td>
<td>3μL</td>
</tr>
<tr>
<td>H2O</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.8 Optimized qPCR GAPDH
Pooled DNA from normal healthy volunteers was used as positive controls; wells were set up with no template as negative controls. 100ng DNA was used in each reaction, and each reaction was run in duplicate. Reactions were carried out in 96-well optical reaction plates (Axygen, Union City, CA) using the ABI Prism™ 7000 Sequence Detector (Applied Biosystems, Foster City, CA).

Thermal cycler conditions were: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 94°C for 20 seconds and 60°C for 45 seconds. All results were normalized to GAPDH amplification (Applied Biosystems, Foster City, CA). Relative expression was calculated using the target threshold (CT) value for reference as a calibrator (Livak and Schmittgen 2001). The formula for this calculation is as follows:

\[
\Delta CT = CT\ (EGFR) - CT\ (GAPDH)
\]

\[
\Delta\Delta CT = \Delta CT - \Delta CT\ Reference
\]

Relative Expression = \( 2^{\Delta\Delta CT} \).

A standard of curve as also constructed for each plate and the copy number of EGFR calculated from that also.
2.2.4 Phase II Clinical Trial

Patients were selected from referrals to St James’ Hospital Thoracic Oncology Service. This is a tertiary referral centre in the Republic of Ireland. All patients were discussed at a multidisciplinary conference, and were approached for screening in the Thoracic Surgery Clinic once they had been deemed potentially clinically and technically resectable.

The primary study objective of this phase II trial was to assess the radiological and pathological complete and objective response rates to neoadjuvant cetuximab + cis/gem in resectable and operable stage IB-IIIA
NSCLC. The secondary end-points were: safety/tolerability, resection rate following therapy, overall survival, relapse-free survival and predictive biomarkers of response.

The inclusion criteria were: male or female patients >18 years old, performance status 0-1, biopsy proven non-small-cell lung cancer (NSCLC), clinical stage IB to IIIA with presence of unidimensionally measurable disease by Response Evaluation in Solid Tumour Criteria (RECIST), adequate end organ function.

The exclusion criteria were: proven or symptomatic brain metastases, stage IIIB or IV disease, major surgery within four weeks prior to study entry, chest irradiation within four weeks prior to study entry, concurrent chronic systemic immune therapy, chemotherapy or hormone therapy not indicated in the study protocol, any prior chemotherapy for the current disease or within five years of the current diagnosis, any investigational agent(s) within four weeks prior to entry, previous exposure to monoclonal antibodies, signal transduction inhibitors or EGFR targeting therapy, known drug abuse, pregnancy (absence confirmed by serum/urine β-HCG) or breast-feeding, legal incapacity or limited legal capacity, any previous malignancy in the last five years or lack of confirmation of complete remission of any previous malignancy, or any medical or psychological condition that in the opinion of the investigator would not permit the patient.

Cisplatin was administered at 80 mg/m² on day 1, and gemcitabine was administered at 1,250 mg/m² on day 1 and 8 of a 21-day cycle.
Cetuximab was administered at standard doses of 400 mg/m\(^2\) on day 1 and thereafter weekly at 250 mg/m\(^2\). There was a two-week wash-out period at the end of treatment.

A total of three cycles of chemotherapy was planned for each patient.

![Figure 2.2 Treatment schema](image)

**Figure 2.2 Treatment schema**

The clinical stage was assessed by CT and PET scanning, and where indicated mediastinoscopy. Post-treatment computed tomography was performed at completion of chemotherapy and this was compared to the pre-treatment scan using RECIST.
At surgery, the patient underwent full anatomical resection and lymph-node dissection for formal pathological staging of the tumour.

Serum, plasma and circulating mononuclear cells were collected on Day 1 of Cycles 1 to 3 of chemotherapy, and also post treatment. Sera were processed within a 45 minute time-window from the time they were collected.

Paraffin-embedded and snap-frozen tumour tissue was collected at the time of surgery.

These biological samples were processed and stored until analysis in the Thoracic Oncology Biobank at St. James’ Hospital at minus 80° C.

2.2.5 Statistical Analysis

Patient characteristics were summarized using frequencies and percentages for categorical variables and medians and ranges for age at surgery. Patient groups were compared using Fisher’s exact test on nominal categorical variables and the exact trend test on ordered categorical variables. The exact trend test looks for evidence of an increasing or decreasing probability of an event (i.e. being KRAS mutation positive) across the ordered categories. The analysis was generated using SAS v 9.2.

Patient survival time was calculated from the date of consent latest follow-up date. Survival rate was estimated using the Kaplan-Meier method, and
compared by the log-rank test. Postoperative mortality was calculated on the basis of patients who died within 30 days of date of surgery. However, all patients were included in the survival analysis.

The first patient received their first treatment in March, 2005 and the last patient received their first treatment in July, 2007. The data reported here was analyzed in September, 2007.

The influence on overall and disease-free survival of all covariates found to be significant in the univariate analysis, and was assessed by means of the multivariate Cox proportional hazards model to identify independent prognostic factors. All P values were two-sided, with a value of <0.05 considered statistically significant. Statistical significance of the association of the composite-predicted tree regression analysis groups with outcome was assessed by the Wald test.
CHAPTER THREE

RESULTS
3.1 Patient Demographics

A total of 197 tumour samples from 197 patients were evaluable. The patient demographics are shown below. Their demographics are fairly typical of a lung cancer population. There were 125 (63.5%) males and 72 (36.5%) females. The majority of patients (65%) were <70 years old. 129 (65.5%) of the patients were ex-smokers and only 11 (5.6%) patients were classified as never smokers, which is highly relevant. Patients with very early stage I disease formed the largest demographic group in terms of stage. 89 (45.2%) patients had adenocarcinomas. The median age at time of surgery was 65.7 years.

Immunohistochemical analysis was carried out on FFPE sections. Genomic DNA was extracted from corresponding FFPE samples as described in materials and methods.

Univariate and multivariate statistics were performed to test for the association of various clinicopathological parameters, with each of the EGFR related biomarkers.
<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>125 (63.5)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>72 (36.5)</td>
</tr>
<tr>
<td>Age at surgery</td>
<td>&lt;70.0</td>
<td>128 (65.0)</td>
</tr>
<tr>
<td></td>
<td>&gt;=70.0</td>
<td>69 (35.0)</td>
</tr>
<tr>
<td>Smoking status</td>
<td>Never</td>
<td>11 (5.6)</td>
</tr>
<tr>
<td></td>
<td>Ex-smoker</td>
<td>129 (65.5)</td>
</tr>
<tr>
<td></td>
<td>Current</td>
<td>57 (28.9)</td>
</tr>
<tr>
<td>Pathological stage</td>
<td>I</td>
<td>111 (56.3)</td>
</tr>
<tr>
<td>Pathological stage</td>
<td>II</td>
<td>45 (22.8)</td>
</tr>
<tr>
<td>Pathological stage</td>
<td>III</td>
<td>41 (20.8)</td>
</tr>
<tr>
<td>Pathological stage</td>
<td>II</td>
<td>45 (22.8)</td>
</tr>
<tr>
<td>Pathological stage</td>
<td>III</td>
<td>41 (20.8)</td>
</tr>
<tr>
<td>Histology</td>
<td>ADENO</td>
<td>89 (45.2)</td>
</tr>
<tr>
<td>Histology</td>
<td>BACC</td>
<td>2 (1.0)</td>
</tr>
<tr>
<td>Histology</td>
<td>LCNEC</td>
<td>5 (2.5)</td>
</tr>
<tr>
<td>Histology</td>
<td>PLEOMORPHIC</td>
<td>7 (3.6)</td>
</tr>
<tr>
<td>Histology</td>
<td>SQAUMOUS</td>
<td>94 (47.7)</td>
</tr>
<tr>
<td>Tumour Size</td>
<td>&gt;=4.0</td>
<td>111 (56.3)</td>
</tr>
<tr>
<td>Tumour Size</td>
<td>&lt;4.0</td>
<td>86 (43.7)</td>
</tr>
<tr>
<td>Tumour grade</td>
<td>POOR DIFF</td>
<td>65 (33.2)</td>
</tr>
<tr>
<td>Tumour grade</td>
<td>MOD DIFF</td>
<td>118 (60.2)</td>
</tr>
<tr>
<td>Tumour grade</td>
<td>WELL DIFF</td>
<td>13 (6.6)</td>
</tr>
</tbody>
</table>

Table 3.1 Patient demographics
Table 3.2 Age at surgery

Table 3.3 shows demographics according to EGFR IHC status. For the purpose of this analysis we included only those cases that were classified as +2 or +3 (grades described in the methods section) positive. Smoking and histology were found to correlate significantly with EGFR staining, with smoking history and adenocarcinoma showing increased levels of staining. Figure 3.1 shows +1, +2, +3 staining as described in the methods section, with negative control on the left of the pictures.

Figure 3.1 EGFR staining
<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>EGFR IHC Positive</th>
<th>EGFR IHC Negative</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N (%)</td>
<td>N (%)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>M</td>
<td>91 (79.8)</td>
<td>23 (20.2)</td>
<td>0.466</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>52 (74.3)</td>
<td>18 (25.7)</td>
<td></td>
</tr>
<tr>
<td>Age at surgery</td>
<td>&lt;70.0</td>
<td>91 (74.6)</td>
<td>31 (25.4)</td>
<td>0.191</td>
</tr>
<tr>
<td></td>
<td>&gt;=70.0</td>
<td>52 (83.9)</td>
<td>10 (16.1)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td>Never</td>
<td>9 (81.8)</td>
<td>2 (18.2)</td>
<td>0.051</td>
</tr>
<tr>
<td></td>
<td>Ex-smoker</td>
<td>98 (82.4)</td>
<td>21 (17.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Current</td>
<td>36 (66.7)</td>
<td>18 (33.3)</td>
<td></td>
</tr>
<tr>
<td>Pathological stage</td>
<td>I</td>
<td>85 (83.3)</td>
<td>17 (16.7)</td>
<td>0.157</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>28 (66.7)</td>
<td>14 (33.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>30 (75.0)</td>
<td>10 (25.0)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>ADENO</td>
<td>58 (68.2)</td>
<td>27 (31.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BAC</td>
<td>1 (50.0)</td>
<td>1 (50.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCNEC</td>
<td>3 (60.0)</td>
<td>2 (40.0)</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>PLEOMORPHIC</td>
<td>6 (100)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SQAUMOUS</td>
<td>75 (87.2)</td>
<td>11 (12.8)</td>
<td></td>
</tr>
<tr>
<td>Tumour Size</td>
<td>&gt;=4.0</td>
<td>78 (74.3)</td>
<td>27 (25.7)</td>
<td>0.215</td>
</tr>
<tr>
<td></td>
<td>&lt;4.0</td>
<td>65 (82.3)</td>
<td>14 (17.7)</td>
<td></td>
</tr>
<tr>
<td>Tumour grade</td>
<td>POOR DIFF</td>
<td>1 ( .? )</td>
<td>0 ( ?.)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MOD DIFF</td>
<td>41 (66.1)</td>
<td>21 (33.9)</td>
<td>0.090</td>
</tr>
<tr>
<td></td>
<td>WELL DIFF</td>
<td>8 (66.7)</td>
<td>4 (33.3)</td>
<td></td>
</tr>
</tbody>
</table>
BAC: Bronchoalveolar carcinoma Table 3.3 Demographics according to EGFR staining
In univariate analysis, both smoking and histology were statistically significantly more likely to test positive for EGFR overexpression. No other features were significantly associated. EGFR expression was detected in 77% of cases in keeping with that observed in recent studies. EGFR overexpression, defined as 3+ expression, was in 87% SCC, a figure higher than that seen in BACC and adenocarcinoma. This finding was statistically significant (p=0.06). No significant association was seen between EGFR protein and any other clinicopathological characteristic examined.

### 3.1.1 Overall survival according to EGFR protein expression

In this series, EGFR protein expression was not a prognostic factor.

![Survival Functions](image.png)

**Figure 3.2. Overall survival – EGFR IHC**
3.2 EGFR Mutations

We tested only for those mutations deemed classical sensitizing EGFR mutations – that is deletions in exon 19 and the L858R point mutation (Figure 3.3). In this population we found 12 of these mutations. The demographics of mutation-positive patients and wild-type mutations are shown below in Table 3.3

![Figure 3.3 The exon 21 point mutation L858R](image-url)
<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>EGFR mut positive</th>
<th>EGFR mut negative</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>7 (5.6)</td>
<td>118 (94.4)</td>
<td>0.761</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5 (6.9)</td>
<td>67 (93.1)</td>
<td></td>
</tr>
<tr>
<td>Age at surgery</td>
<td>&lt;70.0</td>
<td>8 (6.3)</td>
<td>120 (93.8)</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>&gt;=70.0</td>
<td>4 (5.8)</td>
<td>65 (94.2)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td>Never</td>
<td>1 (9.1)</td>
<td>10 (90.9)</td>
<td>0.583</td>
</tr>
<tr>
<td></td>
<td>Ex-smoker</td>
<td>6 (4.7)</td>
<td>123 (95.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Current</td>
<td>5 (8.8)</td>
<td>52 (91.2)</td>
<td></td>
</tr>
<tr>
<td>Pathological stage</td>
<td>I</td>
<td>7 (6.3)</td>
<td>104 (93.7)</td>
<td>0.364</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>5 (11.1)</td>
<td>40 (88.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0 (0.0)</td>
<td>41 (100)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>ADENO</td>
<td>7 (7.9)</td>
<td>82 (92.1)</td>
<td>0.462</td>
</tr>
<tr>
<td></td>
<td>BACC</td>
<td>0 (0.0)</td>
<td>2 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCNEC</td>
<td>0 (0.0)</td>
<td>5 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLEOMORPHIC</td>
<td>1 (14.3)</td>
<td>6 (85.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SQAUMOUS</td>
<td>4 (4.3)</td>
<td>90 (95.7)</td>
<td></td>
</tr>
<tr>
<td>Tumour Size</td>
<td>&gt;=4.0</td>
<td>7 (6.3)</td>
<td>104 (93.7)</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>&lt;4.0</td>
<td>5 (5.8)</td>
<td>81 (94.2)</td>
<td></td>
</tr>
<tr>
<td>Tumour grade</td>
<td></td>
<td>0 (.)</td>
<td>1 (.)</td>
<td>0.795</td>
</tr>
<tr>
<td></td>
<td>POOR DIFF</td>
<td>4 (6.2)</td>
<td>61 (93.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MOD DIFF</td>
<td>8 (6.8)</td>
<td>110 (93.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WELL DIFF</td>
<td>0 (0.0)</td>
<td>13 (100)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.4 Demographics according to EGFR mutational status
Sixty-six percent of patients with a mutation had adenocarcinoma. No clinical feature was significantly associated with carrying a mutation. The survival analysis for mutation positive patients versus non-mutation positive patients is shown below (Figure 3.4). There was no significant survival advantage for patients carrying a mutation, although the curves appear to separate, it does not reach statistical significance.

**Survival Functions**

![Survival Functions](image)

Figure 3.4 Overall survival according to mutation status
3.2.1 *KRAS* Gene Mutations

We tested the same DNA for mutations in exon 2 of *KRAS* (Figure 3.5). We did not observe any significant association with any of the demographics examined with mutation in *KRAS*.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>EGFR mut positive</th>
<th>EGFR mut negative</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>7 (5.6)</td>
<td>118 (94.4)</td>
<td>0.761</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>5 (6.9)</td>
<td>67 (93.1)</td>
<td></td>
</tr>
<tr>
<td>Age at surgery</td>
<td>&lt;70.0</td>
<td>8 (6.3)</td>
<td>120 (93.8)</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>&gt;=70.0</td>
<td>4 (5.8)</td>
<td>65 (94.2)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td>Never</td>
<td>1 (9.1)</td>
<td>10 (90.9)</td>
<td>0.583</td>
</tr>
<tr>
<td></td>
<td>Ex-smoker</td>
<td>6 (4.7)</td>
<td>123 (95.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Current</td>
<td>5 (8.8)</td>
<td>52 (91.2)</td>
<td></td>
</tr>
<tr>
<td>Pathological stage</td>
<td>I</td>
<td>7 (6.3)</td>
<td>104 (93.7)</td>
<td>0.364</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>5 (11.1)</td>
<td>40 (88.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>0 (0.0)</td>
<td>41 (100)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>ADENO</td>
<td>7 (7.9)</td>
<td>82 (92.1)</td>
<td>0.462</td>
</tr>
<tr>
<td></td>
<td>BACC</td>
<td>0 (0.0)</td>
<td>2 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LCNEC</td>
<td>0 (0.0)</td>
<td>5 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PLEOMORPHIC</td>
<td>1 (14.3)</td>
<td>6 (85.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SQAUMOUS</td>
<td>4 (4.3)</td>
<td>90 (95.7)</td>
<td></td>
</tr>
<tr>
<td>Tumour Size</td>
<td>&gt;=4.0</td>
<td>7 (6.3)</td>
<td>104 (93.7)</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>&lt;4.0</td>
<td>5 (5.8)</td>
<td>81 (94.2)</td>
<td></td>
</tr>
<tr>
<td>Tumour grade</td>
<td>POOR DIFF</td>
<td>0 ( . )</td>
<td>1 ( . )</td>
<td>0.795</td>
</tr>
<tr>
<td></td>
<td>MOD DIFF</td>
<td>4 (6.2)</td>
<td>61 (93.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>WELL DIFF</td>
<td>8 (6.8)</td>
<td>110 (93.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 (0.0)</td>
<td>13 (100)</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.5 *KRAS* mutations and demographics
Twelve patients were found to carry a mutation in exon 2 of KRAS. In univariate analysis, no clinical features significantly correlated with the chance of having a mutation (Table 3.5).

Patients in this series who had a KRAS mutation did not have significantly altered survival (Figure 3.6).

<table>
<thead>
<tr>
<th>EGFR Status</th>
<th>N</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>185</td>
<td>64.9</td>
<td>41.4</td>
<td>86.1</td>
</tr>
<tr>
<td>Positive</td>
<td>12</td>
<td>69.0</td>
<td>53.0</td>
<td>72.4</td>
</tr>
</tbody>
</table>

Table 3.6 Age at surgery according to EGFR status
3.3 qPCR

In univariate analysis, the only demographic feature that was significantly associated with the likelihood of detecting amplification was smoking, with 91% of never smokers not displaying amplification. Amplification was seen in 23% of current smokers, and 9% of never smokers, $p=0.35$. No other demographic factor was associated with EGFR amplification.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Category</th>
<th>EGFR ampl positive</th>
<th>EGFR ampl negative</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>17 (13.6)</td>
<td>108 (86.4)</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>10 (13.9)</td>
<td>62 (86.1)</td>
<td></td>
</tr>
<tr>
<td>Age at surgery</td>
<td>&lt;70.0</td>
<td>17 (13.3)</td>
<td>111 (86.7)</td>
<td>0.830</td>
</tr>
<tr>
<td></td>
<td>&gt;=70.0</td>
<td>10 (14.5)</td>
<td>59 (85.5)</td>
<td></td>
</tr>
<tr>
<td>Smoking status</td>
<td>Never</td>
<td>1 (9.1)</td>
<td>10 (90.9)</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>Ex-smoker</td>
<td>13 (10.1)</td>
<td>116 (89.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Current</td>
<td>13 (22.8)</td>
<td>44 (77.2)</td>
<td></td>
</tr>
<tr>
<td>Pathological stage</td>
<td>I</td>
<td>15 (13.5)</td>
<td>96 (86.5)</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>7 (15.6)</td>
<td>38 (84.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>5 (12.2)</td>
<td>36 (87.8)</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td>ADENO</td>
<td>12 (13.5)</td>
<td>77 (86.5)</td>
<td>0.597</td>
</tr>
<tr>
<td></td>
<td>BACC</td>
<td>0 (0.0)</td>
<td>2 (100)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.7 qPCR demographics

3.3.1 Overall Survival and qPCR

In an overall survival analysis, the presence of amplification in the \( EGFR \) gene was not associated with survival. There was a difference at three years in median survival estimates of 387 days, although this did not reach statistical significance, \( p = 0.25 \).
Figure 3.6 Overall survival according to qPCR
3.4 Multivariate Analysis

Although no factors were found to have significant statistical significance with survival in univariate analysis the multivariate analysis revealed no biomarker in this study to be significant (Table 3.8).

<table>
<thead>
<tr>
<th>Multivariate</th>
<th>Survival</th>
<th>HR</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>125</td>
<td>1.22</td>
<td>0.807</td>
<td>1.845</td>
</tr>
<tr>
<td>Female</td>
<td>72</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>adeno</td>
<td>84</td>
<td>0.893</td>
<td>0.427</td>
<td>1.867</td>
</tr>
<tr>
<td>squam</td>
<td>94</td>
<td>0.847</td>
<td>0.403</td>
<td>1.78</td>
</tr>
<tr>
<td>other</td>
<td>19</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neg</td>
<td>185</td>
<td>1.859</td>
<td>0.683</td>
<td>5.059</td>
</tr>
<tr>
<td>pos</td>
<td>12</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRAS</td>
<td></td>
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<tr>
<td>neg</td>
<td>185</td>
<td>0.875</td>
<td>0.374</td>
<td>2.047</td>
</tr>
<tr>
<td>pos</td>
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<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR Amplification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>neg</td>
<td>0.67</td>
<td>0.395</td>
<td>1.136</td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td>------</td>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>pos</td>
<td>170</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGFR IHC</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0</td>
<td>9</td>
<td>0.803</td>
<td>0.288</td>
<td>2.237</td>
</tr>
<tr>
<td>32</td>
<td></td>
<td>0.907</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1+</td>
<td>54</td>
<td>0.64</td>
<td>0.527</td>
<td>1.561</td>
</tr>
<tr>
<td>2+</td>
<td>89</td>
<td>1</td>
<td>0.39</td>
<td>1.048</td>
</tr>
<tr>
<td>3+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.8 Multivariate analysis
3.5 Neoadjuvant Chemotherapy in Non-small-cell Lung Cancer (NSCLC)

3.5.1 Patients and Treatment

From March 2005 to August 2007 32 patients were recruited. Patient characteristics are illustrated in Table 3.9. Thirty patients received chemotherapy after entry. Two patients did not have chemotherapy as, after providing consent, they failed screening when updated imaging revealed them to be inoperable.

<table>
<thead>
<tr>
<th>Demographic</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
</tr>
<tr>
<td>Female</td>
<td>12</td>
</tr>
<tr>
<td>Performance Status</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>13</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Histology</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>11</td>
</tr>
<tr>
<td>Squamous</td>
<td>14</td>
</tr>
<tr>
<td>Adenosquamous</td>
<td>1</td>
</tr>
<tr>
<td>NSCLC NOS</td>
<td>3</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>IB</td>
<td>23</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
</tr>
<tr>
<td>IIIA</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3.9 Patient demographics
3.5.2 Treatment Delivery

A total of 86 of a planned 90 (95%) cycles of chemotherapy were administered. There were treatment delays of one week with 23 of 86 (28%) administered chemotherapy cycles.

3.5.3 Efficacy

Efficacy data are summarised in Table 2. The overall response rate was 76% (n=22). One patient displayed a complete pathological and radiological response. Following appraisal of the pre- and post-treatment scans using RECIST, 15 out of 30 patients (50%) were down-staged. One patient experienced biopsy-proven progression in a distant site during induction. Pre-treatment, six patients had positive N2 nodes on CT scan but a negative mediastinal node biopsy. After surgery, three of these patients were found to have pathologically-confirmed mediastinal lymph node involvement, while two had no mediastinal involvement. The sixth patient progressed with brain metastases before surgery. The median survival was 29.2 months with patients demonstrating 1st, 2nd and 3rd year survival rates of 70%, 53% and 48% respectively.
### Table 3.10 Response to treatment

<table>
<thead>
<tr>
<th>Response</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall response</td>
<td>79% n=23</td>
</tr>
<tr>
<td>Complete response</td>
<td>3% n=1</td>
</tr>
<tr>
<td>Partial response</td>
<td>76% n=22</td>
</tr>
<tr>
<td>Stable disease</td>
<td>21% n=6</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>3% n=1</td>
</tr>
<tr>
<td>Complete pathological response rate</td>
<td>5% n=1</td>
</tr>
<tr>
<td></td>
<td>(1/22 resected patients)</td>
</tr>
</tbody>
</table>

**Scan demonstrating PR**

Figure 3.7 Objective response to treatment (PR)
3.5.4 Safety

With the exception of cardiovascular toxicity, there was an acceptable and expected level of toxicity with this treatment regimen (Table 3.11). However, there appeared to be a higher than expected number of serious adverse cardiovascular events including four patients (five episodes) with Cardiovascular Accidents (CVA), three patients with peripheral arterial ischemia, one patient who experienced both a CVA and peripheral arterial ischemia, and two patients with pulmonary emboli.

<table>
<thead>
<tr>
<th>Toxicity</th>
<th>Number of patients (n)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutropenia</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>12</td>
<td>25</td>
</tr>
<tr>
<td>Febrile neutropenia</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Anaemia</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Nausea</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Constipation</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Dyspnoea</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Elevated creatinine</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Abnormal liver function tests</td>
<td>1</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 3.11 Clinically significant toxicity experienced on study
Safety

- The most common toxicity was skin rash seen in 100% patients

Figure 3.8 Photograph of typical acneiform skin rash
3.5.5 Progression to Surgery

There were 22 patients who underwent surgical exploration following neo-adjuvant treatment. One patient declined surgery, one patient was removed from trial for non-compliance as a result of relapsed alcoholism, one patient had worsening Pulmonary Function Tests (PFTs), two patients had post-chemotherapy mediastinoscopy that showed a persistent tumour in mediastinal nodes, and one patient had progressive disease. One patient underwent thoracotomy, but was found to have an unresectable tumour. All the other patients had complete resections with negative margins.
Patient outcomes following treatment

**Figure 3.9.** Survival curves for patients on study.
CHAPTER FOUR

DISCUSSION
4.1 Discussion of EGFR Biomarker Work

No association was found in this series between any of the EGFR-related biomarkers and survival. This is consistent with the existing literature on the subject. EGFR expression has been extensively examined in NSCLC and the general consensus is that when evaluated alone, the growth factor is not a robust prognostic factor. A previous review found that only 10% of studies published between 1985 and 2000 showed an association between increased EGFR expression and OS (28). Furthermore, an extensive review of 16 studies (1989-2001) by Meert et al, (29) found that EGFR expression was not prognostic in 12 of those studies. In addition EGFR expression does not predict response to chemotherapy in NSCLC (30). Our findings, that no cut-off level of EGFR expression could be associated with prognosis, are in keeping with these observations. We know that EGFR mutation tends to confer survival advantage in a treatment-naive population. Much of our knowledge of the prognostic and predictive nature of EGFR biomarkers though comes from studies of patients with advanced disease. Although there have been some surgical series revealing favourable prognostic outcomes in patients harbouring an EGFR sensitizing mutation, and the converse for those harbouring a mutation in the \textit{KRAS} gene. Our numbers of patients with EGFR mutations were small, which may be why there was no demonstrable survival advantage. Our results were consistent, however, with a recently presented work of one thousand patients from Memorial Sloan Kettering Hospital (D'Angelo, Janjigian et al.)

The clinical features that were associated with having a mutation in our series were consistent with the published literature also. An important feature of this surgical series is that it was racially homogenous. Over the
past five years since the inception of this project, race has been seen to be hugely important in both the molecular configuration and the clinical behaviour of NSCLC. Ireland during the time these patients were operated on was a racially homogenous country and all of the patients in this series to the best of our knowledge were Caucasian. This probably impacts both the low numbers of mutations seen in this series (but consistent with the literature in Caucasian patients) of mutation-positive patients, and also leads to the relatively limited impact demonstrated on survival.

It is interesting that a third of the mutations in this group were in squamous cell carcinomas, as this is relatively rare in the literature. Lung cancer in non-smokers is a disease more likely to harbour EGFR sensitizing mutations. This fact also impacts this study as the majority of patients in this series smoked, and there were only 11 lifelong never-smokers.

Regarding the significance of our findings with IHC and qPCR, the conclusions we can draw are less clear. A recent editorial drew attention to the fact that biomarkers in EGFR are difficult to understand (Shepherd and Tsao). In addition our study utilized qPCR rather than the now more widely accepted FISH to assess gene dosage or amplification. At the time of inception of this work the value of FISH was unclear, and so we investigated the value of qPCR instead, which has since proven to be less studied and less useful. In this series it was not found to be prognostic.

In summary, then, we found that EGFR protein expression, gene expression and mutational analysis were not useful markers when standing alone. Subsequent work done in our laboratory has shown, however, that EGFR in conjunction with other markers (such as Insulin-like growth factor 1) may be prognostic.
This was an important work as it uniquely characterizes the EGFR profile of an Irish population. This information is crucial, as practising oncologists follow international guidelines and evidence-based medicine, and as discussed above, this cohort of patients was racially homogenous, and therefore molecularly quite a different cohort from those cohorts studied in the clinical trials testing EGFR-related therapies, upon which practice is based. One of the key findings of this body of work is the relatively low (but consistent with published literature) rate of EGFR mutation in the Irish population – now established as a key predictive factor in the treatment of advanced NSCLC.
4.2 Discussion of Phase II Clinical Trial

Although this series is small, single arm and from a single institution, the response rate compares favourably to historical controls at our institution and to other reported studies (Pisters, Vallieres et al. 2005). The response rate in the Chemotherapy in Early Stage Trial (ChEST) study that administered neoadjuvant cisplatin and gemcitabine was only 35% (Scagliotti, Pastorino et al. 2008), and in the LU22, which allowed several platinum-based induction regimens, the response rate was 49%. Even in the Southwest Oncology Group (SWOG) S9900 study that administered both chemotherapy and thoracic radiotherapy pre-operatively, the response rate was only 41%. Our study suggests that cetuximab may potentiate the effect of cytotoxic chemotherapy, an observation consistent with preclinical data and data from at least four trials in advanced disease (Butts, Bodkin et al. 2007; T. J. Lynch and Weber 2007; Pirker, Szczesna et al. 2008; Rosell, Robinet et al. 2008).

The treatment was in the main, well tolerated with the majority of patients in this study receiving the planned or the majority percentage of planned dose. However, the cardiovascular toxicity that our patients experienced is concerning. It should be noted, though, that all patients who experienced a grade 3 or greater cardiovascular event had some degree of pre-existing cardiovascular condition. Given that atherosclerosis and lung cancer share the major etiological factor of cigarette smoking, it may be simply that the sample size was small with clustering of cardiovascular comorbidity that was clinically silent on study entry. A more worrying explanation may be that cetuximab potentiates the cardiovascular toxicity of cisplatin. This is an unlikely explanation when much larger samples
randomised to treatment with platinum doublet with or without cetuximab failed to show statistically significant differences between cardiovascular toxicity on the two arms. However, in the FLEX study there were double the amount of grade 4 pulmonary embolic events (4% versus 2% on chemotherapy alone arm), this observation failed to reach statistical significance with a p-value of 0.26 (Pirker, Szczesna et al. 2008).

To date we have no definitive predictive marker of response to therapy with cetuximab in NSCLC. A recent publication correlates EGFR gene copy number as defined by fluorescence in situ hybridization (FISH) in response to cetuximab (Hirsch, Herbst et al. 2008), but, like the current study, all patients received cetuximab. Molecular analysis of samples from the FLEX and BMS 099 have both failed to reveal any robust predictive markers (Khambata-Ford S and Dakhil S 2008; O'Byrne, Bondarenko et al. 2009).

Overall, this treatment regimen seemed to be relatively well tolerated with an encouraging response rate. The feasibility of neoadjuvant chemotherapy is well established. Neoadjuvant studies are technically more difficult to accrue to than adjuvant studies for reasons that are multifactorial. Despite this, neoadjuvant chemotherapy may confer a significant survival advantage, although this remains a controversial area. With the positivity of the adjuvant trials, this may well never be definitively answered, and may be easier to deliver than adjuvant. The role of combining targeted therapies with either adjuvant or neo-adjuvant chemotherapy is as yet undefined and warrants further study.
CHAPTER FIVE

CONCLUSION AND FUTURE DIRECTION
5 Future Direction

Biomarker research is a clear priority to facilitate patient treatment decisions. A recent editorial (Shepherd and Tsao) entitled “Epidermal Growth Factor Receptor Biomarkers in NSCLC: A riddle wrapped up in a mystery, inside an enigma” sums up the current state of play with epidermal growth factor receptor biomarker work. The classic sensitizing mutations of $EGFR$ seem to have a clear role in terms of predicting which patients will benefit most from treatment with the EGFR TKIs. To date, however there is no validated biomarker for predicting which patients might benefit from treatment with the EGFR MAbs such as cetuximab. Unravelling these mysteries and exploring the interplay the EGFR pathway has with other pathways is a clear priority for the future.

Regarding future direction for trial designs such as the one discussed here – neoadjuvant chemotherapy with monoclonal antibodies in early stage NSCLC, the future is less defined. There is no doubt that it is an attractive model, allowing molecular reconfiguration to be examined following exposure to an agent of interest. As adjuvant chemotherapy is now a standard of care, it is difficult to see how this type of study design will be utilised in ongoing drug-development. Rather, so-called “window of opportunity” studies might be utilised, as this type of study design does not preclude the patient from treatment with standard adjuvant chemotherapy.
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APPENDIX C

ABBREVIATIONS USED IN THE TEXT
CHAPTER ONE

IASLC – International Association for the Study of Lung Cancer
SBRT – Stereotactic Body Radiotherapy
VEGF – Vascular Endothelial Growth Factor
EGFR – The Epidermal Growth Factor Receptor
RTK – Receptor Tyrosine Kinase
TKs – Tyrosine Kinases
TKIs – Intracellular TK domain
IHC – Immunohistochemistry
FISH – Fluorescent In Situ Hybridization
qRT-PCR – Real Time quantitative Reverse Transcription PCR
CISH – Chromogenic In Situ Hybridization
qPCR – Real-time quantitative Polymerase Chain Reaction
ISEL – Iressa survival evaluation in lung cancer trial
INTEREST – Iressa in non-small-cell lung cancer trial evaluating response and survival versus taxotere study.
INVITE – Gefitinib (Iressa) versus vinorelbine in chemo-naive elderly patients with advanced non-small-cell lung cancer.
IPASS – Iressa Pan-Asia Study
RAS-GTPase – RAS-Guanosine Triphosphatase
GDP – Guanosine Diphosphate
TRIBUTE – The Tarceva response in conjunction with paclitaxel and carboplatin study.
CHAPTER TWO

**DAB** – Diaminobenzidine Tetrahydrochloride

**GAPDH** – Glyceraldehyde-3-phosphate dehydrogenase

CHAPTER THREE

**CVA** – Cardiovascular Accidents

**PFTs** – Pulmonary Function Tests

**ChEST** – Chemotherapy in Early Stage Trial

**SWOG** – Southwest Oncology Group
APPENDIX D

PRESENTATIONS AND PUBLICATIONS


investigator award from International Association for the Study of Lung Cancer (IASLC).


APPENDIX E

REFERENCES


Niho, S., Y. Ichinose, et al. (2007). "Results of a randomized phase III study to compare the overall survival of gefitinib (IRESSA) versus docetaxel in Japanese patients with non-small cell lung cancer who failed one or two chemotherapy regimens." J Clin Oncol (Meeting Abstracts) 25(18_suppl): LBA7509-.


