TYROSINE KINASE GROWTH FACTORS IN GASTRIC CANCER

ALISON KERR LANNIGAN

MB. ChB, FRCS

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Department of Surgery (Royal Infirmary)

University of Glasgow

ABSTRACT

In the West of Scotland in the last thirty years there has been a definite decrease in the incidence and mortality from gastric cancer but the survival of patients who develop adenocarcinoma of the stomach remains poor.

This thesis is introduced by an audit of gastric cancer in a single centre over a period of twenty years. This aims to describe changes in the presentation, diagnosis and management of the condition during the period of study. Overall, despite improvements in investigation the disease still presents at an advanced stage and rates of curative resection are low. Advances in surgical technique and post-operative care have not improved long term survival with little significant additional benefit from current adjuvent medical therapy.

The tyrosine kinase growth factor receptors have been investigated in a number of human cancers including breast, colon, ovarian and squamous carcinoma. In gastric cancer, the level of expression and prognostic value of EGFr and c-erbB-2 have been reported with some considerable variation using standard immunohistochemistry. To assess the use of growth factors as tumour markers or potential therapeutic targets, reliable quantitative and reproducible measurements of expression must be established.

This study reports the quantitative expression of EGFr and c-erbB-2 using radioimmunohistochemistry (RIHC) and compares the results with expression using conventional immunohistochemistry (IHC). The correlation of established clinico-pathological factors with EGFr, c-erbB-2 and the gene amplification of c-erbB-2 using fluorescence in situ hybridisation is investigated.

In both EGFr and c-erbB-2 expression there is good overall correlation analysis using RIHC and IHC ($p<0.0005$) but the separation of results is significantly better with RIHC particularly in the higher expressing tumours. In comparing tumour to mucosal samples, 22.4% of tumours expressed c-erbB-2 at a higher level, using RIHC and 43.3% with IHC. EGFr was expressed in 22.4% of cases more than the mucosal range with RIHC and 41.8% with IHC. There was no correlation of EGFr and c-erb-2 ($r^2 = 0.004$, $p = 0.594$).

In this study there is significant correlation of c-erbB-2 with well and moderately well differentiated tumours ($p=0.008$) and no serosal involvement ($p=0.066$). EGFr in this study

also appears to correlate with well and moderately well differentiated tumours (p=0.079). There was no correlation between EGFr, c-erbB-2 and patient survival.

This thesis has demonstrated IGF-I induced cellular proliferation with a dose dependent inhibition by the tyrophostin RG13022. IGF-I is found to be a less potent stimulator of the MAP kinase pathway than EGF and RG13022 is less able to inhibit this pathway.

ACKNOWLEDGEMENTS

In the clinical study of gastric cancer, I was encouraged in this work by Professor Colin McArdle and Mr. John Anderson, whose interest and support in this project remains enthusiastic and considerable.

I am fortunate to have been introduced to this fascinating area of research by Professor Peter Stanton and Dr. John Bartlett with the support and commitment of Professor Tim Cooke, in a department where the diversity of scientific background brings an exceptional appreciation of the need for research in modern surgical practice.

In the experimental work of this thesis I am grateful for the knowledge and patience of Dr. John Reeves in the field of immunohistochemistry; Alison Rae and Vivienne Morrison in cell culture and immunoblotting, and Dr. Nicol Keith in fluorescence in situ hybridisation.

In the Department of Pathology, I must commend Dr. Colin Stewart for his tuition on gastric cancer pathology and review of the specimens in the study and the patience of Mr. David Hole from the Cancer Surveillance Unit in our analysis of the audit data.

I would like to thank the patients and surgeons of Glasgow Royal Infirmary for the use of tumour specimens and those of St. James Hospital in Leeds.

I would also like to express my thanks to David, Gavin and Fiona and my parents for the constant encouragement, support and belief in the completion of this manuscript.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any tertiary institution. To the best of my knowledge and belief it contains no material previously published or written by another person, except where due reference is made in the text.

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PART I

GASTRIC CANCER: AN INTRODUCTION

Chapter 1

Gastric cancer – The Clinical Problem

The survival of patients diagnosed with gastric cancer in the West of Scotland is poor. In our institution over twenty years the five year survival has reduced from 11% to 6% despite the improvements in endoscopic diagnosis, surgical technique and post-operative care. A decline in the incidence of gastric cancer has been recognised with a number of factors involved in the decline but we have yet to fully determine an explanation for this and the variation in the incidence of gastric cancer between countries throughout the world.

In the introduction to this thesis I will outline a number of factors which are recognised as contributing to the variability in incidence and presentation of gastric cancer in areas of variable risk. I hope to set the scene of gastric cancer diagnosis and treatment in the West of Scotland in the time period studied. In an attempt to describe the clinical problems of gastric cancer in the West of Scotland, particularly the advanced stage at presentation and difficulties thereafter, in managing the disease, I will present original observations from the West of Scotland population.

In subsequent sections, the current knowledge of tumour biology, prognostic factors and tumour markers in gastric cancer will be reviewed. Following on from recent advances in the understanding of the molecular biology of cancer and improved techniques of investigation, I will describe a series of investigations which may further our knowledge of the behaviour of gastric cancer. In particular, an analysis of the level of expression and gene amplification of c-erbB-2 and of EGFr expression will be described in a series of gastric cancers with a discussion of the merits of the various methods of detection. I will co-ordinate this information with knowledge of in vitro inhibition of gastric cancer cell growth using novel inhibitors to the EGFr and c-erbB-2. In more detail I will describe the IGF-I receptor and preliminary proliferation studies of gastric cancer cells exposed to IGF-I in vitro and demonstrate inhibition of this activity.

Epidemiology

Section i: The changing incidence of gastric cancer

The incidence of gastric cancer has slowly declined in Scotland over the last three decades as it has worldwide but there remains a significant number of patients developing the disease. In Scotland it is the fourth most common cause of death from a malignant disease (Smans 1985). The incidence in the Scottish population has reduced from 22.1 to 18 per 100,000 in males and 12.1 to 7.6 per 100,000 in females in the period from 1960-1990 with a further reduction to 14.5 per 100,000 males in 1997 and 6.8 per 100,000 females. In 1960-1990 there has been a more pronounced decrease in mortality from 27.6 to 15.7 per 100,000 in males and from 23.7 to 11.2 per 100,000 in females. Despite this the overall five year survival has shown only a 2% improvement in the last thirty years (McKinney 1993). In Scotland this equates to five year survival of 11.5% between 1991 and 1995 (NHS Publications 2001).

Section ii: Geographical variation of gastric cancer

The frequency of gastric cancer shows marked differences when comparing the population in Western Europe and in the U.S.A. with the much higher risk areas of Japan, China, Eastern Asia, Asiatic Russia and the Andean South America (Correa 1991). The predominant histological type of gastric cancer in these areas is the intestinal type and is particularly prone to temporal and regional variation (Lauren 1965, Correa 1973).

Section iii: The influence of genetic factors

The incidence of the less common diffuse type is more or less equal in high and low risk areas but corresponds to a higher proportion of cases in areas with a lower incidence and overall risk of gastric malignancy. There is an association between the diffuse type tumour and blood group A (Correa 1973). Migrants from high risk countries retain a higher risk and subsequent incidence of disease if they settle in a country of lower incidence and this can be retained for several generations (Haenszel 1972, Waterhouse 1983). Therefore, geographic, environmental and genetic factors are all implicated.

Section iv: Dietary factors

Dietary factors are involved in the development of gastric cancer and have been evaluated with regards to their promotional and protective effects. In Japan, milk has been shown to be protective possibly involving better utilisation of Vitamins A and E (Hirayama 1968). Diets, which are low in animal fat and protein intake are found to occur in both high and low risk areas but in general those areas at high risk have low dietary intake of fat and protein. A similar picture is found with the intake of complex carbohydrates and it may be that cooking of grains, prominent in the diet in high risk countries, may be involved. A low content of Vitamin C in the diet is thought to be a risk (O'Toole 1996) and a lack of fresh fruit and vegetables (Correa 1983). Vitamin C is known to be a scavenger of mucosal damaging reactive oxygen metabolites and inhibits nitrosation in the production of potentially mutagenic N-nitroso-nitorosamines. Gastric juice ascorbic acid is reduced by Helicobacter pylori infection.

A high salt intake is associated with increased risk of gastric cancer possibly as a consequence of the induction of chronic gastritis and this has been demonstrated in animal models (Sato 1959). Finally nitrate consumption has long been thought to be a factor increasing the risk of gastric cancer (Natl Acad Sciences 1981). It is not, however, the amount of dietary nitrogen that is responsible but the carcinogens produced when

nitrate is reduced by intragastric bacteria in an environment of reduced gastric acidity such as is found with bacterial overproliferation in chronic atrophic gastritis (Correa 1975). In contrast to this theory, atrophic gastritis may not be associated with bacterial overproliferation but achlorhydria may be a marker of abnormal mucosa consequent to a chemical reaction between nitrate and amine at an acid pH (Cunningham 1987). Nitrosation can occur at an acid pH (Reed 1993) although the rate of acid catalysed nitrosation is dependent on nitrate concentration. This may be a way in which the varying amount of dietary nitrates are important in providing a possible method for the regional variation in incidence which corresponds to dietary differences.

Section v: Chronic gastritis and dysplasia

Chronic gastritis and atrophy are linked with protein malnutrition, chemical irritation by spices and smoked fish, osmotic stress due to high salt intake, pernicious anaemia and Polya gastrectomies (Hill 1987). In addition to gastric atrophy these conditions have been found in association with cases of intestinal metaplasia of the gastric mucosa and dysplasia. Bile reflux following gastric surgery is also known to cause dysplasia of the gastric mucosa. Intestinal metaplasia and dysplasia are recognised precursors of gastric cancer particularly of the intestinal type which accounts for the majority of gastric cancer in countries such as Japan. In the United Kingdom, the effect of dietary and environmental factors has been difficult to satisfactorily evaluate. There is an association between gastric cancer and low socio-economic groups which may be in some way related to dietary factors and Helicobacter infection (Eurogast study group 1993, Sitas 1991).

Section vi: Helicobacter pylori infection

The role of Helicobacter pylori (Hp) infection and the development of chronic inflammatory conditions of the stomach has stimulated investigation into its role in carcinogenesis. A high incidence of Helicobacter infection is found in high risk areas and is now considered a significant risk factor in gastric cancer (Parsonnet 1991, Forman 1991). Further acid suppression for symptomatic control of dyspepsia without eradication of Helicobacter pylori may further exacerbate the carcinogenic potential.

Section vii: Gastric cancer - how can we improve?

The incidence of diffuse tumours is relatively uniform worldwide but represents a larger proportion of tumours in low risk areas. Although the development of intestinal type tumours from chronic gastritis to dysplasia and invasive carcinoma is recognised, the development of diffuse tumours is less clear. In all tumours the aggressiveness and metastatic potential are unpredictable. In an area of relatively low incidence of gastric cancer such as the West of Scotland, screening programmes for the disease such as those in Japan (Oshima 1986) would not be economically viable. Therefore, our efforts must focus on the early detection and treatment of the cancer possibly by screening high risk groups such as in 'dyspepsia' clinics (Allum 1986). The establishment of better methods of outcome prediction and new treatment programmes are also essential.

In order to focus on the difficulties in establishing a diagnosis and management strategy at an early stage, I have brought together a review of the literature and a series of original observations on the pattern of presentation and treatment in gastric cancer over twenty years in a Glasgow hospital. The catchment area of the hospital is in a significantly deprived area of the city which is an important factor in the analysis of outcomes in the survey. The general health of the population is poor with significant

cardio-respiratory disease and poor nutrition. Health education is less effective, with disease presenting at an advanced stage. There are high levels of smoking, drug and alcohol abuse and each of these factors contributes to the poor outcomes which are described. I will describe the demographics of the population, the clinical symptomology, treatment, prognostic factors and outcome. Together with findings from a number of other studies I will highlight the difficulties in the management of gastric cancer in the east end of Glasgow and the need to establish new methods of intervention.

Chapter 2

Changing trends in gastric cancer in the West of Scotland Results of an audit over twenty years

During the two periods of 1974-1984 and 1986-1994, audits of the treatment and outcome of gastric adenocarcinoma in one Glasgow teaching hospital have been undertaken. The purpose was to outline areas of potential improvement in treatment (Cunningham 1987) and observe trends within factors which may aid prediction of outcome. During the time periods outlined, 328 and 369 patients respectively were diagnosed as having histologically confirmed gastric adenocarcinoma in Glasgow Royal Infirmary. The data was gathered from case notes and pathology reports using similar data collection forms in both groups. The information was processed in the Cancer Surveillance Unit which was also the source of the patient list of reported cases of gastric cancer in the hospital. The earlier period of data was reviewed, analysed separately and then analysed in conjunction with the later set. The later data set was reported for the first time.

The first series of data from 1974-1984 was collected by a Research Fellow in the Department of Surgery at Glasgow Royal Infirmary in the last two years of that period with the project supervised by Professor C. McArdle in both periods of study. Data collection forms were developed and of the 330 patients identified from the Cancer Register, 328 had sufficient clinical data to be included in the analysis.

In the later set, the Cancer Surveillance Unit identified 406 patients with gastric cancer during the time period. However, 37 patients (9%) were excluded because there was no histological diagnosis of gastric cancer. Clinical data for the remainder of the patients was included. There is obviously a potential for bias when a group of patients are excluded from an analysis and using the criteria in this study, the bias would apply to both time periods. Within the group excluded there are certain to be patients who were suffering from gastric cancer and obviously no histological type could be determined.

Diagnosis from clinical examination or barium studies with no surgery performed; rapid deterioration and death making endoscopy inappropriate or patient frailty would mean exclusion from this study. However, there are those patients who present with weight loss, coffee-ground vomiting or an abdominal mass who deteriorate prior to investigation and are labelled as a potential gastric cancer without documented evidence. The diagnosis of gastric cancer in these cases is by no means certain and inclusion would be inappropriate. Using the same criteria in both time periods allows comparison in these groups. Bias from potentially excluding elderly and advanced disease is recognised but the analysis of those patients having potentially curative surgery would remain focused.

Ensuring the quality of data collected in the earlier series is more difficult than in the later series where the case-note survey was completed by myself. The sets of data were held by the Cancer Surveillance Unit for both time frames and had been analysed in co-operation with Professor D. Hole, Professor of Epidemiology at Glasgow University.

Demographics

Section i: Age distribution of patients

There appears to be little difference between the proportion of patients in each age group (Table 1) and the peak age group between 65 and 74 reflects that of other centres in the UK and Europe (Lindahl 1988, Irvin 1988, Athlin 1995). The diagnosis continues to be uncommon under 55 years and a higher proportion of male patients present under the age of 75. Between 1974 and 1984, 84% of males and 72% of females were under 75 years and between 1986 and 1994 the same difference remained.

In the latter time period, females predominate over the age of 75 and this reflects, in part their improved life expectancy and the increased risk of cancer with age (Figures 1 and 2).

The age distribution of patients is summarised in Table 1 and Figures 1 & 2

Table 1

Age group	**1974-1984**	**1986-1994**
<55	52 (16%)	34 (9%)
55-64	83 (25%)	107 (29%)
65-74	125 (38%)	128 (35%)
75+	68 (21%)	100 (27%)

Figure 1 **Age of patients 1974-1984**

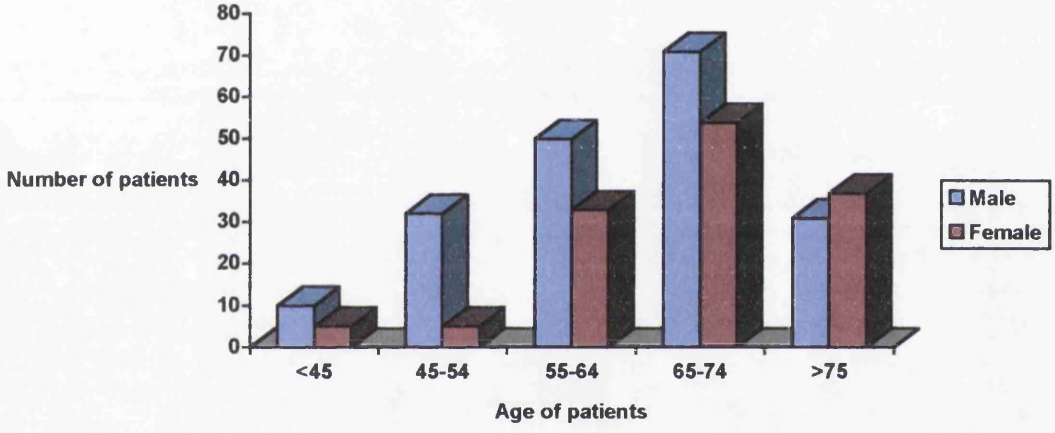
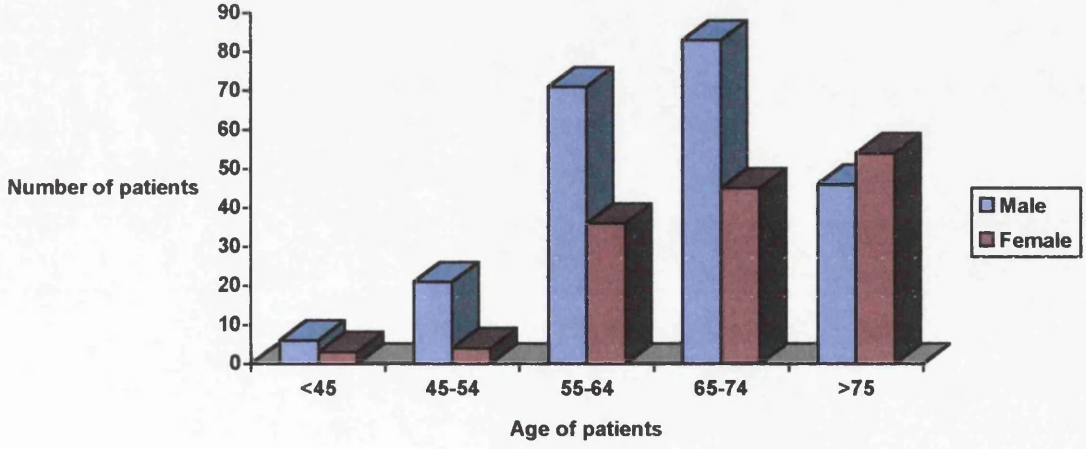


Figure 2 **Age of patients 1986 -1994**



Section ii: Distribution of patients by sex

The male to female ratio has changed from 1.45:1 to 1.6:1 (Table 2) and is at the lower end of the ratio of 1.5-2.5:1 found in most European and North American centres (Cady 1989, Viste 1986).

Table 2 **Distribution of patients by sex**

Sex	**1974-1984**	**1986-1994**
Male	194 (59%)	227(61.5%)
Female	134 (41%)	142 (38.5%)

Symptomology

Section iii: Presenting symptoms and duration of onset

Little change was recorded in the relative frequency of presenting symptoms from the patients over the two decades (Table 3). There has been no defining symptom to emerge to separate the process of underlying gastric malignancy from benign disease.

Table 3 **Presenting symptoms**

	1974-1984	**1986-1994**
Anorexia / weight loss	272 (83%)	226 (61%)
Dysphagia / vomiting	210 (64%)	190 (51%)
Dyspepsia / Abdominal pain	211 (64%)	205 (56%)
Anaemia	92 (28%)	60 (16%)
Emergency bleeding / perforation	55 (17%)	54 (15%)

This difficulty in distinguishing benign and malignant disease symptomatically, until the cancer is advanced has, in high risk countries, led to population screening. One such

approach involves the use of barium studies with photofluorographic imaging to examine the population over 40 years with suspicious cases having endoscopy. In Japan, this amounted to screening 5.2 million people annually in 1985 and has resulted in the proportion of early gastric tumours increasing. The detection of increasing numbers of gastric cancer in the early stages appears to improve survival in the disease (Yamazaki 1989). However, the lower incidence of the disease in Scotland would not make this an economically viable option and in addition, formal evaluation of screening for gastric cancer with randomised trials has not, and is now unlikely to be performed. The accelerated decline in mortality with respect to incidence which is attributed to screening in gastric cancer must take account of figures which show that the population invited for annual screening has never reached the target of 30%. In addition the effect of lead time bias and detection of pre-malignant lesions must be further investigated (Pisani 1996).

Dyspepsia clinics and programmes to improve the early detection of gastric cancer (Allum 1986) have had some success. Co-operation between GP practices and specialised units for early referral of symptomatic patients in this initiative with good compliance in attendance for endoscopy achieved an increase in the percentage of early lesions. Unfortunately, the non-specific nature of symptoms (Meyers 1987) implies that substantial resources would be required and this option is probably only achievable in central specialist centres (Humphreys 1995).

There are a number of issues which must be addressed when one examines the duration of patient symptoms prior to presentation (Table 4). The health education of the population must improve to alert patients with dyspepsia to the potentially sinister nature of their symptoms in particular when there are more than 20% of patients presenting after more than 6 months. Interestingly, although we continually strive for early detection to ensure more successful surgery and longer survival, those patients in our study who presented with symptoms of more than 6 months duration had a longer five year survival (Table 5). This was a consistent finding in both decades studied and on multivariate analysis of both sets of patients there was no relationship with clinico-

pathological factors used commonly to predict outcome such as tumour differentiation, serosal, nodal or resection margin involvement or with age, sex or tumour site. When we examined all the resected cancers over both decades (Tables 8 and 9) those patients who presented with symptoms for longer than 6 months had significantly better survival (p=0.001). Many groups have suggested a prolonged period of symptoms prior to presentation leads to poorer prognosis (Brookes 1965, Barber 1961) but as many have agreed with our findings and suggested a population of slower growing tumours for some as yet unknown reason (Fujita 1978). Consistently, younger patients with longstanding symptoms have a poorer outcome in published series (Tso 1987, Siewert 1995).

Table 4 **Duration of symptoms**

	1974-1984	**1986-1994**
< 3 months	122 (38.9%)	212 (57%)
3 - 6 months	117 (37.3%)	77 (21%)
> 6 months	75 (23.9%)	80 (22%)
missing	14	

Table 5 **Relationship between patient survival and duration of symptoms**

Duration of symptoms			**% survival**	
	No. of patients (%)		**2**	**5**
<3 MONTHS	212	(57)	19	6
3-6 MONTHS	77	(21)	13	2
>6 MONTHS	80	(22)	36	24

Section iv: Location of tumour

With the decline in the incidence of gastric cancer worldwide there has been a proximal shift of disease although antral tumours remain the most common (Hansson 1993,Antonioli 1982, Bizer 1983, Harrison 1995). This change is less pronounced in

Japan (Kampschoer 1989). Between the two periods of time of this study there has been an increase in both the antral and proximal tumours, with a corresponding decrease in the unspecified tumours but overall little difference in the relative percentages of each site (Figures 3 and 4). Although the number of antral tumours in both series is in agreement with other hospital and population based series, the proportion of proximal tumours was initially higher than other series (Lauren 9.3%, Allum 5.6%) and the subsequent increase in proximal tumours was less. (Paterson 18% to 39%; Kampschoer 17% to 27%,Meyers 21% to 44%,Husemann 6.6% to 37.5%).

Figure 3 **Location of tumour 1974-1984**

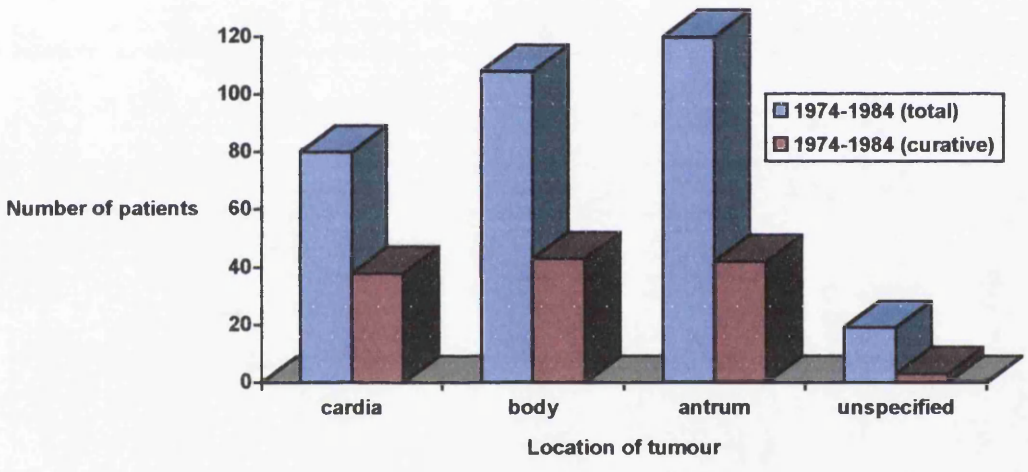
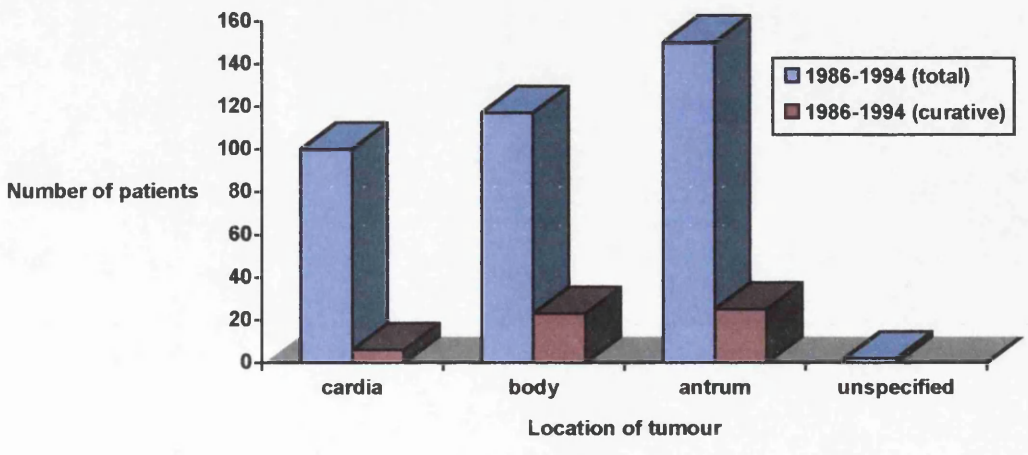


Figure 4 **Location of tumour 1986-1994**



Figures 3 and 4 show that the proportion of tumours in each location of tumours is similar in each time period but there are fewer proximal tumours in the curative resection group in the last decade.

There may be several reasons to explain these changes. The initial high incidence of cardia tumours may be due to mis-classification of lower oesophageal adenocarcinomas to the stomach. We have cross-checked the patients in our survey to oesophageal adenocarcinomas on the Cancer Register and found no correlating patients. However, this register relies on the initial assessment of site by the investigating surgeon and in the earlier series there may have been a tendency to assume lower oesophageal adenocarcinomas tumours were oesophageal extensions of cardia tumours. Within the time periods outlined the incidence of adenocarcinomas as a proportion of total tumours of the oesophagus in this centre has increased from 27% to 37% (Hole 1997). This would suggest that the incidence of proximal gastric adenocarcinomas although initially higher than expected when compared to other studies, may be accurate. The dramatic increase in the incidence of gastric and oesophageal adenocarcinomas over the last twenty years in many series (McKinney 1993, Meyers 1987, Powell 1990, Lund 1989, Blot 1991) may have occurred at an earlier time in our population and an explanation for the higher initial figure may be that the increase in incidence pre-dated our study. A common aetiology may be involved in these increases of tumour in both sites. In Sweden where there has been a definite coding for cardia tumours since 1970 (Hansson 1993) there has been a steady rise in the incidence of cardia tumours with a male preponderance. In the West Midlands and Birmingham study (Allum 1986) the percentage of patients with cardia tumours was 5.6% .The criteria for inclusion in the study of cardia tumours was such that any tumour extending beyond the cardia itself was excluded. If we reclassify our series we have 18% of tumours in this confined group. Other studies have included resected but not all biopsy proven tumours (Paterson 1991) and may reflect changes in resection.

We examined all resected cases over twenty years and found that 24% curative resections were of proximal tumours and that 24% of all tumours resected were proximal in site (Table 8). As a hospital based survey with a specialist upper gastrointestinal unit, the tertiary referral pattern may also influence the relative numbers

of tumours at each site. There remains, however, a need for acknowledged definitions of proximal tumours as highlighted in German studies (Siewert 1995).

Survival and location of tumour

Figure 5 **Survival in curative resections 1974-1984**

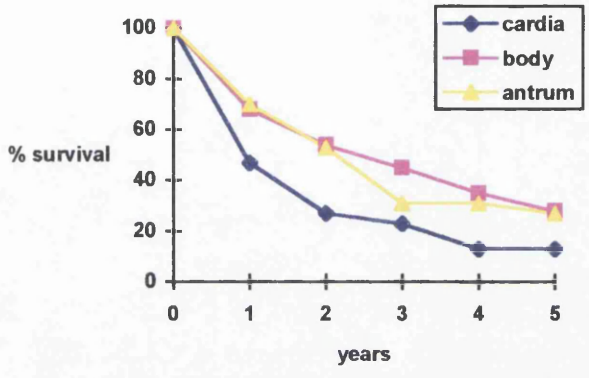
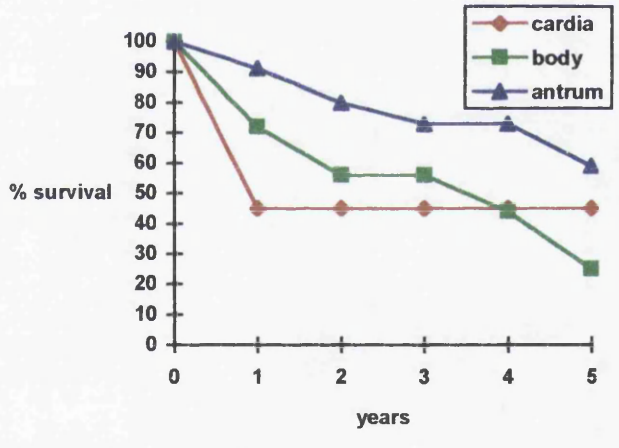


Figure 6 **Survival in curative resections 1986-1994**



Long term survival related to the site of the tumour (Figures 5 and 6) in the 1974-1984 group showed that the poorer outcome was associated with tumours of the cardia. Five year survival was 13% compared to 28% and 27% for body and antral tumours respectively ($p<0.05$). This is not the case in the later set where only 11% of the curative resections were of cardia tumours as opposed to 30% in the earlier series and the five year survival was 45%. However, in this cohort of patients there were only six patients with cardia tumours who underwent curative resection. When we examine survival (Tables 8 and 9) of the resected cardia tumours over the twenty year period,

the five year survival appears lower at 8% (p=0.11) compared to 17% and 21% for the body and antrum (p=0.96) although the differences are not significantly different.

Section v: Surgical procedures

In the period from 1974-1984, 15.5% of patients had no surgical treatment increasing to 39% in the later group. The total resection rate reduced from 49% to 46%; curative resections from 39% to 14.6% and palliative resections increased from 9.8% to 31.2%.With the introduction of a YAG laser to the department in 1986 there appears to have a decline in intubation procedures in consequence. The types of procedures are in Table 6, Figures 7 and 8

Figure 7 **Surgical procedures 1974-1984**

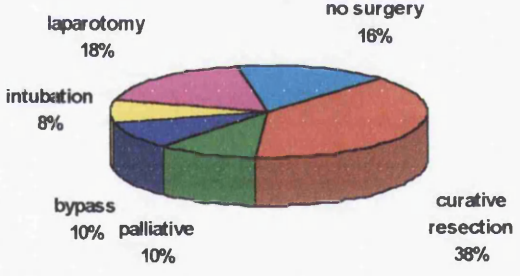


Figure 8 **Surgical procedures 1986-1994**

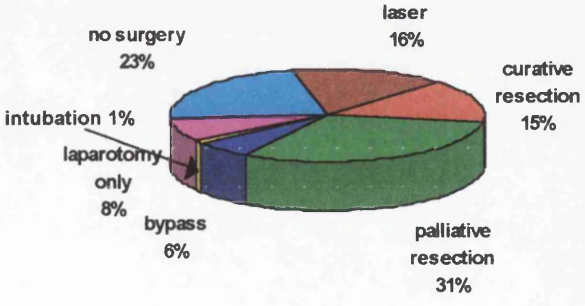


Table 6 **Surgical Procedures and Findings**

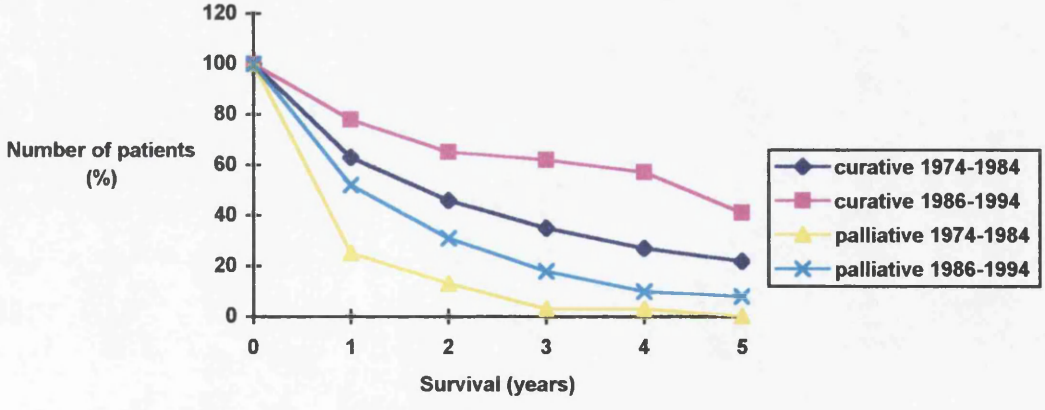
Primary treatment	1974-1984	1986-1994
Curative resection	128 (39%)	54 (14.6%)
Palliative resection	32 (9.8%)	115 (31.2%)
Bypass	33 (10.1%)	23 (6%)
Intubation	26 (7.9%)	4 (1%)
Laparotomy only	58 (17.7%)	28 (7%)
Laser	–	59 (16%)
No surgery	51 (15.5%)	84 (22.8%)
Regional chemotherapy	–	2 (0.5%)
Resection	161 (49.1%)	169 (45.8%)
No resection	167 (50.9%)	200 (54.2%)
Site		
Fundus / cardia	80 (26%)	100 (27%)
Body	108 (35.1%)	117 (32%)
Antrum	120 (39%)	150 (41%)
Direct Invasion (resected cases only)		
Serosa	67(47.2%)	7(4.2%)
Colon	10(6.6%)	15(18.8%)
Pancreas	30(19.6%)	2(1.2%)
Liver	4(2.6%)	
Liver secondaries	11 (7%)	11 (6.5%)
Histological findings (resected cases only)		
Serosa involved	111 (76%)	127 (77.9%)
Resection margins +ve	38 (25.7%)	36 (22.1%)
Lymph node +ve	72 (64.3%)	99 (63.1%)
Mean number of nodes sampled	3	7
30 day mortality		
Resected cases	18 (11.2%)	17 (10%)
Curative resections		
1974-84	12.9%	
1974-79	17.6%	
1980-84	8.4%	
1986-94	7.4%	
1986-90	11.4%	
1991-94	0%	

The direct invasion data has not been analysed formally. Although it may have given some reasoning to labelling a resection as palliative rather than curative, the availability of this information in the later series was inconsistent. In addition, misinterpretation of, for example, invasion of pancreas rather than adherence could not be represented reliably using this information.

Section vi: Survival in the treatment groups

As one would expect, changes in the relative numbers of patients undergoing palliative procedures did not result in any significant improvement in length of survival (Figure 9 and Table 1, Appendix A). In contrast there has been an apparently significant increase in the survival of patients undergoing curative resections. The 5 year survival increased from 22% to 41% (p=0.079) and for palliative resection there was a small improvement (0 to 8%). On closer examination of the data it is apparent that there has been a shift in the numbers in each group due to a stricter definition of a potentially curative resection. This not only raises survival of the 'curative' group but by adding to the palliative group those patients who are borderline palliative also raises the survival in that cohort. This theory is reflected in the analysis of the total number of resected patients which remains unchanged over time. The resected patients had a five year survival of 18% and 16% with a median survival of 14 and 15 months in the time periods 1974-84 and 1986-94 respectively.

Figure 9 **The changes in survival between the two decades as determined by operative procedure**



The operative procedures displayed are curative and palliative resections as determined by the surgeon.

Section vii: Histopathological factors and survival

In the later group, 41% of patients undergoing potentially curative resection survived five years from diagnosis. The apparent improvement in 5 year survival from 22% in the 1974-1984 group probably reflects a change in the criteria, from the surgeon's perspective, for curative resection, rather than a reduction in the biological aggressiveness of the cancer. These criteria and the clinical assessment of gastric cancer may vary between surgeons. However, improved pre-operative staging with conventional and spiral CT scanning, laparoscopy with peritoneal washings and laparoscopic ultrasound have more accurately identified the stage of disease. When a surgeon describes the surgical procedure as curative or palliative he is using a combination of clinical skills, operative experience and the results of advanced investigations. This is one aspect of the stage migration phenomenon which allows us to place a patients tumour in a stage group with more accuracy. Subsequently the survival of patients stage for stage reflects this contribution of additional, valuable information.

Therefore, I examined the effect on survival of tumour differentiation, involvement of resection margins, serosa and lymph nodes in the curative resection group during both time periods.

There was a tendency to a better outcome in those patients with well differentiated tumours who had curative resections, but this was not statistically significant. There was no difference in the proportion of patients with each type of tumour in the group of patients having a curative resection or the total number of patients in time period.

Figure 10 **Survival of patients in curative resection group as expressed by serosal involvement**

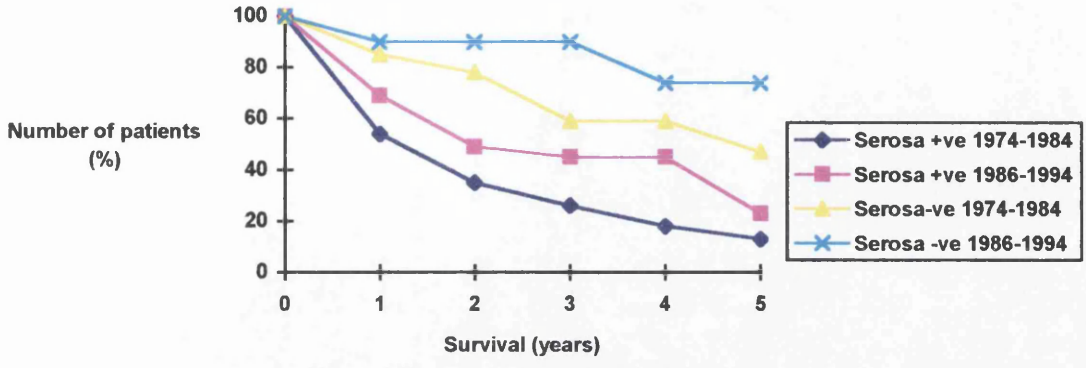
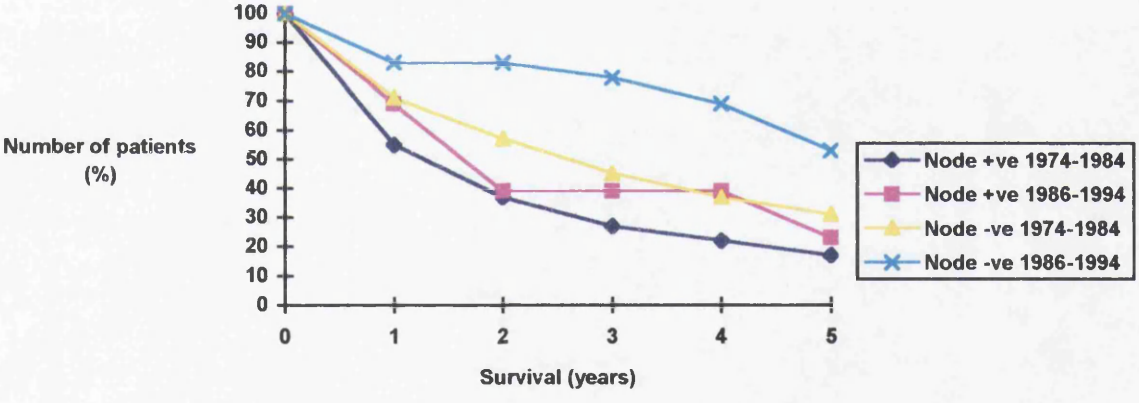


Figure 11 **Survival of patients in curative resection group as expressed by nodal involvement**



In both sets of patients, those with resection margin involvement relapsed and died (Table 2,Appendix A). Although serosal and lymph node involvement can never be considered in isolation the contribution of each to prognosis has been sought. Serosal involvement remained the most important predictor of survival (Figure 10). The patients with serosal involvement had significantly poorer survival ($p<0.005$). The involvement of lymph nodes associated with serosal involvement further reduced survival as observed in the first series of patients. Lymph node status itself showed a different trend (Figure 11). For the earlier patients it was only important when serosa was not involved but in

the later group lymph node involvement alone had no effect on survival, although the number of patients with no lymph node involvement was very small.

In order to overcome some of the difficulties of small numbers we analysed these factors in the group of resected tumours (Table 7). An analysis of the factors in combination showed that the involvement of either serosa or nodes influenced survival to a similar degree. On multivariate analysis, lymph node involvement was significant when over 50% of the nodes examined were involved by tumour. Serosal involvement and resection margin disease were also statistically significant factors on multivariate analysis (Table 9).

Table 7 **Survival of resected cases by degree of involvement**

(based on 331 resections over the period 1974 -1994)

Extent of involvement			Number of cases	% surviving 2 years	5 years	(median survival)
Lymph node	serosa	resection margin				
neg	neg	neg	37	78	58	>60m
neg	pos	neg	48	54	20	29m
pos	neg	neg	21	52	21	32m
neg	neg	pos	0			
pos	pos	neg	100	34	8	15m
neg	pos	pos	12	17	0	4m
pos	neg	pos	2	0	0	
pos	pos	pos	37	5	0	7m

Table 8 **Survival of resected cases by other factors**

(based on 331 resections over the period 1974 -1994

Factor	Number of cases	% surviving 2 years	5 years
Site			
Cardia	78	26	8
Body	121	37	17
Antrum	129	45	21
Differentiation			
Well	30	43	30
Moderate	95	46	24
Poor	178	34	11
Not known	28	29	14
Duration of main symptom			
< 3 months	152	34	12
3 - 6 months	87	30	9
> 6 months	79	56	37
Age group			
<55	59	44	20
55 - 64	92	38	20
65 -74	121	39	17
>75	59	31	8
Sex			
Male	201	35	16
Female	130	42	19
All resected patients			
	331	38	17
Serosa			
Negative	71	69	49
Positive	238	30	8
Resection margin			
Negative	237	49	22
Positive	74	7	0
Lymph nodes			
Negative	98	59	33
<50%	48	44	11
>50%	117	21	5

Table 9 **Multivariate analysis of survival time for resected patients**

Factor	Number of cases	RHR	95%c.i.	p value
Serosa				
-ve	71	1		
+ve	238	2.56	(1.79-3.65)	0.001
Resection margins				
-ve	237	1		
+ve	74	2.24	(1.675-3.03)	0.001
Nodal status				
-ve	98	1		
1-50%+ve	48	1.22	(1.75-3.39)	0.33
51-100%+ve	79	1.99	(1.54-3.17)	0.001
Duration of symptoms				
< 3 months	152	2.44	(1.75-3.39)	0.001
3 - 6 months	87	2.21	(1.54-3.17)	0.001
< 6 months	79	1		
Site of tumour				
Cardia	78	1.29	(0.94-1.78)	0.11
Body	129	1		
Antrum	129	1.01	(0.76-1.33)	0.96

Chapter 3

Discussion

This study examined the changes in the treatment of gastric cancer and the resultant improvement in the survival of patients in one centre over a twenty year period. The initial results indicated that far from improving the outcome in patients with gastric cancer the five year survival had apparently reduced, from 11% in the first ten years to 6% in the second decade (p=0.013).

Section i: Changes in surgical approach to gastric cancer

The total resection rates of gastric tumours in published series shows considerable variation from 29.3% to 75.9% (Lindahl 1988, Irvin 1988, Allum 1989, Cady 1989, Gall 1985).The total resection rate in this series is around 50% but contrary to the increase in resection rate observed in many hospital based series in Europe and the USA, the rate in this study is lower in the most recent decade .There is a more marked difference in the number of resections considered to be curative. Although some centres suggest as Cady (1989) that the rate of curative resection has remained constant at 34% which is consistent with our observations from 1974-1984, the majority of centres have seen an increase in curative resections to between 45% and 60% (Meyers 1987, Haugstvedt 1993, Stipa 1994, Boku 1990). This was not reflected in our later series in which we found a substantial decrease. A possible explanation for this may be the catchment area of the hospital. This series of patients represents a population rather than simply a hospital based study despite a degree of tertiary referral status. The area is significantly deprived with co-morbid disease amongst the highest in the U.K.

With a change in the proportion undergoing curative resection there was a corresponding increase from 9.8% to 31.2% of patients having a palliative resection which is significantly higher than other comparative series (Lindahl 1988,Allum 1989, Cady 1989, Gall 1985). Palliative resection is known to give an improved survival over

intubation in advanced disease (Cunningham 1987, Allum 1989) and this may have prompted a change in the surgical policy towards palliative resection. More likely it is the changing perception amongst the surgeons that resections previously described as curative are now in reality palliative based on experience and sophisticated investigations. This leads to difficulties when comparing groups in older published reports of curative resections. If we were to define this group in terms of the Japanese histological criteria (Japanese Research Society 1981) it is only possible to show 'relative' curative resections. These are patients with no serosal involvement but we cannot comment on the whether there is adequate lymph node clearance as a D2 lymphadenectomy was not routinely performed in many of the earlier patients. On this basis 42% of the total number of curative resections and only 6% of all patients are in this category (which coincidentally corresponds to the five year survival of the curative resection and total patient groups).

The laparotomy rate in our study has also fallen from 85% to 60% when other studies report rates around 80% to 90% (Lindahl 1988, Irvin 1988, Lund 1989, Gall 1985) and the proportion of patients who underwent an exploratory laparotomy has almost halved. Again this reflects improved staging by CT scanning and diagnostic laparoscopy (Ajani 1995, Stell 1996) particularly in proximal tumours where diagnostic laparotomy rates are only 9%. The introduction of laser therapy has had a considerable impact on the management of proximal tumours with good palliation and a median survival of 3.6 months compared to 2 months and 0.8 months for intubation in both series.

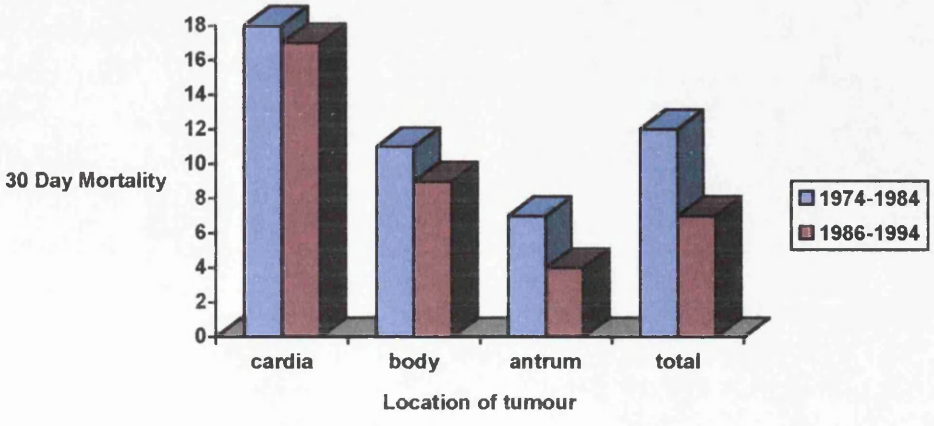
Section ii: Variation in survival and operative mortality

The overall five year survival has decreased from 11% in the 1974-1984 group to 6% in the 1986-1994 group despite an increase in the five year survival in the curative resection group from 24% to 41%. Between 1970 and 1990 the five year survival for curative resection in the Japanese experience was around 60% and in non-Japanese

centres 40% (Akoh 1992).In a selected group from the late 1980s the curative resection five year survival varies between 45% to 55% (Cady 1989, Stipa 1994) and overall survival shows a wide range from 4.5% to 30% in Europe and the USA (Lindahl 1988, Irvin 1988, Allum 1989, Cady 1989, Gall 1985, Roder 1993, Akoh 1992). Over twenty years the resections which are considered curative histopathologically in as much as there is no serosal, lymph node or resection margin involvement have a five year survival of 58%.

The 30 day mortality in resected cases was stable at 11.2% and 10% (Table 6). In curative resections there has been a decrease of 5% and in the last five years the 30 day mortality was 0%. In the palliative group the rate was unchanged (9% to 11%). This contributes to the improved survival in the curative resection group of patients and also to the more significant decrease in mortality from gastric cancer with respect to the decrease in incidence. There was considerable variation in the operative mortality with site (Figure 12 and Table 3, Appendix A). Cardia tumours have almost twice the operative mortality of body and antral tumours for both curative and palliative resections.

Figure 12 **30 day mortality of patients undergoing curative resection expressed according to the location of tumour**



Section iii: The influence of histopathological factors

The influence of factors known to effect prognosis have been examined to establish an explanation for the changes in the survival after resection. The poorer prognosis related to the involvement of resection margins (British Stomach Cancer Group 1984) and poorly differentiated tumours (Yu 1995) is consistent with other studies but the influence of lymph node involvement requires further comment (Pacelli 1993). The majority of Japanese centres consider the involvement of the loco-regional nodes to be the most important prognostic factor in curative resections and the surgical clearance of these nodes is the aim of the standard practice of extended lymphadenectomy (Roder 1995, Tsuchiya 1995). However, in Europe the majority of patients present at a more advanced stage, 70-80% at stage 3 and 4 (Fielding 1984), and the involvement of serosa seems to be more important (Athlin 1995, Bozzetti 1986, Yu 1995). One must also consider the influence on survival of the Japanese methods of pathological reporting, in particular, the detailed sampling of nodes for micro-metastases and the correction this makes to staging of the disease.

In the earlier series of patients serosal involvement was a significant factor in survival ($p=0.004$) and lymph node involvement was only of importance when there was no serosal involvement. The number of patients in the curative resection group is limited in the later series but confirms that serosal involvement has an over-riding effect on survival. In multivariate analysis of all resected cases, serosal involvement significantly reduces prognosis ($p=0.001$).

In resected cases there is a significant effect on survival when more than 50% of resected nodes are involved by tumour ($p=0.001$). The value of information on nodal status in our practice may be important but the proportion of nodal involvement is dependent on the extent of lymph node resection. The eventual relationship between nodal involvement and prognosis may be the same when a radical lymph node resection is performed.

A number of trials in the U.K. and Europe have attempted to determine the benefit to prediction and actual survival of extended lymphadenectomy. Initial results suggested an increase in morbidity and mortality which may be related to adjusting to new surgical techniques, patient selection and the need to modify the practice of splenectomy and distal pancreatectomy (Roder 1995, Lee 1995, Cuschieri 1996, Bonenkamp 1995, Sue-Ling 1995). The conclusion from Bonenkamp et al (1999) suggests that there is no survival advantage in Dutch patients undergoing extended lymphadenectomy and the MRC trial tended to concur. Siewert, however, in selected groups showed improved survival in particular Stages 2 and 3a.

The influence on survival of tumour site reveals that proximal tumours have a poorer prognosis with five year survival in both decades of 5%. The resection rate (33%) is less than at other sites with only 6% considered curative and although the five year survival in this group is 45% in the total resection group it is only 2.7%.

Section iv: Summary of findings

Despite the initial impression that there has been no improvement in the treatment of gastric cancer over the last twenty years, it would seem that there have been some small advances. Those patients undergoing curative resection are more likely to survive the initial post-operative period and in the long term. Palliative resection is more effective in prolonging life although we cannot comment on quality of life. Fewer patients undergo unnecessary operations and this is particularly important in an ageing patient population. However. the majority of patients continue to present with advanced disease and only early detection of disease would seem to improve overall outcome.

The need for more radical surgery and the development of specialist centres are still a matter of great debate. The apparently changing nature of the disease with proximal tumours showing similarities with the increasing oesophageal adenocarcinoma

population and antral tumours in decline may reflect the results of treatment of Helicobacter pylori, dietary, socio-economic or cultural changes. The surgical workload from gastric cancer is not changing (Sedgwick 1991) and conventional therapy is producing minimal improvement in prognosis. We must concentrate expertise and resources to detect and treat tumours at an early stage, provide markers for high risk groups, implicate those tumours with poor prognosis and advance novel therapies for the disease.

This retrospective study of gastric cancer patients from one district in Glasgow has served to illustrate the difficulties in treating gastric cancer. An ageing, deprived population which presents late and often with advanced disease would benefit from early intervention and innovative surgery. However, the principle aim of this thesis is to bring together the study of gastric cancer at a molecular level to discover alternative therapies for the disease. In particular, if the recent development of monoclonal antibodies to c-erbB-2 for the treatment of advanced breast cancer can be considered for use in gastric cancer there is a need for reliable methods of c-erbB-2 detection. With this in mind, I have aimed in the forthcoming chapters to outline the role of c-erbB-2 in carcinogenesis; describe its role, if any, in predicting prognosis in gastric cancer and highlight the importance of reliable measures of receptor expression. I have similarly examinated EGFr and will proceed to study the level of expression of these growth factors in gastric cancer specimens, comparing methods and expression of each factor.

In addition, I have sought to examine whether the high level of IGFr in foetal gastric mucosa has bearing on the behaviour of gastric cancer. Using proliferation studies I have detailed preliminary work which may determine whether the inhibition of IGFr is a worthwhile target to develop further in gastric cancer.

PART II

TYPE I GROWTH FACTORS IN GASTRIC CANCER

Background information and historical review

Chapter 4

The role of growth factors and their receptors in carcinogenesis

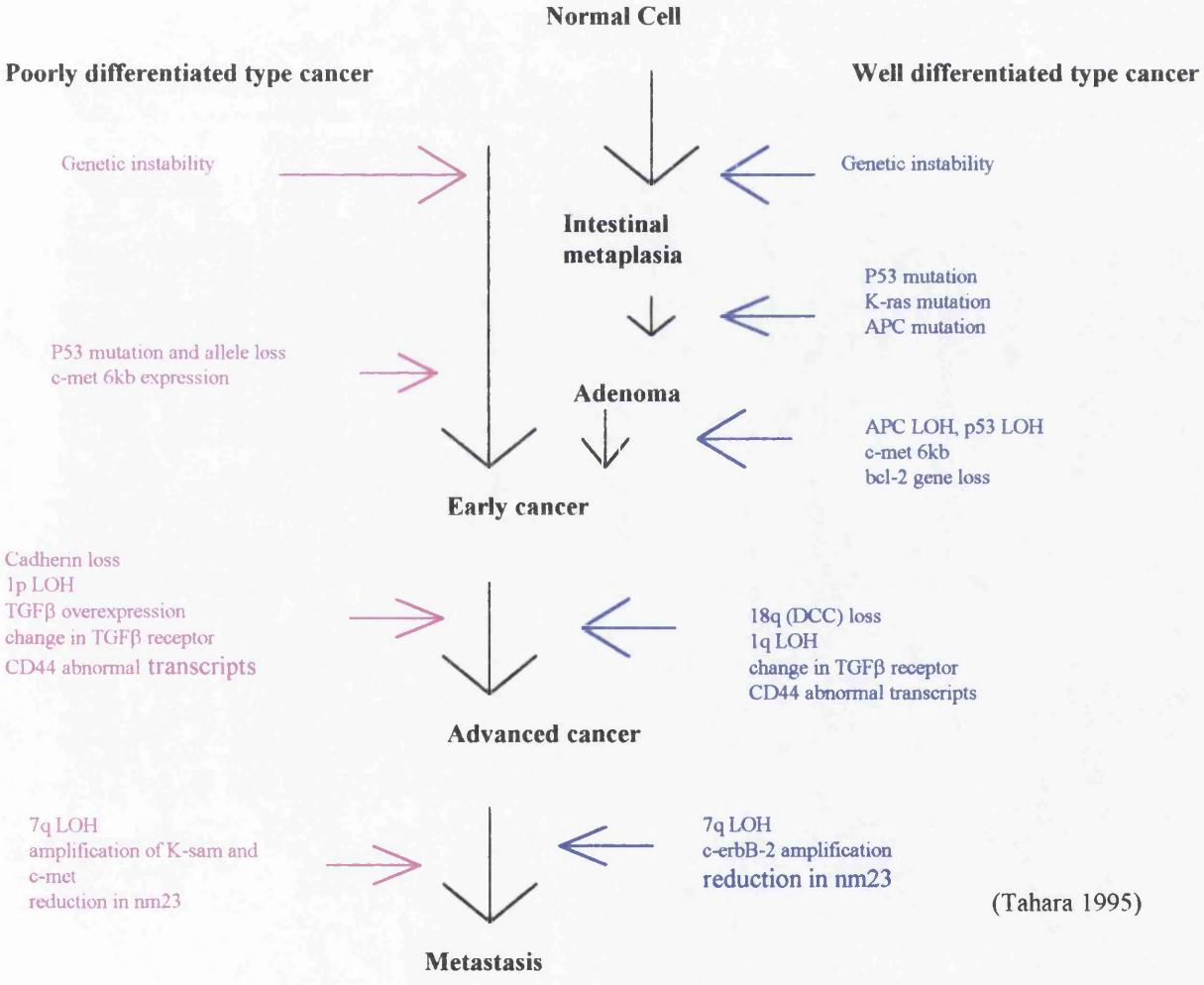
Section i: Introduction

In the progression from normal epithelial cells to invasive cancer, it has been suggested that gastric cancer follows a series of well-defined histological steps particularly in the intestinal type of tumour. The natural history of the disease and in particular the diffuse type of gastric tumour is not entirely clear (Correa 1991). The advances in molecular biology in recent times have allowed identification of many of the molecular changes involved in tumour initiation and progression (Tahara 1995). Tumour formation occurs when there is an imbalance within the normal physiological processes of cellular proliferation and cell death (Harrington 1994). Gastric cancer displays multiple gene alterations including oncogenes, growth factors or cytokines, cell cycle regulators, tumour suppresser genes, cell adhesion molecules and genetic instability (Figure 13).
In the preceding chapters where I have described the population from which the specimens of gastric cancer have been obtained, there is a high presentation of advanced cancer with poor outcome. I was interested to determine whether higher expression of c-erbB-2 was implicated in advanced and metastatic disease which the genetic pathway suggests.

In the development of malignant cells, the alteration of the expression of normal cellular genes by mutation, translocation, amplification or other mechanisms may contribute, in particular in those genes involved in cellular differentiation and proliferation. Among the molecular changes implicated in the aetiology of carcinogenesis are those mediated by growth factors (Stemmerman 1994). These are small polypeptides which are involved in the regulation of cellular proliferation (Carpenter 1979) and regulate cellular proliferation by acting on specific target tissues. Their receptors are found on the

plasma membrane of cells and the expression of receptors found in each cell varies among between tissues (Gullick 1991). Binding of the growth factor transmits the signal within the cell to modify the metabolic activity and stimulate the cell cycle (Kazlaukas 1994).

Figure 13 **The genetic pathways in the development of gastric cancer as proposed for different histological types**



(Tahara 1995)

Genetic mutation within the cells results in alteration of protein products and this can occur at any stage in the signal transduction pathway from receptor to nuclear proteins. Receptors and intracellular proteins altered in this way are capable of transforming normal cells and promoting the proliferation of malignant cells (Tahara 1995).

The genes encoding the growth factors, the growth factor receptors and many of the elements in cellular signalling have been identified. When mutation in one of these genes with stimulatory function occurs it results in an alteration in activity. This is a dominant effect and only one copy of the gene need undergo mutation. This is called an oncogene. The normal allele is called a proto-oncogene. Inactivation of an inhibitory gene to cause increased activity and the possibility of uncontrolled cellular proliferation is a recessive effect and the gene is termed a tumour suppresser gene. Many of the proteins involved in cellular signalling have been identified as proto-oncogenes and produce stimulatory or inhibitory changes in cellular activity.

Oncogenes were identified in the early part of the twentieth century when it was found that sarcoma could be transmitted between chickens and the causative agent was found to be an RNA virus. Subsequently investigators found that sequences homologous to the oncogene region of the virus were present in the DNA of all tissues of virus -free chickens and the concept of the proto-oncogene was developed. The viral oncogene v-erb-B is homologous to the gene encoding EGFr in humans (c-erbB-1).
In comparing the proto-oncogene which has been identified for EGFr and the receptor protein product of the oncogene there are both intra and extracellular regions which are missing. The activity of EGFr is altered in several ways. On binding of EGF the normal receptor internalises the growth factor/receptor complex to regulate receptor numbers and prevent overstimulation. This process does not occur in the altered gene state. The binding of EGF is required for receptor activation in unaltered receptors but the

oncogenic EGFr would seem to be constantly stimulated due to the lack of much of the extracellular regulatory domain (Hunter 1985).

Section ii: Structure and function of growth factors

The growth factor receptors are transmembrane proteins with an extracellular domain containing the NH_2 terminus which binds the growth factor and is called the 'ligand' binding domain. There is a transmembrane alpha helical domain which spans the lipid bilayer and the intracellular area where the events leading to signal transduction take place. This is the "catalytic" domain and contains the COOH terminus (Ullrich 1990).

On binding of the growth factor, the receptor undergoes a conformational change which activates the intracellular catalytic domain of the receptor (Ullrich 1984). This leads to the activation of a series of cytosolic enzymes called protein kinases which catalyse the transfer of the terminal phosphate group of ATP to serine, threonine or tyrosine residues on specific intracellular proteins initiating a complex cascade of intracellular events. The protein kinases are an interacting group. Tyrosine kinases are found on the catalytic domain of the transmembrane receptors and their kinase activity produces phosphorylation on the tyrosine residues of intracellular proteins (Cantley 1991). The others phosphorylate serine or threonine residues and are cAMP-dependent protein kinase, the diacylglycerol-dependent cGMP-dependent protein kinase, protein kinase C and Calcium-calmodulin dependent protein kinase.

The growth factors are classified for convenience into major groups determined by the initial type of phosphorylation . Thereafter the sub-groups are determined by grouping factors with similar extra and intracellular domain sequences (Fantl 1993). Within the tyrosine kinase group of growth factor receptors of which this work is particularly concerned, the Type I group includes epidermal growth factor receptor and a group of

similar proteins c-erbB-2,c-erbB-3 and c-erbB-4. The Type II receptors include insulin-like growth factor receptor I and the insulin receptor.

In Type I receptors, ligand binds to the extracellular regulatory domain and activates the catalytic domain by effecting a conformational change in the receptor. In receptors such as EGFr, receptor pairing is promoted by ligand binding and alignment of the receptors causes activation and the transfer of the terminal (gamma) phosphate group of adenosine triphosphate (ATP) to the hydroxyl group of tyrosine residues located on specific target proteins on the partner receptor and within the cell.

Tyrosine kinase receptors undergo auto-phosphorylation to enhance signal transduction and to regulate the concentration of receptor and ligand at the cell surface (Hunter 1984). This involves the transfer of a phosphate group from the receptor but rather than to another intracellular protein the phosphate group moves to another area on the receptor to enhance or regulate the function of the receptor. This process also initiates internalisation of the receptor to regulate the number of receptors present in a tissue type. The receptors are internalised into coated vesicles and degraded in the lysosomal compartment (Schlessinger 1988). Truncated receptor may be unable to internalise and cause increased transforming activity.

Type II receptors such as insulin-like growth factor I receptor appear to be covalently linked prior to ligand binding rather than requiring ligand binding to initiate the change but the tyrosine kinase activity thereafter follows a similar pattern (Keller 1993). Tyrosine phosphorylation initiates a phosphorylation cascade involving secondary messenger pathways the result of which is a change in genome transcription and cell division. The complex method of signalling allows amplification of the receptor signal and interactions with the intracellular events from other growth factor receptor signalling (Fantl 1993).

Section iii: Receptor ligands

In the Type I growth factor receptor group we are principally concerned with the activity of EGFr and c-erbB-2, also called neu, the human epidermal growth factor 2 or simply HER2. More recently the group are described as the HER family. EGFr (HER1) interacts with soluble polypeptide growth factors which act as ligands. There are a number of cognate ligands to the EGF receptor including EGF, TGFα, heparin binding EGF, amphiregulin and cripto all of which share a common structure.

EGF is a 53 amino acid peptide which binds exclusively to the EGF receptor and does not directly interact with the other Type I tyrosine kinase receptors. There is evidence that EGF stimulates the growth of skin and corneal epithelial cells, cells of the gastrointestinal tract and inhibits gastric secretion. In epithelial tumour cells, EGF has been shown to enhance growth, in vitro, in skin, bladder, breast, gastrointestinal tract and gynaecological malignancies (Hamburger 1981).

In gastric cancer the overexpression of both EGFr and EGF in the same tumour specimen, produced pathological findings suggestive of more proliferative and invasive activity than in specimens with overexpression of only one or no overexpression (Sugiyama 1989, Yonemura 1992). This suggests an autocrine mechanism exists between EGFr and its ligands. This is also seen with combined over expression of TGFα and EGFr in advanced gastric cancer (Bennett 1989).

TGFα is a 50 amino acid peptide which exhibits 40% homology to the EGF molecule and also functions exclusively through the EGF receptor. An increase in the synthesis and secretion of TGFα occurs in a number of carcinoma cell lines including skin (Coffey 1987), breast (Bates 1988) and gastric (Tahara 1990). Synchronous expression of TGFα and EGFr without gene amplification may indicate a transcriptional abnormality which would repress expression of mRNA for EGFr and TGFα (Kitadai 1993). The coexpression of high levels of TGFα and EGFr are also found in breast cancers (Lundy

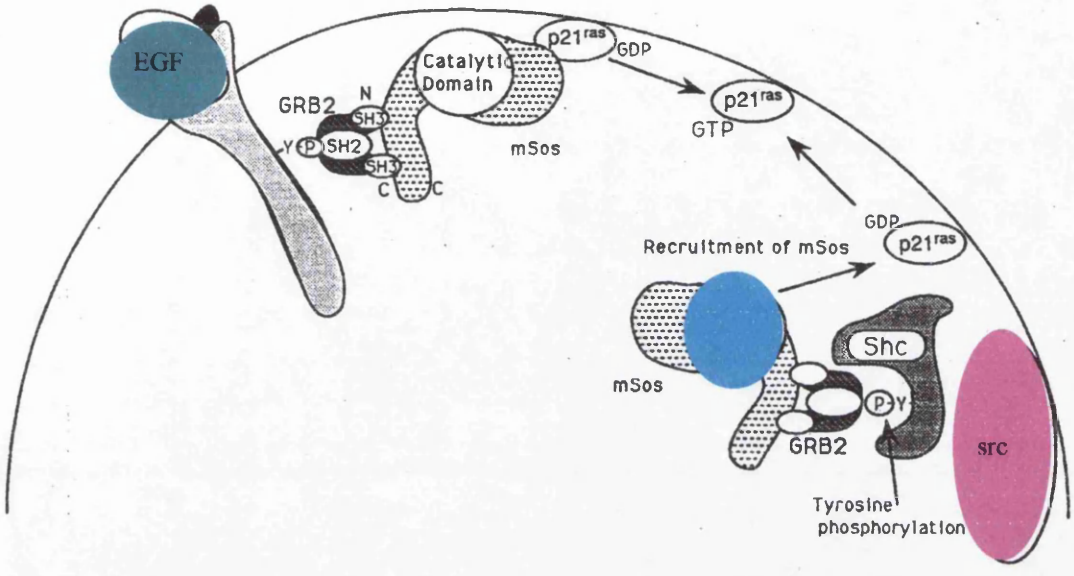
1991). Cripto expression is associated with gastric cancer and intestinal metaplasia and there is evidence of good correlation between tumour stage, prognosis and cripto expression (Kuniyasu 1994).

There is no cognate ligand for c-erbB-2. However, cell surface monomers form homodimers with the same receptor or heterodimers with other members of the HER family (Alroy 1997). C-erbB-2 can be stabilised and transactivated in heterodimers by ligand interacting with the partner in the heterodimer such as EGFr (Pinkas-Kramarski 1998). This allows c-erbB-2 to participate in signal transduction in the absence of a cognate ligand. Heterodimers containing c-erbB-2 are highly potent and are the preferred heterodimerisation partner in the HER family (Graus-Porta 1997).

Section iv: Growth factor signal transduction

In the Type I receptor pathway, binding of the ligand leads to a complex series of interacting signalling pathways at the level of the cell membrane which precipitate nuclear signals activating transcription of genes (Figure 14). The ability of the cell to respond to different ligands, the interaction of ligands and heterodimerisation of receptors may influence signal transduction. In addition, positive and negative feedback within the complex cytoplasmic activity described below may explain the diversity of processes effected by growth factors which include cell growth, differentiation, migration and viability.

Figure 14 The EGF receptor signal transduction pathway



Binding of ligand to the type I receptor leads to receptor dimerisation and autophosphorylation. This creates a binding site for Grb2 which is an 'adaptor', protein to connect a series of signalling proteins.

Grb2 binds through the SH2 (Src homology-2) domain which is a specific binding site for tyrosine phosphoproteins. Thereafter the SH3 domain binds SoS1 which acts as a Ras activator. Ras functions as a guanine nucleotide releasing protein and converts inactive RasGDP to the active GTP bound form by nuceotide exchange. Activation of the G protein Ras has a number of interacting consequences including the activation of the kinase Raf1 leading to the activation of the mitogen activated protein kinase (MAP kinase) pathway and its subsequent activation of nuclear transcription factors (McCormick 1993, Egan 1993, Batzer 1993,Schlessinger 1994). Mutations in the Ras oncogenes disrupt the GTP-ase activity of the G-protein in affected cells which leads to abnormal stimulation and cellular proliferation (Gibbs 1984).

In Type II receptors the 185 kilodalton cytoplasmic protein insulin receptor substrate I (IRS-I) acts as an intermediary signalling protein but continues to activate the src-homology 2 domains including those of Grb-2 and subsequently phosphatidyl inositol 3 kinase (Siddle 1992). In studies using IRS I deficient mice the activation of the phosphadidyl inositol 3 kinase mechanism is effected by an alternative substrate to a lesser degree. It is slightly larger and immunologically distinct but not fully investigated as yet.

Another method of intracellular signalling involves the enzyme phosphatidyl inositol 3 (PI3) kinase. Following tyrosine phosphorylation the 85 kilodalton sub-unit of PI3 kinase causes a conformtional change in the p110 sub-unit leading to increased phosphorylation of phosphotidyl inositol phosphate. This modifies cellular function by transcription factor induction or calcium release. The MAP kinase phosphorylation cascade is also activated by the cleavage of phosphatidyl inositol diphosphate by phospholipase C to produce inositol triphosphate and diacylglycerol which activates the protein kinase C proteins (Blenis 1993). These in turn phosphorylate RAF-1 in the MAP kinase pathway. G proteins such as Ras also act in both stimulatory and inhibitory ways by interaction with adenyl cyclase and phospholipase C (Kolch 1993).

The interaction between growth factors, oncogenes in tumour growth and the effect on signal transduction is under investigation in a wide variety of tumours in order to identify the mechanisms of tumour growth and induction. The role of the individual growth factor, its interactions and the use of this information for tumour markers, assessment and therapeutic intervention is important in the future of oncology.

In gastric cancer there is some knowledge of the expression of EGFr, c-erbB-2 and their relationship to prognostic factors. The role of insulin-like growth factors in malignancy is less reported, particularly in gastric cancer. In the next section of the thesis is a

review of our current understanding of the expression of EGFr, c-erbB-2, insulin-like growth factor and its receptor in gastric cancer.

Chapter 5

Epidermal growth factor receptor and gastric cancer

In studying the process of tumour formation, the role played by growth factors, growth factor receptors and abnormalities in their expression and function will be crucial. Much of the interest has attempted to correlate the clinical course of various cancers with the level of expression in order to develop prognostic indicators in malignancy. In gastric cancer, specifically, the analysis of growth factor biology may be of clinical value in assessing tumour aggressiveness, metastatic potential and pre-malignant conditions (Wright 1992).

There are a number of well recognised clinico-pathological factors associated with outcome in gastric cancer, including tumour differentiation, nodal involvement, serosal involvement and metastatic disease (Yu 1995). However, if there was a method of predicting the metastatic potential or aggressiveness of a tumour from a pre-operative biopsy specimen this would have advantages in staging and treatment plan. There may also be the potential to use the information on growth factor density and signal transduction to develop novel treatments for established gastric cancer (Tokunaga 1994). Earlier studies looking at gastric cancer and potential relationships with growth factor receptors, in particular EGF have addressed some of these areas.

Section i: Relationship to tumour stage

In gastric cancer, tumours which express increased levels of EGFr are found to be at a more advanced stage with greater depth of invasion. Of 156 tumours studied by Yasui (1988), 130 were advanced lesions and 26 were cases of early gastric cancer according to the Classification of the Japanese Research Society for Gastric Cancer. Only one of the 26 early tumours showed EGFr immunoreactivity (3.8%). In the advanced tumour group, EGFr expression was found in 33.8% of tumours. The difference between these

groups was statistically significant ($p<0.01$). When related to clinico-pathological factors, the level of EGFr expression correlated with the depth of invasion of the tumour ($p<0.05$). EGFr levels in metastatic lymph nodes from 45 tumours were found to be consistent with the primary tumour.

Sugiyama in 1989 assessed 220 gastric cancer specimens where 32% exhibited EGFr expression. This study correlated depth of invasion and and increased lymph node metastases with increased EGFr expression. In advanced disease Hirona (1995) found overexpression of EGFr was related to tumour size ($p<0.05$).

Other studies have found no relationship to pathological variables. Lemoine (1991) showed 18% overexpression of EGFr with no association with tumour stage, lymph node involvement, site of tumour or growth pattern. Similar results were published by Lee et al (1994) with 43% of cases demonstrating staining for EGFr but there were no significant relationship to clinico-pathological factors.

Section ii: Relationship to survival

Hirona (1995) found overexpression of EGFr was associated with significantly poorer survival ($p<0.05$) but in an earlier study (Yasui 1988) there was a non-statistically significant trend to poorer survival which increased with synchronous EGF expression. There are few other studies in which duration of survival allows adequate analysis and although Lee (1994) showed a tendency to shorter survival in positive cases.

Section iii: Relationship to tumour differentiation

Expression of EGFr is higher in intestinal type and well differentiated tumours. In the group of tumours studied by Lemoine (1991) expression was 18% and was higher in intestinal type tumours (27%) than in diffuse type tumours (12%). In the advanced tumour group from Yasui (1988) 51% of well differentiated, 22.9% of poorly

differentiated and 24.2% of scirrhous tumours were EGFr positive. Well differentiated tumours had a significantly higher level of EGF and EGFr than the poorly differentiated type ($p<0.05$). However, of the 26 early tumours 85% were well differentiated tumours and one of these showed EGFr immunoreactivity (3.8%). In contrast, Sugiyama (1989) found the level of expression was higher in poorly differentiated tumours.

Section iv: Relationship to non-malignant and pre-malignant tissue

The difficulties encountered in comparing tumours and adjacent 'normal' mucosa are highlighted in a study from Guys Hospital (Filipe 1995). Expression of EGFr, EGF and TGF-alpha was compared in established gastric cancer and in premalignant conditions in order to determine at what stage these factors appeared to influence tumour progression. Formalin fixed, paraffin embedded samples from 16 early gastric cancers, 9 advanced (19 intestinal type,4 diffuse and 2 mixed) and biopsies from patients with benign disease were studied. The 'normal' biopsies had morphology varying from normal to intestinal metaplasia and the biopsies from the mucosa of cancer patients had the same range of appearance. Results of immunohistochemistry were scored independently by two observers without prior knowledge of clinico-pathological factors relating to each specimen. A semi-quantitative scoring system was established to grade the intensity of staining, the extent of expression and location of staining within the glands.

EGFr expression was moderate to weak in most types of lesion and there was no difference between histologically normal mucosa from cancer fields than non-cancer controls. However, in areas of intestinal metaplasia from cancer fields there was a significantly higher expression than non-cancer controls ($p<0.1, z=3.8$ vs $z=1.07$). The intensity of staining was found to increase serially from normal through intestinal metaplasia to dysplasia and EGFr staining extended throughout the glandular field.

Using a polyclonal EGFr antibody and immunohistochemistry with formalin fixed paraffin embedded resection specimens, Lee et al (1994) noted significant staining on benign gastric tissue and that the staining tended to be diffuse. This was not confirmed by Hirona et al (1995) who found that all the normal gastric epithelium and non-tumour cells were negative.

Section v: Measuring EGF receptor expression

Although the study from Yasui (1988) clearly shows a significant difference between the level of EGFr in early and advanced tumours, it also highlights some of the difficulties in comparing studies of this nature. Firstly, even with the high incidence of gastric cancer in Japan the collection of 156 tumours took 11 years. Secondly, there is difficulty in comparing studies which use different criteria for determining EGFr expression specifically as to whether membrane or cytoplasmic staining is measured and the stratification to assess the extent of staining. Finally, the study used fixed tumour in paraffin blocks. Although the authors determined that the antibody used was capable of staining frozen and fixed tissue, the condition and antigen integrity of the tissue itself may differ with fixation.

Resected tissue from the patients was fixed in formalin and serial sections taken from paraffin blocks. A mouse monoclonal antibody to the EGF receptor which was developed using A431 epidermoid carcinoma cells as an immmunogen was used with a standard method of immunohistochemistry to determine the level of expression of EGFr. This antibody had previously been used in paraffin and frozen sections with 90% agreement in results (Tahara 1986).

Within this study a specific anti-EGF antibody was also used to determine EGF levels. A scoring system for the levels of EGF within tissue samples was developed depending on the percentage of tumour cells stained. However, a similar system was not used with the EGFr staining and the authors state that any staining of tumour cells was determined to be a positive result without significance given to the extent, intensity or position of

staining. There was no discussion of whether membrane or cytoplasmic staining was considered to be relevant. Most studies suggest that positive staining occurs on the cell membrane but that many positive cells exhibit cytoplasmic staining possibly as a result of internalised receptor-ligand complexes.

Sugiyama (1989) used paraffin fixed specimens and the control sections which were used for comparison were human submandibular gland tissue for EGF and A431 cells for EGFr. Although A431 cells stain only on cell membranes the carcinoma cells seemed to also stain in cytoplasm and both were taken as positive. Using frozen and fixed preparations to study expression Lemoine (1991) employed a variety of antibodies to EGFr and found each reliable. For ease of interpretation he recommended in frozen sections EGFR1 (Gullick) which we have used in our work and the 12E rabbit anti-EGFr antibody for fixed/paraffin block preparations as used in the previous study. Overexpression was defined as staining of tumour cell membrane often accompanied by cytoplasmic staining.

Reports vary on the pattern of staining with Lee (1994) finding that staining for EGFr was always on the cytoplasm with accentuation on the cell membrane (9 diffuse and 15 intestinal type). However, Hirona (1995) using an anti-EGFR monoclonal antibody (Biogenex.CA) determined positivity to be only for those cells which were definitely membrane stained.

These studies show wide variation in the level of expression of EGFr. There is a lack of a standardised definition of positivity in immunohistochemistry both in terms of membrane or cytoplasmic staining and a scoring system which would demonstrate intensity and extent. Subsequent analyses are difficult to compare with regard to percentage positivity, not only from the observer variability but this is compounded by discrepancies in the scoring systems used.

Section vi: Ligand binding to determine EGFr expression

To determine the EGFr expression of gastric carcinoma, Pfeiffer (1990) examined the binding capacity of EGF receptors using 125-iodine labelled EGF binding assays. This was at the time the only available method of quantitatively determining the receptor level of EGFr. Frozen sections of tumour and adjacent mucosal tissue from the specimens were selected. The tissue was prepared using a homogeniser, filters and centrifuges in a standard way. Incubation of the tissue with radio-labelled EGF and binding determined by the equilibration-saturation method. The results were analysed using a programme to correct for non-specific binding but the heterogeneity of gastric tumour within blocks of tissue makes binding studies more difficult to accurately determine.

Specific binding of labelled EGF was observed in all 15 carcinomas and 13 of 15 samples of normal mucosa. In 9 of 15 cases and 8 of 10 fundic carcinomas, statistical comparison of the paired individual binding curves suggested a significantly higher binding capacity in the carcinomas than in mucosa from the same patient. Overall comparison of the binding capacities showed no significant difference.

Of the nine cases with increased binding capacity, 8 cases had a 5.1+/_1.2 fold increase with one case of a young patient with a metastatic tumour having a 320 fold increase. Samples of mucosa were taken from patients undergoing surgery for benign disease and the binding capacity was found to lie within the same range as the mucosa taken from those patients with tumour. On further experiments the mucosa from tumour patients exhibited a 2 fold regional variation.

The difficulties with interpretation of ligand binding studies on gastric cancer specimens are two-fold. Ligand binding uses preparations of tissue which include malignant, non-malignant and potentially premalignant tissue. Contamination of the preparation in this way may be important but in addition, gastric cancer is recognised as having a heterogenious appearance in terms of differentiation and on immunohistochemical staining. A method of measurement of EGFr expression which would recognise these variables would be of some advantage.

Section vii: EGF receptor gene amplification

The reported gene amplification of EGFr is 2 to 6%. Using Southern blotting Lemoine (1991) found 2 cases of gene amplification from 30 tumours analysed (6.6%) with one of these not overexpressing EGFr. Yoshida (1989) using the same technique in 37 gastric carcinomas found amplification in one case (2.7%). This was a poorly differentiated tumour which showed positive EGFr staining.

In summary, EGFr expression in gastric cancer ranges from 18 to 43%. There is increased expression in advanced disease and associated with a greater depth of invasion. In those studies with detailed follow-up, survival tends to be poorer in those patients with tumours expressing increased levels of EGFr. These are often advanced tumours. In general, increased expression of EGFr is found in differentiated tumours. Intestinal metaplasia and dysplasia from cancer fields showed increasing expression compared to intestinal metaplasia in non-cancer fields and this suggests EGFr may be a possible marker to determine the potential invasiveness of lesions.

There is considerable variation among studies in the method of scoring expression used with immunohistochemistry. The most important determinant of any relationship is the measurement of EGFr expression and I will attempt to use a novel method of detection to get over some of the inconsistencies that seem to have arisen. The method of immunohistochemistry is reproduceable but the different interpretations of images in terms of scoring area and intensity can be difficult. Differences in expression between frozen and fixed sections may also be an important factor in level of expression particularly in mucosal samples. I will attempt to bring together the elements if immunohistochemistry using frozen tissue sections with a quantitative method of scoring; the aim being to exclude the observer variables as much as possible.

Chapter 6

C-erbB-2 and gastric cancer

The c-erbB-2 gene which has been mapped to chromosome 17 at q21 (Fukushige 1986) encodes a polypeptide with a sequence homologous but distinct from the kinase domain of EGFr. The gene product has been identified as a 185 kiloDalton glycoprotein with tyrosine kinase activity. However, kinase activity is not stimulated by EGF and a growth factor ligand has not been identified for c-erbB-2 (Akiyama 1986, Yamamoto 1986). Although there is widespread expression of c-erbB-2 in fetal tissues the level of expression in adults is much lower and tends to be confined to epithelial tissues. The expression of c-erbB-2 has been studied in adenocarcinoma arising in a number of tissues, in particular breast tissue. Much interest has been related to its use as a prognostic indicator and overexpression in breast tissue has been correlated with poor outcome (Slamon 1987).

In gastric cancer, overexpression occurs in adenocarcinoma but to a varying degree. The relationships between overexpression, prognosis and clinico-pathological indicators of outcome have also demonstrated a number of inconsistencies between studies. The expression of c-erbB-2 in gastric carcinoma has been reported at a level of 5.4% (Livingstone 1995) to 72% (Kim 1993) but the majority of studies have found around 25 to 30% positivity using immunohistochemistry (Table 11)

Section i: Relationship to tumour differentiation

Increased levels of c-erbB-2 have been consistently found in well differentiated gastric adenocarcinoma. Using a sample of 93 patients with advanced gastric adenocarcinoma who underwent gastrectomy between 1979 and 1989, Jain et al (1991) found 18 (19%) positive cases. All of the c-erbB-2 membrane positive cases were of the intestinal type ($p<0.02$) and a higher frequency of c-erbB-2 membrane staining was found in tubular

and well to moderately differentiated tumours ($p<0.05$) and those with an expansile growth pattern ($p<0.05$). Further evidence of a relationship between expression of c-erbB-2 and differentiated tumours was found in papillary carcinomas ($p<0.01$), well differentiated cancers (Mizutani 1993) and intestinal type tumours (Lemoine 1991). In the intestinal type tumours reported in the latter study from London, 53% exhibited positive staining in comparison to 8% in diffuse type tumours. Despite the patient numbers being small in a number of studies there is a consistency in reporting this association between c-erbB-2 and well differentiated tumours from both British and Japanese groups (Hilton 1992, Sasaki 1992 and Motojimi 1994). In finding a significant relationship between these factors ($p<0.01$) Uchino (1993) was prompted to extend investigation of c-erbB-2 expression to a larger group of differentiated tumours with no loss of significance in the association.

Section ii: Relationship to tumour stage

A significant relationship was found between increased c-erbB-2 expression and serosal invasion, lymph node metastases, venous invasion and stage of disease ($p<0.05$) by Yonemura (1993) who studied 164 patients having subtotal or total gastrectomy with lymph node dissection. C-erbB-2 positive patients had a poorer prognosis ($p<0.05$).

In early tumours Mizutani (1993) found 4 out of 7 positive tumours had lymph node metastases but tumours without expression, were node negative. In this series, there was increased c-erbB-2 positivity correlating with depth of invasion. C-erbB-2 was detected more often in cancers with lymph node metastases but not to a significant extent The incidence of c-erbB-2 positivity in lymph nodes was found to be higher than in the primary tumour ($p<0.01$) particularly in well differentiated tumours. Overexpression of c-erbB-2 in the primary tumour with corresponding levels in lymph node metastases has been described (Hilton 1991) but, interestingly, increased intensity of staining in lymph node metastases was found and positive staining in lymph nodes even when the primary tumour was negative (Ohguri 1993, Mizutani 1993).

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When c-erbB-2 positivity was compared with tumour stage by Motojima (1994), advanced tumours showed significantly increased immunoreactivity ($p<0.001$). There was also a correlation with depth of invasion ($p<0.005$) and lymph node metastases ($p<0.01$). Of the patients undergoing potentially curative resection, nodal invasion ($p<0.003$) and c-erbB-2 positivity ($p<0.005$) were found to be independent prognostic variables.

The relationship between tumour stage and c-erbB-2 expression appears to be significant but has not been found in all studies (Uchino 1993, Jain 1991, Lemoine 1991).

Section iii: Relationship to metastases

Higher c-erbB-2 expression in lymph node metastases may suggest a role for c-erbB-2 in the metastasis of gastric cancer and a significant correlation between c-erbB-2 expression and the presence of liver metastases has been described (Mizutani 1993).

In the small group of Japanese patients (Sasaki 1992), attempts to relate expression to clinico-pathological factors were difficult due to the small numbers but of interest was the observation that the overexpressed tumours were well differentiated tumours and of those tumours with peritoneal metastases, 2 of the 6 had c-erbB-2 gene amplification ($p<0.1$). Of the 2 out of 9 tumours with peritoneal metastases, they were well differentiated tumours both with amplification ($p<0.05$).

Section iv: Relationship to survival

Reports of an association between c-erbB-2 expression and survival are variable. Some investigators have found prognosis is better in patients with tumours overexpressing c-erbB-2 (Jain 1991, Hilton 1991) often associated with well differentiated tumours but as

disease but may be of importance in the survival of patients with early tumours (Mizutani 1993).

Section v: Measuring c-erbB-2 expression

Differences in outcome for published studies of the significance of c-erbB-2 in gastric cancer may be explained by variations in testing methods. The most frequently used method of measuring c-erbB-2 is by protein immunohistochemistry (IHC). This method is widely available in most routine pathology laboratories and most experience in the field is using this method. IHC can be specific, rapid and requires few reagents but the sensitivity of the assay can vary depending on the particular monoclonal antibody used in the assay and some antibodies may occasionally reveal cytoplasmic immunoreactivity which is considered a non-specific pattern by most investigators (Busmanis 1994).

The description of the staining distribution which was seen in the tumours in one of the initial studies by Falck and Gullick (1989) is particularly valuable in outlining some of the difficulties in defining positivity in gastric sections and expressing that information in a form which can be compared to other studies. The antibody 21N was developed by this group to recognise the residues 1243-1255 on the carboxy terminus of the protein and was used in immunohistochemistry experiments. The observations of staining patterns show there was no significant staining in non-neoplastic epithelium whether histologically normal or showing metaplastic or dysplastic changes. Membrane staining was regarded as most specific, although in positive cases there was accompanying cytoplasmic staining. The few cases which were highly positive were easily recognisable but the majority of positive cases stained weakly. Patchy staining was common, and this is one of the particular difficulties in studying gastric cancer. Often there are well demarcated areas of positive staining but these are interspersed with negative areas in the same tumour field.

Positive and negative neoplastic tubules were also found in the same area of tumour by Ohguri (1993). Two immunoreactive staining patterns were identified; predominantly

cytoplasmic staining and a membranous type where staining was found on the lateral and basal sides of tumour cells with occasional weak cytoplasmic staining. Cytoplasmic and membranous staining together were classified as membranous but in both staining patterns the proportion of positive cells varied considerably.

A large proportion of tumours which were found to have positive cytoplasmic staining but no membrane staining were excluded in further experiments using the 21N antibody from Hilton (1991).

It has been suggested that cytoplasmic staining is caused by cross-reactivity of the antibody to a 155kD cytoplasmic protein similar to, or a product of, the c-erbB-2 oncogene (De Potter 1989). This was identified by immunoblotting in a small sample of their tumours in order to determine that the cytoplasmic staining was a 185kD protein. Further support for the significance of cytoplasmic staining came with a report from Kumar (1991) which suggested that 19% +/-4% of de novo synthesis of p185 HER2 (c-erbB-2) was expressed on cell membranes and the remainder in the other cellular compartments, in a human breast tumour cell line.

Comparison to a known positive breast control was used by Hilton (1991) and by Ohguri (1993) who, in addition, employed an antibody dilutional method to semi-quantify the strength of immunoreactivity.

Jain (1991) used the 21N antibody with formalin fixed specimens embedded in paraffin. Staining was scored as -, +, ++, +++ and although this study used a scoring system to semi-quantify the results there was no comparison with cell lines known to express elevated levels of c-erbB-2.

Standardisation of the control tissue used and the scoring systems which have been developed has led to production of commercially available kits for c-erbB-2 detection. The aim, with these, and other methods of c-erbB-2 detection which have been developed is primarily to decrease inter-laboratory differences.

There is certainly confusion in some cases in recognising which tumour specimens are considered to express c-erbB-2. If more than 20% of the tumour cells are stained this has been considered positive by some (Motojima 1994). C-erbB-2 protein detected was

usually membranous but cytoplasmic staining was also found in most positive cases. Although 33 (28%) of the gastric tumours were determined to be positively stained, the study found that most of the tumours exhibited some degree of staining for c-erbB-2. However, since this was less than 20% of cells these tumours were classified as negative. No explanation was given for using 20% as the cut off level of expression. There was also lack of clarity as the investigators state "strong staining was easily recognisable, although most positive tumours were weakly stained".

Comparable results have been found in both paraffin and frozen sections of gastric cancer specimens (Lemoine 1991) and there is no doubt that with great care in the immunohistochemical technique and the use of control tissue IHC is a valuable tool. However, the methods used to process tissue, the time and nature of fixation and the temperature of paraffin embedding all may affect c-erbB-2 protein antigen loss or intensity of immunostaining in the specimens (Penault-Llorca 1994).

Staining of c-erbB-2 protein in non-neoplastic mucosa was detected by Ohguri (1993) and by others (Uchino 1993). Tumour fixation and subsequent methods of antigen retrieval,such as microwave use, may explain the variable range of expression within a tumour series and in mucosal samples.

In 1990 Kameda et al (1990) compared the level of c-erbB-2 expression in 34 gastric carcinomas and corresponding mucosa. The monoclonal anti-body used in this case also recognised the cytoplasmic domain without residue specificity (Nichirei,Tokyo) and the results were determined by a scoring system from - to +++ depending on the number and intensity of cells stained although details of this were not given. Well differentiated tumours tended to exhibit cell membrane staining whilst in poorly differentiated tumours it tended to be cytoplasmic. Western blotting of tumour lysates demonstrated low levels of c-erbB-2 in more of the poorly differentiated group with higher levels in the well differentiated tumours expressing membrane c-erbB-2.

However, in some poorly differentiated tumours with low levels on Western blotting there was positive immunostaining. Unfortunately different commercial monoclonal antibodies were used in these experiments. The study suggested a higher level of expression of immunoreactivity in both tumour and 'normal' mucosa but the results were given as raw scores and are difficult to interpret. In 23 of 34 cases (68%) staining of the tumour scored higher than the corresponding mucosa but some samples of mucosa with metaplasia and regenerative epithelium expressed high levels of c-erbB-2.

Finally, Lim et el (1993) demonstrated expression of c-erbB-2 in 72% of tumours. There were no details of the intensity or distribution of staining or criteria of positivity, and no indication of controls, levels in normal mucosa and any use of a scoring system. Further interpretations of this data and any correlations made would obviously be misleading. In particular the suggestion that patients in this area of South-East Asia have tumours which are biologically different.

Section vi: C-erbB-2 expression in different populations

McCulloch (1994) and Livingstone (1995) have examined specimens of tumours from patients undergoing resection of gastric carcinoma in Japan and the U.K. These patients have been matched for age, time of operation, tumour differentiation, stage and site. In Liverpool, 89 tumours were studied from each group using a polyclonal antibody (Dako plc.Bucks,UK). No difference in the level of expression was observed between the two populations. A scoring system -, +(<5%), ++(<50%) and +++(>50%) was employed and no staining of normal epithelium was observed. UK tumours expressed c-erbB-2 in 13% of cases and 11% in Japanese tumours. In contrast, Livingstone using a similar scoring system and commercial antibody (NC-004,Novocastra) found that UK tumours expressed c-erbB2 in more cases with a level of 54% compared to 21% ($p<0.01$) and this difference was demonstrated across the spectrum of stage of disease.

Section vii: C-erbB-2 gene amplification

Gene amplification of c-erbB-2 has been assessed in small numbers of tumours and ranges from 5 to 13%. Correlation with immunostaining is found but is not universal and further explanation of c-erbB-2 overexpression is required. In the assessment of c-erbB-2 status the use of techniques to measure protein expression and gene amplification will need to be compared. For research purposes, most studies have used Southern blotting to determine c-erbB-2 amplification. However, the use of fluorescence in situ hybridisation (FISH) is becoming more routine despite being relatively time consuming and complicated to perform.

ErbB-2 gene amplification was assessed using Southern blot analysis on 3 out of 34 tumours which expressed high levels of c-erbB-2 (Kameda 1990). Two of the three cases showed a corresponding gene amplification (5.9%). In one of the tumours, although differentiation was the same throughout the tumour field, the expression and amplification showed considerable variation from site to site.

The absence of gene amplification in the third tumour prompted a search for transcriptional causes of higher protein expression. Within the promoter region of the erbB-2 gene is an area termed the TATA box. The levels of binding proteins to this and other promoter areas were examined by gel retardation assays and in general found to be higher in tumour than mucosa. However, the binding proteins to the TATA box showed a significantly higher level in the tumour identified as having high c-erbB-2 expression without gene amplification. Using gastric cancer cell lines, the cell lines with a single copy gene and high expression of c-erbB-2 were found to have higher levels of TATA binding proteins than cell lines with poor c-erbB-2 immunoreactivity. This suggests a possible explanation for varying levels of c-erbB-2 expression, that in some cases it may be due to amplification but increased expression in some instances is due to transciptional changes.

Overexpression of c-erbB-2 and amplification were also studied by Sasaki (1992). Of the 24 tumour specimens which were analysed using Southern blotting and immunohistochemistry 3 showed overexpression and 2 showed amplification (8.3%) although only one of these tumours overexpressed c-erbB-2. Southern blotting was also used in a group of forty tumours and revealed four tumours with gene amplification, all of which demonstrated c-erbB-2 overexpression with over 80% of the cells stained and 3 of the 4 were intestinal type tumours.

Gene amplification has been demonstrated in several small studies of gastric carcinomas (Gutman 1984, Houldsworth 1990) occurring in 5 to 13% of cases with up to a 32 fold overexpression of erbB-2 mRNA. Some correlation of c-erbB-2 amplification with tubular or well differentiated tumours (Park 1989, Tal 1988, Yoshida 1989) has been described.

Section viii: Relationship between EGFr and c-erbB-2

There was no relationship found between expression of EGFr and c-erbB-2 (Lemoine 1991) and this was supported by no obvious correlation in gene amplification (Yoshida 1989).

Table 10 **Review of published data on the relationship between c-erbB-2 and prognostic factors**

REFERENCE	POSITIVE	*STAGE*	*NODES*	*GRADE*	*GROWTH PATTERN*	*SITE*	*METASTASES*
Falck&Gullick	19	-	-	well/tub/pap	-	-	-
Kameda et al	55	no	-	well	-	-	-
Lemoine et al	26	no	no	intestinal	no	no	-
Hilton et al	9	-	yes	intestinal	-	-	-
Jain et al	30	-	no	int / well	expansile	-	-
Sasaki et al	17	no	no	moderate	-	-	peritoneal
Tateishi et al	12	-	-	papillary	-	-	no
Motojima et al	28	yes	yes	no	-	no	-
Kim et al	72	-	no	no	-	-	no
Yonemura et al	19	yes	yes	no	-	-	no
Uchino et al	8	no	no	well/mod/pap	-	-	no
Ohguri et al	25.7	no	no	no	-	-	
Mizutani et al	14.2	yes	no	papillary	expansile	-	liver

Table 11 **Review of published data on the expression of c-erbB-2, with the tissue used and the method of immunohistochemical counting.**

REFERENCE	YEAR	METHOD	FIXATION	NUMBER	%POSITIVE	STAINED AREA COUNTED
Falck&Gullick	1989	IHC	Paraffin	126	19	membrane
Kameda et al	1990	IHC	Par/frozen (mixed)	34	55	membane/cytoplasm (well/poorly differentiated)
Lemoine et al	1991	IHC	Par/Frozen	40	26	membrane (every tumour)
Hilton et al	1991	IHC	Paraffin	87	9	membrane
Jain et al	1991	IHC	Paraffin	93	30	membrane(11%) cytoplasm(19%)
Sasaki et al	1992	IHC	Paraffin	24	17	membrane
Tateishi et al	1992	IHC	Paraffin	179	12	membrane
Motojima et al	1992	IHC	Paraffin	120	28	membrane
Kim et al	1993	IHC	Paraffin	100	72	unspecified
Yonemura et al	1993	IHC	Paraffin	164	19	membrane
Uchino et al	1993	IHC	Paraffin	106	8	membrane (+108 papillary)
Ohguri et al	1993	IHC	Paraffin	136(92 early 44 advanced)	25.7	(48.6% early 51.4% adv)
Mizutani et al	1993	IHC	Paraffin	226(92 early 134 advanced)	14.2 7.6% early 18.7% adv	membrane
McCulloch et al	1994	IHC	Paraffin	89 UK 89 Japan	30 31	unspecified
Livingstone et al	1995	IHC	Paraffin	33 UK 40 Japan	5.4 21	membrane

The literature review is summarised in tables 10 and 11. The level of expression of c-erbB-2 varies from 5 to 72 % with the majority of studies suggesting a level of around 30%. Gene amplification ranges from 5 to 13%.

There is evidence of increased expression of c-erbB-2 in well differentiated tumours and a significant relationship exists between increased c-erbB-2 expression, lymph node metastases and stage of disease ($p<0.05$). In some studies, c-erbB-2 positivity is found in lymph node metastases in the presence of c-erbB-2 negativity in the primary tumour. To a lesser extent the presence of liver metastases is found more often in tumours with increased levels of c-erbB-2.

Correlation of c-erbB-2 with survival is found for high and low expressing tumours and may be related to positivity in a particular histological type or stage of disease.

There is evidence that c-erbB-2 expression will be important as a prognostic factor in gastric cancer. Inconsistencies in the published studies are most likely to be due to differences in c-erbB-2 measurement. In particular, the variable scoring of sections, membrane and cytoplasmic staining, the use of different antibodies, the patchy nature of staining in adjacent areas of tumour and the effect of differing methods of fixation.

In order to overcome some of the recognised problems, a novel approach to measuring c-erbB-2 has been developed and I will report in detail its use in gastric cancer. Frozen tissue sections are used to overcome the variables in the fixation process and preserve antigenicity in tumour and mucosal samples. The technique of radioimmunohistochemistry, utilises a radio-iodinated anti-c-erbB-2 monoclonal antibody to label the receptors and a computor-assisted image analysis to quantify the bound receptor. Sections of cell pellets with known c-erbB-2 levels are processed with each batch of samples as internal calibration standards and comparison with expression in samples of non-neoplastic mucosa is made.This technique combines the objective quantification of ligand-binding analysis with the specificity of immunohistochemistry.

Chapter 7

Insulin-like growth factors and receptors

Insulin-like growth factors are a group of structurally related polypeptides which effect the proliferation and differentiation of a wide range of cell types.

The insulin-like growth factors (IGFs) were initially called somatomedins (Daughaday 1972). They were discovered as a group of substances mediating the actions of growth hormone (somatotropin) but better understanding of the structure, function and mechanism of action of the IGFs has shown widespread synthesis and activity in many tissues.

Section i: Structure of insulin-like growth factors

IGF- I (somatomedin-c) and IGF-II (somatomedin-a) are single chain polypeptides with 70 and 67 residues and molecular weights of 7649 and 7471 respectively. There is 60% amino acid sequence homology between IGF- I and IGF-II (Daughaday 1989) and 43% sequence homology between IGF-I and pro-insulin which is reflected in the insulin activity of these factors which is not inhibited by anti-insulin antibodies (Froesch 1963). Homology is in the A and B domains but there is none between the C-peptide retained in IGF and the C-peptide present in pro-insulin but cleaved in the formation of mature insulin. In addition, the IGFs have a D domain. Each IGF is the product of a single complex gene (Rotwein 1991). The human IGF- I gene is on chromosome 12q and the IGF-II gene on chromosome 11p close to the insulin gene.

Section ii: Function and regulation of IGFs

IGFs are synthesised throughout the body in most, if not all tissues and synthesis is determined by the specific tissue and a number of regulatory factors. IGF expression

often occurs in tissues at specific stages in development and in tissues undergoing rapid growth. Tissues which maintain rapid proliferation of cells into adulthood often exhibit high IGF- I expression. IGF- I circulates in the blood at readily detectable levels and hepatic synthesis is the main contributor to the serum concentration. The IGFs have insulin-like anabolic activity in adipose tissue, liver and skeletal muscle (Zapf 1986). The hormone acts in an endocrine fashion and is regulated by nutritional and endocrine factors (Underwood 1986). Nutritional deficiency reduces IGF- I and causes resistance to GH. GH stimulates IGF- I expression and in their specific target tissues, PTH, FSH, TSH and oestrogen (Murphy 1988) stimulate expression. IGF- I also has autocrine and paracrine activity in the tissues of synthesis (D'Ercole 1996). IGF-II also has detectable blood levels but seems to act more in the tissues of synthesis rather than in an endocrine function. The factors which regulate expression of IGF- I, influence IGF-II expression but to a lesser degree.

IGF expression often occurs in specific tissues at developmental stages when these tissues are undergoing rapid growth. IGF- I and IGF-II are important in embryonic and fetal development. Postnatally, IGF-II is expressed much less than IGF-1 which continues to play an important developmental role (Baker 1993). IGF- I and IGF-II are present in all species and have been isolated in several including rat (Whitfield 1984), mouse (Bell 1986)and bovine (Honnegger 1986) forms with minimal variation in amino acid sequence.

Section iii: IGF Binding Proteins

IGFs are secreted soon after synthesis. The free form of IGF- I has a half life of 10 to 12 minutes. The vast majority of IGFs are complexed to binding proteins (IGFBPs) which can prolong the half life to 12 to 15 hours (Guler 1989). These are synthesised by the IGF producing or nearby cells and modulate actions by inhibiting their activity at

receptors, protecting IGFs from degradation and delivering them to the tissue cell surface.

There are 6 recognised high affinity IGFBPs (Baxter 1989) most of which bind IGF- I and IGF-II with similar affinity and at least a further seventh IGFBP of low affinity (Ellis 1998). IGFBP 3 is a 150,000MW complex and binds most IGF. The smaller binding proteins are of 24,000 to 34,000MW. The IGFBPs are synthesised in the tissues producing IGFs. The role of IGFBPs is to deliver and modify the activity of IGFs. With the exception of IGFBP 4 they can potentiate the activity of IGFs possibly by a slow release mechanism which prevents down regulation of the receptors. Phosphorylation of the binding proteins may explain why in some circumstances they inhibit and in others potentiate the action of IGFs.

Section iv: Type I IGF receptor

IGFs bind to cell surface receptors. The Type I IGF receptor is most likely to be responsible for the mitogenic effects of IGF- I and IGF-II (Czech 1989).

The Type I IGF receptor is a heterotetrameric glycoprotein of 300,000MW composed of paired disulphide linked alpha and beta subunits of 130,000MW and 95,000MW respectively. The particular configuration with disulphide bonding distinguishes the receptors from other tyrosine kinase growth factor receptors (Siddle 1993).

The Type I insulin-like growth factor receptor and the insulin receptor show 80% homology in their tyrosine kinase domains and 40-60% homology in other areas (Ullrich 1986).In cells expressing both receptors a proportion of the receptors will be hybrids of each receptor (Siddle 1993).The Type I IGF receptor has 2 to 3 times more affinity for IGF- I compared to IGF-II and 100 times more affinity for IGF- I than for insulin (Czech 1989). The subunits are derived from a single gene and subsequently a common precursor which is dimerized then the individual subunits cleaved. The homology with the insulin receptor suggests a common primordial gene (Herington 1991).

The ligand binds to the alpha subunit, induces a conformational change in the receptor and the tyrosine kinase activity induces autophosphorylaton of the beta subunit. Thereafter phosphorylation of intracellular proteins ultimately leads to progression of the cell cycle and cellular differentiation. Activation of gene transcription is effected by signalling pathways similar to those of EGFr. There is phosphorylation of SH2 domains on intra cellular signalling proteins and binding to the Grb2/Sos complex as previously described. However, rather than direct binding to SH2, the beta subunit also phosphorylates 'docking proteins' IRS-1 and IRS-2 which when phosphorylated are recognised by SH2 domains (LeRoith 1996).

Section v: Type II IGF receptor

The type II insulin-like growth factor receptor has no kinase activity. It is found on nearly all cell types and binds IGF-II with more affinity than IGF- I with negligible binding of insulin. The receptor is a single polypeptide of molecular weight 250,000Da and has close homology with the mannose-6-phosphate receptor (Morgan 1987).

Section vi: Physiological role of IGFs

The physiological role of insulin-like growth factors involves the mediation of cellular proliferation by stimulation of DNA, RNA and protein synthesis. When proliferation is complete IGFs are potent stimulators of cellular differentiation. In most tissues IGFs stimulate the synthesis of extracellular matrix and in endocrine tissue IGFs effect hormone secretion promoting steroid synthesis in ovarian granulosa cells and Leydig cells. IGFs stimulate specialised cell function in the immune, haemotological and neuronal system and may inhibit apoptosis to enhance cell survival (Ellis 1998).

Insulin-like growth factors in malignancy

Components of the IGF signal transduction pathway have been implicated not only in the proliferation of malignant cells but in the transformation of cells. With IGF II targeted to the mammary gland of transgenic mice, an excess of mammary gland tumours has been noted (Bales 1995) and in the transformation of cells by the src oncogene the Type I IGF receptor has been implicated as an intermediary (Westley 1995). IGF- I, IGF-II, the IGF receptors and binding proteins are expressed in a wide variety of cancer cell line and tumour specimens including those of ovary, breast, colon, bone, lung and brain.

Section vii: IGFs in breast cancer

IGF- I and IGF-II have been shown in vitro to be potent mitogens in breast cancer cell lines (Macauley 1992). Some studies have suggested that IGF- I is not expressed by tumour cells (Yee 1989) but by the supporting stroma in breast and its function is by endocrine or paracrine means. In contrast it is felt that breast cancer cells secrete IGF-II (Lee 1994). However, Gebauer (1998) found mRNA for IGF- I and IGF-II in malignant tissue but not cancer cell lines and Giana (1995) proposed a stromal origin for IGF- II. The type I IGF receptor is expressed in most human breast cancer (Cullen 1990, Foekens 1989) and using ^{125} I-IGF- I binding studies (Jammes 1992), the receptors are present on epithelial cells with binding higher in the malignant cell population.

There are a higher concentration of IGFBPs 1-5 in malignant compared to normal epithelium (Pekonen 1992), with all breast cancers expressing IGFBPs 1-6. In oestrogen negative tumours there is increased expression of IGFBP 2 and 4 with increased IGFBP 3 in oestrogen receptor positive tumours (Helle 1995).

While the contribution of IGFs produced in the malignant tissue may be small in comparison to the plasma level, the availability of IGF- I and IGF- II to the receptor may be increased by reduced IGFBP 3 binding. Increased levels of IGFBP 3 protease have been found in the plasma of breast cancer patients with metastatic disease (Frost 1996).

This is an enzyme which proteolytically transforms IGFBP 3 into a low affinity binding protein with increased release of IGFs to the tissues and the consequent stimulation of tumour growth (Helle 1996).

There are important interactions between the IGF system and endocrine activity in breast cancer. Oestradiol increases the expression of IGF- II and increases the cellular concentration of mRNA for IGF- I receptor. Oestrogens stimulate IGFBP 3 proteases. In addition it is now apparent that the IGFBPs interact with the cell membrane directly and in the absence of IGFs, modulate cellular function (Adamo 1992, Dubois 1995, Oh 1998).

In vitro IGF- I and oestradiol synergistically enhance growth in the MCF-7 breast cancer cell line. The interaction between IGF and oestrogen occurs at the oestrogen receptor (Lee 1997). The type I IGF receptor promotes the transcriptional activity of the oestrogen receptor by MAP kinase dependent- ER phosphorylation (Kato 1995).The effect of oestradiol stimulation can be reduced with antibodies to IGF- I (Stewart 1990) and in vitro the effect of oestrogens on the IGF system can be reversed with antioestrogens (Kawamura 1994, Lee 1997). In vivo, tamoxifen reduces plasma IGF- I and increases IGFBP 1. With megestrol acetate IGF- I is increased but the reduction in IGFBP 3 protease may reduce the bioavailability of IGF- I to the tissues (Helle 1996).

Section viii: IGFs in ovarian cancer

In ovarian cancer, primary epithelial cell lines derived from untreated ovarian cancers have shown IGF-I mRNA expression (Yee 1991, Conover 1998) and a significant amount of IGF-I secreted. Cell lines expressing IGF-II mRNA have been found with protein expressed into the media in high concentrations. Affinity cross-linking experiments documented abundant type I IGF receptors and there was also significant ^{125}I-IGF I binding to IGFBPs on the surface of some ovarian cancer cell lines (Conover

1998) and type I receptor mRNA found in all cell cultures and malignant ovarian tissue (Yee 1991). In all cultured cells there was expression and secretion of IGFBPs with IGFBP 2 the most abundant in all but one culture. These were associated with advanced, aggressive tumours and not expressed in the lowest grade/stage tumour.

In other studies on ovarian cancer cell lines there was cellular proliferation in response to addition of exogenous IGF-I like peptides (LR3-IGF-I) which do not bind to IGFBPs but activate the IGF receptor. This proliferation was not enhanced by addition of oestrogen. Proliferation of ovarian cancer cells in serum free media was increased by addition of IGF-I. This effect was inhibited by incubation of cells with anti-sense oligonucleotides to the type I IGF receptor (Resnicoff 1993).

Section ix: IGFs in pancreatic cancer

In pancreatic cancer, IGF-I mRNA is abundant and present in both cancer cells and the surrounding stroma in contrast to normal pancreas which has low levels of IGF-I only present in the stroma. IGF-I stimulates the growth of pancreatic cells in culture. This effect is inhibited by the IGF-I monoclonal antibody alpha-IR3 and IGF-I receptor antisense oligonucleotides. IRS-1 is overexpressed in pancreatic cancer but IGF-II and insulin are not detected. However, insulin and IGF-II are produced by the islet cells and may influence the growth of the cancer cells by endocrine means (Korc 1998).

Section x: Lung cancer and IGF-I

Most of the effects of the IGFs in lung cancer are mediated by the type I IGF receptor which is expressed in normal bronchial epithelial cells and primary cancer with high density binding sites especially prominent in squamous cell carcinoma and small cell lung cancer (Shigematsu 1990). IGF-I is secreted into the media of cultured cells from both small cell (SCLC) and non-small cell lung cancer (NSCLC) (Macauley 1990) with

strong staining for IGF-I in squamous carcinoma. IGF-I is detected in primary lung tumours at higher levels than in normal lung (Minuto 1986). In NSCLC IGFs bind predominantly to the Type I IGF receptor but in SCLC binding was primarily to IGFBP 2 on the cell surface.

Section xi: IGF-I in colonic carcinoma

IGF-I has been shown to stimulate the growth of colonic cancer cells using cell proliferation studies (Durrant 1991,Guo 1992). In contrast to the findings in breast cancer the concentration of Type I IGF receptor in colonic cancer cells using ligand binding studies has shown no statistical significance between receptor concentration in malignant and normal colorectal tissue (Adenis 1995). Forty six percent of normal tissue and 70% of malignant tissue was considered positive with no association between receptor concentration and prognostic variables.

Section xii: IGF-I in gastric cancer

In the human foetus the stomach and the placenta contain the highest levels of IGF-1 mRNAs which suggests that the growth and development of the stomach may be highly dependent on IGF-1(Guo 1993). Using the gastric cancer cell lines MKN 45,St 42 and St 16, Durrant et al (1991) studied the growth of cells incubated with IGF-I and the density of IGF-I receptor. IGF-I was reported as significantly mitogenic for St 42 and MKN 45 (St 16 was not tested). The concentration of receptor was found to be 250 IGF-I receptors per cell for St 16, 190 per cell in St 42 and 310 per cell in MKN 45.This is in contrast to the much higher expression of EGF receptor with 7780 receptors per cell in St 42 and 39,700 receptors per cell in MKN 45.There was no correlation between IGF-I and EGFr expression, and mRNA for IGF- II was present in both cell lines.

Cellular proliferation in gastric cancer cell lines AGS cells and SIIA cells was stimulated only in the SIIA cells by IGF-I. Ligand binding studies confirmed the presence of IGF-I receptors on the AGS cells at a concentration of 4.27+/- 0.4 x 10^4 sites per cell and the alpha subunit was of MW 130,000Da. Northern blotting showed no mRNA for IGF-I in the AGS tumour cells and therefore no blocking of the receptors by endogenous IGF-I. Although IGF-I produced no growth response in AGS cells, truncated IGF-I which has affinity for and activates IGF-I receptors but has less affinity for IGFBPs produced a growth response. Gel electrophoresis confirmed the presence of proteins consistent with IGFBPs and it was concluded that the lack of growth response was due to the activity of IGFBPs sequestering IGF-I and preventing receptor interaction.

The experimental evidence which shows the importance of the activity of IGF-I and its receptor in tumour growth has prompted attempts to investigate whether blocking these effects would be inhibiting. Tamoxifen inhibits the IGF-I induced growth of breast cancer cell lines and this activity may explain the success of tamoxifen in oestrogen receptor negative tumours. In a number of experimental studies the monoclonal antibody (alpha IR3) to the Type I IGF receptor used in breast cancer cell lines inhibited anchorage-independent growth in the presence of serum but could not block serum-free growth. In athymic mice the antibody inhibited the growth of MDA-MB-231 cells when injected at the time of tumour inoculation but not in established tumours (Werner 1996). Suramin is a polysulfonated naphthylurea which inhibits growth-factor induced mitogenesis. It is cytostatic in osteosarcoma cells (Pollak 1990) and reduces serum levels of IGF-I and IGF-II in breast, prostate and lung cancer.

Summary

There appears to be evidence that IGF-I and its receptor have a role in the proliferation of cancer cells. There is high IGF-I expression in rapidly proliferating cells and foetal stomach. In ovarian, breast and colon cancer, accelerated cellular proliferation has been demonstrated with IGF-I with most but not all tissues having increased levels of the IGF-

I receptor. In malignancies of breast and lung, in particular, drugs and monoclonal antibodies have inhibited the growth of cancer cells in vitro and in animal studies.

In gastric cancer, often presenting at an advanced stage, the study of growth factors such as EGFr and c-erbB-2 is important to understand the mechanism of the disease process, develop markers of aggressive disease and prognostic indicators. It is also important to study whether these factors have a potential role in cancer therapy. In conjunction with the study of level of expression of EGFr and c-erbB-2 in gastric cancer, information has been obtained from studies of cellular proliferation in vitro and inhibition of the cell growth. Progression of this research to other potential growth factor targets in cancer cell growth is important and to this end I have studied the role of IGF-I in proliferation of gastric cancer cell lines.

Within this thesis I plan to outline the preliminary proliferation studies of gastric cancer cells exposed to IGF-I. In addition, I have sought to demonstrate that this activity can be inhibited by tyrophostins, principally developed to inhibit EGFr activity but also effective in this setting. There are potential clinical implications with this work to develop further novel therapeutic agents but also a number of difficulties to overcome which I will also discuss further.

STATEMENT OF AIMS

In the following studies the role of both Type I growth factors and the IGF-I pathway in gastric cancer will be studied. The review of evidence suggests a role for both EGFr and c-erbB-2 in gastric cancer. However, the results of studies are conflicting and this may be in part due to the differing methodology used for measuring the growth factor receptors. The methods of study most commonly used have been immunohistochemistry and ligand binding studies but there are technical difficulties with each method. Immunohistochemistry gives cellular localisation but is not quantitative and as discussed there were problems with reproducibility related to fixation and processing. Ligand binding is quantitative but does not give localisation and is unsuitable for c-erbB-2. To overcome these problems the technique of radioimmunohistochemistry has been developed for gastric cancer. The purpose of the current programme of experiments is to evaluate radioimmunohistochemistry in determining EGFr and c-erbB-2 expression in gastric cancer. The results will be compared to standard immunohistochemistry. The results will be related to conventional prognostic factors to assess them in predicting tumour behaviour and prognosis.

These aims are effected by examining a retrospective series of 67 gastric cancers in which EGFr and c-erbB-2 expression are measured using radioimmunohistochemistry, immunohistochemistry and c-erbB-2 amplification using fluorescence in situ hybridisation.

The effect of novel inhibitors of growth factor receptors in the N87 and MKN45 gastric cancer cell lines will be assessed in the presence of IGF I. Cellular proliferation and tyrophostin inhibition in IGF-I stimulated gastric cancer cell lines and subsequent changes in signal transduction will be demonstrated.

The defined aims are as follows:

Assess the validity and reproduceability of radioimmunohistochemistry for EGFr and c-erbB-2 in gastric cancer cell lines and tumour specimens.

Evaluation of radioimmunohistochemistry in comparison to standard immunohistochemistry.

The determination of any relationship between the type I growth factor receptors EGFr, c-erbB-2 and clinico-pathological prognostic factors.

The assessment of the use of IGF-I inhibition in cell culture and the effect on downstream signal transduction with reference to clinical application in gastric cancer.

PART III

EXPRESSION OF GROWTH FACTOR RECEPTORS IN ASSESSMENT OF GASTRIC CANCER

Introduction to Experimental Parts of Thesis

Expression of the EGFr and c-erbB-2 varies widely in the studies we have reviewed and much of this may be due to methodological rather than tumour biology. In an attempt to more accurately measure growth factor receptor density in tumour specimens, we have sought to improve on the methods of investigation currently available.

Ligand binding involves the exposure of the tissue in question to radiolabelled ligand which subsequently binds to the receptor being studied. The membrane preparations are derived from tumour biopsies and include malignant and non-malignant cells. In tumours with high receptor levels or cell pellets from cultured cells which express high levels of receptor, this may not represent a significant problem but for cancers where receptor expression may be close to, or less than, normal tissue, these contaminants may be important.

Immunohistochemistry also uses receptor antigen binding detected by a complex of reactions resulting in staining at the site of activity. This allows localisation of receptors to tumour cells but is inherently subjective and at best semi-quantitative. By combining the localisation of immunohistochemistry in tumour specimens with a method to correlate this information to ligand binding we have sought to develop a quantitative, tumour cell specific method of determining growth factor receptor density.

The level of expression of EGFr and c-erbB-2 in specimens of gastric adenocarcinoma and normal gastric mucosa was measured by radio-immunohistochemistry. The quantitative aspect of this method of study is determined by comparison with receptor density in cancer cell lines as determined by ligand binding studies. In addition, tumour tissue was stained using standard immunohistochemistry and c-erbB-2 amplification measured using fluorescence in-situ hybridisation (FISH).

Chapter 8

Methodology

EGFr and C-erbB-2 Expression

Section i: Tumour Specimens

The majority of tissue used in this series of investigations was collected from patients undergoing surgery in Glasgow Royal Infirmary during the period 1990 to 1995 where the operating surgeon was a member of the University Department of Surgery team. The patients included those undergoing both curative and palliative resections and several patients having palliative procedures without resection in whom laparotomy was performed for intubation or bypass procedures. The patients were not consecutive but there were no exclusion criteria and those tumours which were collected depended on staff availability only. Ethical approval for the study was given by the Ethical Committee of the Eastern Unit of Greater Glasgow Health Board (Glasgow Royal Infirmary). The collection of tumour specimens and data did not compromise or influence the patient treatment or routine pathological analysis.

At the time of surgery, the resected specimen was taken immediately to the pathologist. The pathologist would make an initial assessment of the fresh specimen with regard to the size and site of tumour. He would then remove a sample of macroscopically normal mucosa at the resection margin of the stomach most distal from the tumour in order to reduce the likelihood of malignant or pre-malignant changes within the mucosal sample which may alter the growth factor receptor staining. A sample of tumour was then removed, the full thickness of the stomach wall in the majority of cases. Both specimens were immediately frozen in liquid nitrogen and transported in flasks of liquid nitrogen to the tumour bank in the department of surgery in Glasgow Royal Infirmary. They were stored in the tumour bank in liquid nitrogen at -70^{O}C. Tumour samples which were obtained from other hospitals were transported to the pathology department and

assessed on site by the resident pathologist and then on to the tumour bank. Around five percent of tumours came from other centres in Glasgow.

Another small group of tumours originated from St. James Hospital in Leeds. The specimens had been collected for several projects in Leeds with ethical approval for tissue collection and subsequent analyses. The clinical details and pathological information were kindly supplied by Professor J. Primrose who was the director of the gastric tumour study at that time and Dr. J Wyatt, Consultant Histopathologist. The tumours were collected from St. James Hospital and Airedale General Hospital over a period of three years with inclusion of the majority of patients undergoing resection and no formal selection or obvious bias. Although samples from a large number of the tumours in this study were in the tumour bank in Glasgow many on sectioning and staining were found to be poorly preserved or had inadequate clinical and pathological information. These samples were therefore unsuitable for inclusion in this study. Those tumour blocks which were poorly preserved could bring a question of selection bias to this study. The larger tumours possibly with areas of necrosis may be less well preserved. However, the preservation of the blocks of tumour was more in question. These tumours had been selected previously as suitable for other studies but mis-handled in transit and this is the source of poor preservation. In addition, lack of information may suggest many influencing factors and potential areas of bias, particularly shortened follow-up in advanced disease. However, the study of these tumours was one of resected specimens.This narrows the bias for those patients excluded in this group in that the tumour group as a whole comprised mainly resected tumours but this in itsel brings selection to those included in this study.The small number of specimens obtained outwith Glasgow amounted to 6%.

Gastric adenocarcinoma specimens from 67 patients were used in the study and all subsequent analyses used only these tumours. Samples of oesophageal adenocarcinoma, adenosquamous gastric cancer and gastric lymphoma were used for observational comparison only.

Section ii: Patient and tumour data noted

The details which were recorded for each patient and specimen of tumour were as follows:

Patient data

- unit record number
- age
- sex
- presentation and symptoms
- type of operation
- clinical outcome including evidence of recurrence, further therapy, survival time and cause of death

Pathological data

- pathology department number
- macroscopic size of tumour
- tumour differentiation and Lauren classification if available
- nodal involvement (number involved/number sampled)
- serosal involvement and tumour stage
- presence of metastases

Section iii: Method of Radio-immunohistochemistry

This method allows a quantitative estimation of antibody binding. By using control tissues with known receptor concentration and determining the antibody binding to these tissues the receptor density measurement of EGFr and c-erbB-2 in tissues has been measured.

Using multiple sections cut from a cancer cell pellet, binding assays were performed; further sections were measured for area then assayed for protein content and measured against known cell concentration standards. Thus, for each binding assay; mean section area, protein content and cell number were estimated and therefore, receptors per area of section could be calculated. To ensure comparable cell lines to those used with tumour specimens in this thesis, these experiments, to calculate receptor numbers, had been performed just prior to the main body of work on the same cell pellets.

The control tissues used for EGFr and C-erbB-2 concentrations were cell pellets of tumour cells grown in tissue culture in which the receptor density has been determined.

The cell lines used for EGFr are:

A431 (2×10^6 receptors per cell)

BT 20 ($1\text{-}4 \times 10^5$ receptors per cell)

EJ (2×10^5 receptors per cell)

SKBR3 ($< 2 \times 10^4$ receptors per cell)

ZR 75 (1×10^4 receptors per cell)

(De Cremoux 1994; Dong 1991; Fitzpatrick 1984; Long 1992; Stanton 1994)

The cell lines used for c-erbB-2 are:

N87 (4.88×10^9 receptors per mm^2)

BT 474(5.81×10^8 receptors per mm^2)

MD 361(1.309×10^8 receptors per mm^2)

ZR 75(1.09×10^7 receptors per mm^2)

MCF 7(2.48×10^6 receptors per mm^2)

(Reeves 1995)

Section iv: Tissue Preparation

Sections were cut from the cell pellets and tissue blocks. The freshly collected tissue specimens which had been frozen in liquid nitrogen were trimmed and placed on the freezing block in the cryostat in order to warm the tissue to approximately -30°C. The tissue blocks were fixed to cork blocks using Tissue-Tek adhesive and returned to liquid nitrogen. The cork was then attached to a cryostat chuck with a drop of water and placed in the mount of the cryostat. 5µm sections were cut for immunohistochemistry, radio-immunohistochemistry and FISH. They were processed at the same sitting to ensure the same area of tumour was compared for all three techniques. Each batch had a haematoxylin and eosin stained reference slide. These were used to check the presence of tumour on sections and direct to the areas of interest for RIHC and IHC. The H and E slides were checked by Dr. Colin Stewart, Consultant in the Pathology Department in Glasgow Royal Infirmary to confirm tumour presence and type.

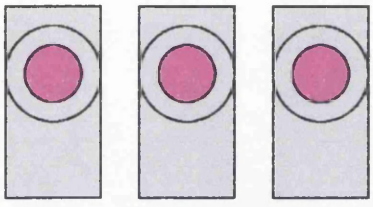
Slides were stacked together in groups according to experiment with mini-dividers between slides to protect the tissue sections. The slides were wrapped in foil to provide an airtight package and stored in a -70°C freezer until required. When the sections were required the foil packages were removed from the freezer and allowed to warm to room temperature to prevent the formation of condensation.

For radio-immunhistochemistry one section was required as a control with two hot antibody sections (Figure 15). Each of the sections was ringed with a water repellent pen, to contain the reagents when applied to the slides. The slides were fixed in acetone for ten minutes and washed in phosphate buffered saline (PBS) for ten minutes through three changes of PBS. Having dried around the sections, 100ul of blocking serum was applied for forty-five minutes.

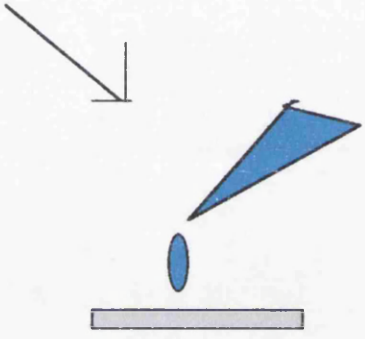
Figure 15

Summary of tissue preparation of the hot and control slides

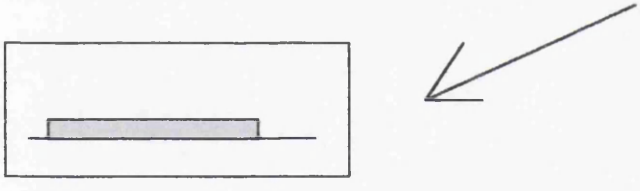
Each tumour has 3 slides treated identically in all but one step. The control slide has a blocking agent of excess antibody to prevent binding of the radio-labelled antibody.



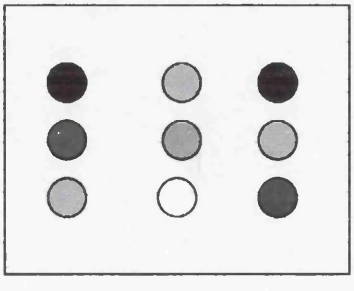
Hot and control sections on slides ringed with water repellent pen



After fixation in acetone and washing in PBS the blocking agent is applied and the radiolabelled antibody



ides are incubated in a humidified chamber for 3 then washed in PBS and fixed in buffered saline



The slides are taped in an X-ray cassette for 96 hours to indicate the time each slide will need to be exposed to emulsion

The blocking serum for the hot sections consists of 50:50 normal rabbit serum to PBS. The cold section blocking agent was normal rabbit serum and the appropriate antibody at a concentration of one hundred times excess of that for the hot antibody.
After this time 10μl of radiolabelled antibody was added to the section without washing off the blocking agents.

Section v: Incubation of slides

After addition of the hot antibody the sections were incubated in humidified chambers for 3 hours with agitation after half the time had passed. The sections were then washed in PBS several times and placed in neutral buffered formalin (50:50 formalin/PBS) for 10 minutes to fix the antibody molecules cross-linked with receptors. After washes in distilled water and air-drying the slides were taped into an x-ray cassette. Having placed films on the slides in the dark room the films were developed after 96 hours. The autoradiographs were used to indicate the time that each slide needed to be exposed to emulsion. For each run of antibody there was a test run using cell pellets and a few tumour sections in order to test the iodinated antibody and other reagents.
In the preliminary studies, the length of exposure for each of the cell pellets was determined which gave the best separation of silver grains for counting. This was reproducible with each cell pellet requiring the same time of exposure. When the tumour samples were exposed in the X-ray cassettes with the cell pellets, one could match the tumours to cell pellets in terms of density of X-ray image and calculate the optimum exposure time for each tumour.

Section vi: Preparation of slides with photographic emulsion

In the dark room using the Kodak number 2 safe light filter all the slides were dipped in autoradiographic emulsion. The Kodak NTB-2 emulsion was diluted 1:1 with water at

43^{o}C and kept at this constant temperature in a water bath. When the emulsion was too cold it was too firm to dip the slides and when the temperature was too high there was silver grain transformation. The slides were allowed to dry in air and placed in racks. These were placed in airtight containers containing silica-gel in order to prevent condensation formation. They were stored at 4^{o}C for varying lengths of exposure as determined by the initial autoradiograghs and arranged in groups at times 4, 24, 48, 96 and 164 hours. The correct exposure time allowed a sufficient density of single grains for counting.

After the appropriate interval the slides were allowed to return to room temperature in the dark room and were developed by dipping in the D19 developer at a dilution of 1:1 with distilled water. This had to be maintained at a constant working temperature of 10^{o}C. In large runs of slides trays of ice were used for this and the temperature constantly monitored. The slides were dipped in developer for 4 minutes then placed but not agitated in distilled water at 10^{o}C for 1 minute. The slides were then placed in fixer at room temperature for 5 minutes and finally washed in distilled water.

The sections were then washed further and placed in Scott's tap water for 1 minute prior to counterstaining with 0.1% safranin for 30 seconds. After washing, the slides were fixed and mounted ready for counting. A safranin counter stain allowed areas of tumour to be identified in the slides and the silver grains were counted within areas of marked tumour. This is illustrated in Figure 16.

Section vii: Silver grain counting

The system used to count the grains was a semi-automated counter, the Joyce-Loebl MiniMagiScan attached to an Olympus BH-2 microscope. The microscope and counter were calibrated to determine the light conditions and appropriate thresholds for detection of the grains and the default settings of the system established and stored in the programme for each session at the counter. For each slide the magnification was set at 40x and the image of the slide captured by the image analysis system to the monitor.

The control cell pellets were counted as full fields of tumour cells when looking at a high power field. In the tumour specimens the area of tumour was identified and marked. Filters excluded the effect of the counter-stain and the counter determined the number of silver grains in the marked area and noted the area counted. The cold sections were used to determine the background activity, which was deducted per unit area. In each section this process was repeated for at least ten screens and the data stored in the system. Prior to counting the tumour sections, several test runs of cell pellet sections were used to ensure the system settings were optimal and the results reproducible.

The automated counting system worked by measuring the optical density of each screen pixel in the captured image (the screen was divided into 512 pixels of 2.54×10^{-8} mm^2 horizontally and 512 vertically). The grains were seen as dense objects on a light screen with the red filter enhancing the effect by excluding the safranin counter stain and at high power the stain and grains were in a different focal plane. The system identified each pixel with a density greater than the preset threshold and then divided the positive pixels into discreet objects. The number of objects counted was the grain count. All touching pixels are counted as one object but since the grains were not all focused in the same plane, the number of pixels occupied varies from one to several. There was, therefore, an optimum density for counting and it was for this reason that the slides had varying exposure times in order to ensure adequate separation of the grains such that the system would recognise them as discreet objects.

Calculations of the grains per unit area per hour of exposure were then made for tumours and cell pellet controls. The cell pellets used were of known receptor density calculated from ligand binding studies. The ratio of receptor to grain density was calculated. It had previously been found by graphical expression of the densities that the mean of the receptor : grain densities should be used as a conversion factor in order to calculate the receptor density in the areas of tumour.

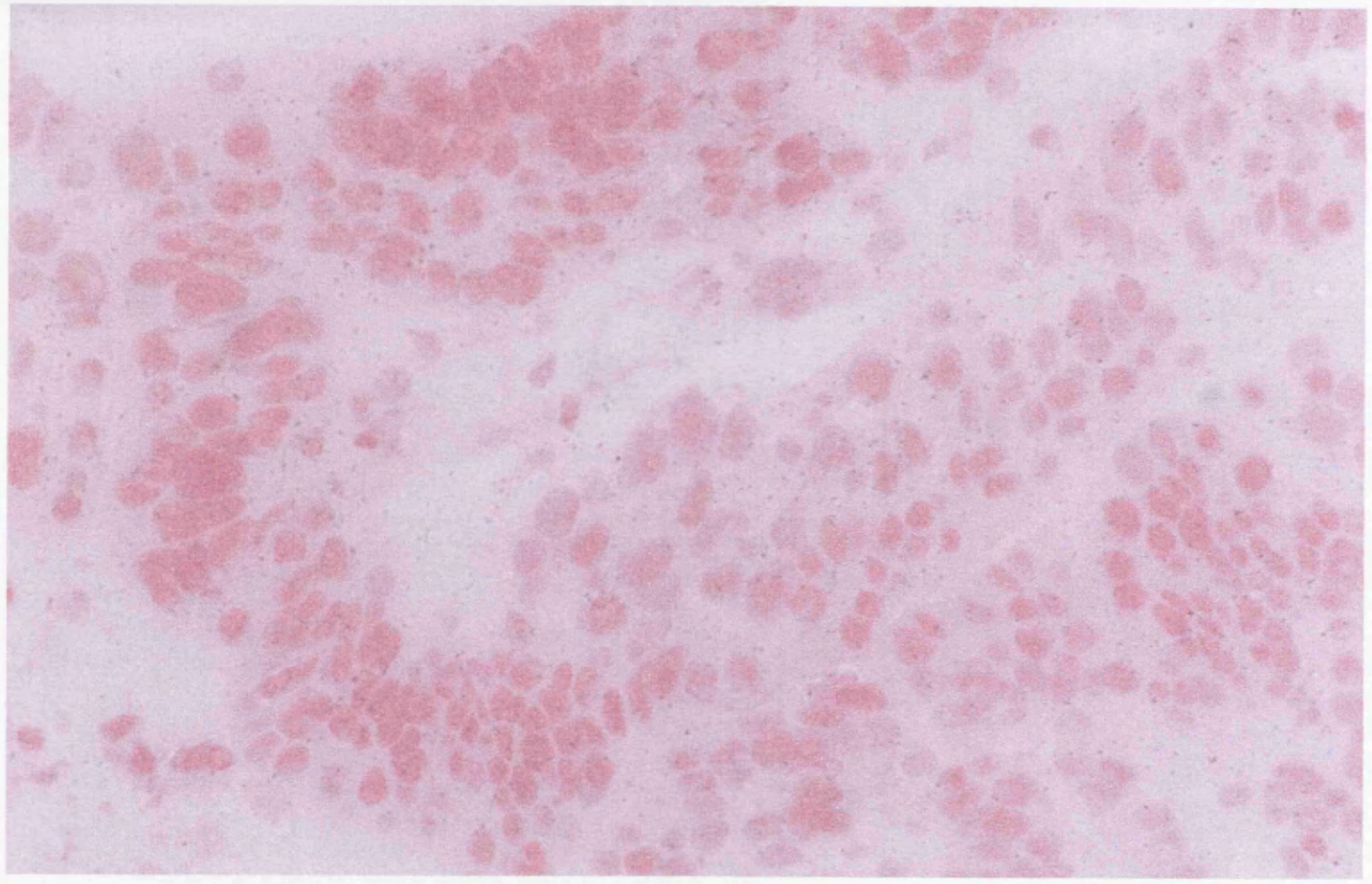


Figure 16. Photomicrograph of a gastric tumour section following the radioimmunohistochemical protocol for c-erbB-2. The nuclei are counterstained red with safranin. Note the high density of silver grains over the areas of tumour cells. Quantification is achieved by comparing the grain density of the tumour section to those of cell pellets with known receptor levels.

Section viii: Radioimmunohistochemical reagents and antibodies

a) **Antibodies**

The ICR12 monoclonal IgG2a antibody was raised to the external domain of the c-erbB-2 receptor and was a gift from C. Dean, Institute of Cancer Research, London (Styles 1990).

The EGFR1 monoclonal antibody was of the IgG2 class and recognised an epitope on the external domain of the EGF receptor (Waterfield 1982) and was available from hybridomas maintained by Dr.Brad Ozanne, Beatson Oncology Unit, Glasgow.

The anti-rat and anti-mouse immunoglobulins were standard preparations purchased from Dako Limited (High Wycombe,UK)

b) **Iodogen coated tubes** (Harlow 1988) from Dr. J. Reeves, University of Glasgow)

Iodine-125 (Amersham) to iodinate the antibodies as described

c) **Autoradiography**

Fuji autoradiograph film

NTB-2 (Kodak) autoradiographic emulsion diluted 1:1 with double deionised water

D19 Developer (2g Metol,8g Hydroquinone,90g Anhydrous Sodium Sulphite,45g Anhydrous Sodium Carbonate and 5g Potassium Bromide per litre of distilled water)

Kodak Fixer

Section ix: Iodination of Antibodies

Iodination of antibody (Harlow 1988) was performed in a hot lab specifically allocated for experiments using radioisotopes adhering to the laboratory safety instructions regarding handling of radioactive materials. Using iodogen coated tubes,15ug antibody

in 125ul PBS and 2.5uL (250uCi) Iodine-125 was placed in the tube and the reactants allowed to mix for 5 minutes. The reaction was stopped by adding 875uL 50%PBS/50%FCS with the dye xylene cyanole to make the total volume to 1mL.The reactants were then passed through an equilibrated Gel Filtration Sephradex G25 (Nap10) filtration column with PBS/FCS and the progress monitored using the dye. The iodinated antibody which passes through the column more quickly was collected and then aliquoted. The iodinated antibody was stored at -20^{o}C in lead pots and defrosted only once thereafter. Free iodine should be retained within the column with the xylene cyanole. The iodogen tube, hot tops and filtration column were disposed appropriately. Aliquots of the radiolabelled antibody were then diluted and placed in scintillation tubes and the activity read in the gamma counter. From this the amount of incorporated activity was detected per unit volume. It was estimated that around 25% of the label was incorporated by this method. The total antibody which was added to each section was 50ng with an activity of 50,000cpm for c-erbB-2 and 200,000cpm for EGFr. The hot antibody was diluted with unlabelled antibody to the required activity and weight per volume.

Section x: Method of Ligand Binding

There were two methods of ligand binding studies used with the tumour cell lines.

C-erbB-2

In developing the quantitative radioimmunohistochemistry for c-erbB-2 expression, tumour cell lines N87, BT 474, MDA-MB 361, ZR 75 and MCF 7 were used. Frozen cell pellets were prepared and sectioned as in the RIHC studies. The c-erbB-2 antibody, ICR 12, was labelled with radiolabelled iodine-125 by the iodogen method. Briefly, 15μg of antibody in PBS was incubated with 10MBq of ^{125}I for 5 minutes in the presence of 50μg iodogen in a total volume of 125uL.The reaction was stopped using 875uL 50% rabbit serum in PBS and the labelled antibody separated from free iodine using a gel filtration column.

Cell pellet sections were rehydrated with 50uL rabbit serum in PBS for 5 minutes and radiolabelled antibody added to each section in concentrations of 2pM to 5nM diluted in rabbit serum/PBS. The control sections assessed non-specific binding with addition of a 200 excess of unlabelled antibody. After incubation at 22^{o}C in a humidified chamber for 4 hours the sections were washed in PBS, dried and solubilised in 0.5% SDS and transferred to gamma counting vials for measurement of bound iodinated antibody. The binding data was analysed with the LIGAND software programme(Munson 1980).

From each of the binding assays 8 sections were used to measure the area by Imaging Research Inc. MCID image analysis system and then the sections dissolved in 0.5M NaOH and the protein content measured using the Bio-Rad reagent and method (Bradford 1976). From these binding experiments the cell number, mean section area and protein content were estimated. This allowed calculation of the receptor number per area used in the calculation of the conversion factor for the silver grain count in the RIHC experiments.

EGFr

In the binding studies using antibody to EGFr the radiolabelled antibody was prepared in the same way as in the RIHC studies above. Cell pellets from the cell lines A431,BT20, EJ, SKBR3 and ZR75 were selected and immersed in a buffer solution (Leake 1993) of 0.01M K_2HPO_4, 0.01M KH_2PO_4, 0.0015M Disodium EDTA, 0.003M NaN_3, 0.01M monothioglycerol and 10% glycerol (v/v).The specimens were homogenated in the Ultraturrax at maximum speed and then centrifuged at 500 rpm for 5 minutes at 4^{o}C. The resulting supernatant of membrane and cytoplasmic elements was further centrifuged at 15,000 rpm for 30 seconds and the resulting pellet resuspended in 0.02M K_2HPO_4, 0.02M KH_2PO_4, 0.15M NaCl and 70ug / ml Bacitracin (Koenders 1991).

After protein estimation of the resulting membrane suspension (Bradford 1976), it was diluted with buffer to 1.1mg of protein/ml and aloquated. Radiolabelled antibody was added in concentrations from 0.72 to 12nM with an excess of unlabelled antibody in the control tubes to a final concentration of 150nM.After incubation at 4^{o}C for 16 to 20 hours HAP slurry (Benradd 1990) (hydroxy apatite powder : Bacitracin containing buffer at a

ratio of 2:3) was added to each tube and incubated for a further hour. HAP absorbs the EGFr protein and therefore separates EGFr bound ^{125}I-EGF from the unbound ^{125}I-EGF. The HAP slurry was centrifuged at 800 rpm for 2 minutes and the supernatant discarded. After washing the pellet with further buffer and centrifuging at 800 rpm for a further 2 minutes the resulting pellet was washed and then placed with the cut tip of the eppendorf in to pony vials and into a gamma counter.

The binding data was analysed using the LIGAND programme (Munson 1980).

Section xi: Method of Immunohistochemistry

Tumour sections were cut and stored as described above. Two parallel sections per slide for control and antibody testing were used. Each section was initially treated with 100% acetone for fixation then washed three times in PBS. Slides were then incubated with 50% egg white for 30 minutes in order to block endogenous biotin (Reeves 1994). They were again washed in PBS and further non specific binding was inhibited with a serum blocking solution containing 25% normal human serum, 25% normal rabbit serum, 20% biotin with PBS and incubated for 10 minutes. This blocking solution was then aspirated and replaced by the antibody and control solutions. For c-erbB-2 the anti-c-erbB-2 antibody was diluted and used at a concentration of 1ug/ml and the control sections incubated with the control antibody non-immune rat IgG at the same concentration. The anti-EGFR and control antibody, IgG2b were also used at a concentration of 1ug/ml. After two hours of incubation in humidified chambers the sections were washed carefully with PBS, ensuring that there was no spillage of antibody onto control slides. The sections were then incubated with the secondary antibody for thirty minutes being biotinylated anti-rat immunoglobulin for c-erbB-2 and anti-mouse for EGFR. The dilution was 1 in 400. Sections were again washed in PBS and the streptavidin biotinylated peroxidase complex added to the sections for thirty minutes. Following washing in PBS the peroxidase signal was developed using a 10 minute exposure to Nickel DAB. This produced a black precipitate and the slides were

counter-stained by red staining safranin at a dilution of 0.1%.The slides were then dehydrated and mounted.

Immunohistochemistry scoring

The scoring system used for the immunohistochemistry was developed in order to achieve a more structured and reproduceable method of describing immunohistochemical staining rather than simply positive or negative. In each section the area of tumour was identified. Within the tumour area, the level of staining was graded as 1,2 or 3 with 3 being the most intense and the percentage of area stained at each intensity was noted. The percentage was then multiplied by the grade and the three scores added together. The maximum score being 100% at grade 3 equalling 300.The tumours were all scored in this way and a random selection scored by a second observer to assess inter-observer variability. A high scoring section is demonstrated in Figure 17.

Section xii: Immunohistochemistry reagents and antibodies

a) **Antibodies**

EGFR1 and ICR12 as described previously

b) **Blocking solutions**

i)Egg White (50:50 egg white and PBS mixed and spun at 2000rpm to remove albumin)

ii)Rabbit and Foetal Calf serum (purchased from Sigma) Human Serum prepared in our laboratory.

iii)Biotin solution (20mg biotin dissolved in 1ml Dimethyl sulphoxide with 19mls PBS added and sodium azide to 0.05%)

c) **Streptavidin biotin peroxidase complex** prepared as for the avidin-biotin peroxidase complex described by Hsu et el (1981) except that N-biotinyl-e-aminocaproic acid N-hydroxysuccinimide ester incorporating a spacer arm was used as the biotinylating reagent.

d) **Nickel DAB**(0.07% NiCl 2 6H2O , 0.025% diaminobenzidine tetrahydrochloride and 0.01%H2O2).

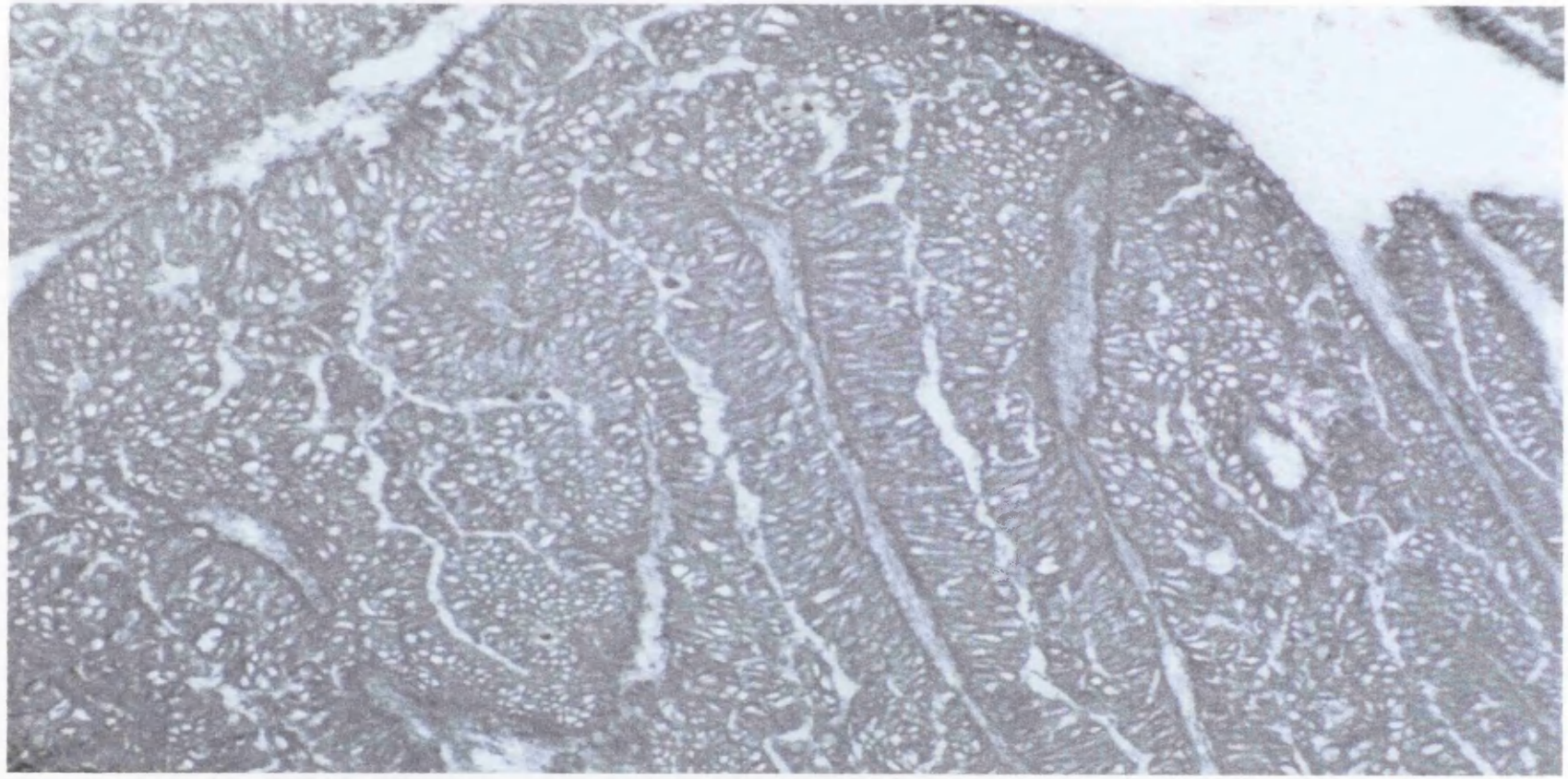


Figure 17. Photomicrograph of a gastric tumour section following the immunohistochemistry protocol for c-erbB-2. The cell membranes are darkly stained with Nickel-DAB and areas of gastric cancer cells are identified. The slide is scored using a semi quantitative system which uses the intensity and extent of staining in a tissue section.

Section xiii: Method of Fluorescence in-situ hybridisation (FISH)

FISH is a technique which uses specific proto-oncogene sequences as probes to enable the detection of the amplified copy number on a cell by cell basis. The labelled probes are detected by a complex of antibodies immunostained with a fluorescent.

Overexpression of c-erbB-2 in tumour cells may be related to changes in the regulation of gene transcription but the most common method is amplification of the c-erbB-2 gene on chromosome 17q12-21.32. Most publications have measured gene amplification by Southern blot hybridisation but although valuable in providing information about genetic alterations which underlie the development of solid tumours there are disadvantages. The spatial relationship between cells is destroyed and there is contamination of the DNA analysis by non-specific cells such as non-malignant or stromal cells during the preparation. The recently introduced technique of fluorescence in situ hybridisation (FISH) allows the detection of the amplified copy number for each cell but preserving the spatial relationship of the cells (Gray 1992). This provides information on amplification which can be correlated with histological information and immunostaining. FISH is therefore ideal to investigate the heterogeneity and histological pattern of c-erbB-2 in gastric cancer.

During the time of the experimental work for this thesis, FISH was a recently developed concept. The protocol which is detailed below was developed by Dr. Nicol Keith at the Beatson Research Laboratories, University of Glasgow and under his close direction I analysed the gastric cancer specimens and together analysed the images to determine those which were amplified for c-erbB-2.

Sections of gastric tumour which had been cut with those for IHC and RIHC were removed from the -30^{o}C freezer, allowed to come to room temperature and removed from the air tight packaging. The sections were digested with pepsin (0.02% pepsin in 10mM hydrochloric acid.) for ten minutes at 37^{o}C then washed five times in distilled

water followed by five washes of PBS (Hopman 1992) They were fixed in Streck tissue fixative (Streck Laboratories Inc. Omaha N.E.) and dehydrated with ethanol.

Using 70% formamide in 2xSSC sections were denatured at 75^{o}C for three minutes, using the Omnislide modular system (Hybaid,UK).The erbB-2 sequences were probed with a commercial erbB-2 probe (Oncor,Gaithesburg,M.D.) and hybridisation was carried out overnight at 37^{o}C. After hybridisation the slides were washed in 50% formamide,1xSSC at 42^{o}C for 20 minutes and in 2xSSC for 20 minutes. Prior to immunocytochemical detection (Kallioniemi 1992) the slides were blocked in 4xSSC-TB (4xSSC 0.05% Tween and 0.5% Boehringer blocking agent) for 30 minutes.

For two colour FISH the first detection layer consisted of FITC-avidin (Vector,Ca) in 4x SSC-TB for 45 minutes at room temperature then washed in 4xSSC-t for 10 minutes and transferred to PN buffer.(PN=0.1M NaH_2PO_4 and 0.1M Na_2HPO_4 at pH8 with 0.05% Tween with PN-B containing 0.5% Boehringer blocking agent)

The second detection layer consisted of biotinylated anti-avidin D (Vector) and sheep anti-digoxigenin in PN-B for 45 minutes. After further washing in PN buffer the third detection layer of FITC-avidin and anti-sheep Cy5 in PN-B for 45 minutes. After further PN washes the slides were counterstained with propidium iodide (0.4ug/ml) and mounted in anti-fade medium (Vectashield,Vector).

Fluorescence was analysed on Bio-rad (Richmond,C.A.) MRC-600 laser scanning confocal microscope equipped with krypton argon laser. Images were processed using edge enhancement algorithms to aid definition of nuclear boundaries and hybridisation sites. Images were merged using Comos and Nexus software (Bio-Rad) and optimal colour balance of the pseudo colour images was achieved using image processing software (Photomagic,Micrografx,Arapaho Richardson,Texas,USA). Final figures were annotated in, and directly printed from Micrografx Draw (Micrografx) using a dye sublimation printer (Colour Ease, Kodak, Harrow, UK)(Murphy 1995).

Method of Proliferation studies and Western Blotting using IGF-1

Section i: Tissue Culture

Cell cultures of primary gastric adenocarcinoma cell lines were used in the proliferation studies and in the biochemical analysis using Western blotting. The cell lines were MKN45 (ATCC), a poorly differentiated adenocarcinoma cell line with known overexpression of EGFr (Ochai 1988) and N87 (kindly provided by Richter King), an adenocarcinoma cell line which is known to overexpress c-erbB-2. The cell lines were routinely cultured in the growth medium RPMI 1640 (Gibco BRL) containing 10% heat inactivated foetal calf serum and supplemented with 2mM L-glutamine, 100U penicillin and 100ug streptomycin/ ml. They were incubated at 37°C in an atmosphere of 5% CO_2 and were routinely tested and throughout the period of study remained negative for mycoplasma contamination.

Section ii: Cell growth determination

Cells were plated in 24 well plates at a density of 2.5 to 5.0 x 10^4 for MKN45 and 3.0 to 4.0 x 10^4 for N87.These were determined after initial experiments had used a range of plating densities from 2.0 to 5.0 x 10^4 and the cell numbers were counted for up to ten days. The cell plating density was varied over time depending on the growth of the cultured cells in check proliferation studies in non-drug containing medium.

After twenty four hours the medium in each well was removed and the wells washed twice with PBS (phosphate buffered saline).The medium was replaced with RPMI 1640 supplemented as before but free of foetal calf serum. Cells were supplemented with 10nM hydrocortisone, 10ug/ml transferrin and 30nM sodium selenite (HTS). Initially the medium was compared to cells grown in medium also containing insulin 5ug/ml (HITS) to show there was inhibition of growth in the insulin free group but that adequate growth

was clearly seen. The insulin supplemented medium was also used to compare growth of cells in this medium with those supplemented by IGF-I.

After the cells were incubated for a further twenty four hours the medium was changed and this was defined as day zero. The culture medium was changed every two days and the selected plates were prepared for counting. On the day of counting the cells were washed with two washes of PBS and incubated with 0.025% trypsin in 0.02M EDTA. An aliquot was taken and diluted in 0.9% sterile saline and the cells counted using a ZM1 electronic cell counter(Coulter Electronics).

Cells were incubated in serum free medium containing IGF-I (Santa Cruz Biotechnology Inc. California) at concentrations of 0.1nm to 100nM.The optimum concentration for subsequent experiments was found to be 10nM and this was used in the inhibitory drug studies.

The inhibitory drug used was RG13022 which is a tyrophostin. These compounds were developed to specifically inhibit EGFr tyrosine kinase (Yaish 1988) and in particular EGFr autophosphorylation (Reddy 1992). RG13022 had been shown to have a cytostatic and reversible inhibition of the growth of breast cancer cell growth (Reddy 1992), squamous cell carcinoma in nude mice (Yoneda 1991) and pancreatic cell growth (Gillespie 1993). In the human gastric cancer AGS cell line, RG13022 produced an antiproliferative effect and a reversible dose dependent antiproliferative effect on MKN45 and N87 cells stimulated with EGF as previously observed in our laboratory (McLaughlin 1995). As yet there have been no experiments to determine the activity of the tyrophostins with cells stimulated by IGF-I. To examine the effect of RG13022 a stock solution of 40nM was prepared in dimethyl sulphoxide and added to culture media (0.1%v/v) on day 0 and thereafter at two day intervals. DMSO was added to all control wells (0.1% v/v).

Western Blotting

Section iii: Cell Harvesting

In the preparation of cell lysates for Western blotting the cells were grown to 70% confluence in 25cm^2 flasks with RPMI 1640 with 5% foetal calf serum and supplemented as in the medium for cell proliferation. After 24 hours cells were washed twice with PBS and the medium changed to the serum free medium as previously described. After incubation for a further 24 hours in serum free medium to ensure there was no residual effect of serum growth factors, drug and IGF-I were added to the flasks. From the proliferation studies it was evident that the optimum concentration of IGF-I for growth in culture of these cell lines was 10nM.Of the twelve flasks used in each experiment RG13022 (in 0.1% DMSO) was added to 4 flasks at a concentration of 100uM and to 4 flasks at a concentration of 10uM with corresponding controls using DMSO 0.1%. After 24 hours incubation the medium was removed, the cells washed in PBS and the medium replaced with the drug containing medium with the addition of 10nM IGF-I to 6 flasks each with a control flask. The cells were harvested after a further incubation of 5 minutes for N87 and 10 minutes for MKN45.

Medium was removed and the cells washed with chilled PBS and 400uL of lysis buffer added (50mM Hepes pH 7.4, 1% Triton X-100, 150mM NaCl, 5mM EGTA, 100ug/ml PMSF, 10ug/ml aprotonin, 100ug/ml benzamidine, 5ug/ml leupeptin and 100um Na_3VO_4).Cells were harvested with a cell scraper and transferred to 1.5ml eppendorfs which were rotated in the cold room for 20 minutes. After centrifugation at 15,000 g for 10 minutes at 4^{o}C, the supernatant was removed, aliquoted and stored at -70^{o}C.

Protein concentration within the cell lysates was determined using the BioRad dye reagent method and 75 ug protein used in the protein blotting for both cell lines.

The N87 and MKN45 cell lysates were denatured by boiling for 4 minutes in an equal volume of Laemmli buffer (0.125 M TrisCl pH 6.7, 2% SDS, 10% glycerol, 2.5% mercaptoethanol) with the samples made to an equal volume with lysis buffer.

The SDS-PAGE gels were prepared with a 4% stacker (30% acrylamide /0.8 bisacrylamide solution, dH_2O, Ammonium persulphate, TEMED, 50% glycerol and stacker buffer-0.5M Tris/HCl pH 6.7 and SDS in solutions of 0.4% and 10%) and a 10% resolving gel (30% acrylamide solution / 0.8 bisacrylamide , dH_2O, Ammonium persulphate, TEMED, 50% glycerol and resolving buffer - 1.5M Tris/HCl pH 8.9 and SDS in solutions of 0.4% and 10%).

After removing the stacking comb the wells were washed with running buffer and the proteins loaded and run through the stacker at 30mA and through the resolver at 10mA overnight. BioRad molecular weight markers were run with the protein lysates.

Section iv: Blotting

The proteins were transferred to PVDF membranes. They were soaked in methanol for 1 minute and then washed in distilled water for 5 to 10 minutes and then soaked in transfer buffer (48mM Tris, 39mM glycine, 0.037% SDS) for 20 minutes. After removing the gel from the plates it was placed in transfer buffer for 20 minutes. Six sheets of Whatman 3M paper were soaked in transfer buffer. The gel was placed on 3 sheets followed by the membrane and a further 3 sheets of filter paper. This complex of gel and membrane was placed in a BioRad SemiDry blotting apparatus and transfer of proteins performed at 13V over 90 minutes. Coomassie staining of the protein remnants on the gels thereafter allowed inspection of protein loading.

Section v: Protein detection

Each of the membranes used with antibody to the IGF-I receptor were blocked in a solution of 5% Marvel in TTBS (0.1M Tris/HCl pH 7.4, 1.5M NaCl, Tween 20) overnight

at 4^{o}C. When using antibodies to IRS-1 the membranes were blocked overnight in 1% bovine serum albumin for the monoclonal antibody and in 5% marvel / TTBS for the polyclonal antibody. With the antiphosphotyrosine antibody (Py54) membranes were blocked overnight in a solution of 5% ovalbumin with 10% normal sheep serum.

Using the IGF-I receptor antibody at a dilution of 1 in 250 in 5% Marvel / TTBS the membranes were incubated in a hybridisation chamber at 26^{o}C for 1.5 hours. After washing three times with TTBS the membranes were incubated with the secondary antibody, a peroxidase linked anti-mouse antibody at a dilution of 1 in 1000 in 5% Marvel / TTBS for a further 1 hour. The excess was drained and the membranes placed in a dry tray where ECL solution was added and the membrane covered in the solution for one minute. Excess ECL was drained on 3M paper and the membranes wrapped in Saran-wrap before exposure to Fuji film for varying times from 10 seconds to 20 minutes.

When using the antiphosphotyrosine antibody the membranes were incubated with antibody at a dilution of 1 in 10,000 in the blocking solution for 1.5 hours and then incubated in a secondary antibody of anti-mouse antibody in 5% Marvel / TTBS for a further hour.

For the monoclonal antibody to IRS-1 the membranes were incubated in antibody at a dilution of 1 in 2500 in 1% B.S.A. for 2 hours and in secondary anti-mouse antibody at a dilution of 1 in 1000 for 1 hour. With the polyclonal antibody the membranes were incubated in antibody at a dilution of 1 in 2500 in 5% Marvel / TTBS for 2 hours then in anti-rabbit antibody at a dilution of 1 in 1000 in 5% Marvel / TTBS for 1 hour.

Chapter 9
Results of analysis of c-erbB-2 and EGFr

In this section, I present the results of EGFr and c-erbB-2 measurement. The details of a quantitative analysis using radioimmunohistochemistry in tumours are compared with normal gastric mucosa and to tissue sections from the same patients examined by conventional immunohistochemistry and FISH. Using the clinico-pathological information detailed below a comparison will be made of receptor density with recognised prognostic factors.

Of seventy five tumours which were originally studied, the results from sixty seven were used in the final analysis. The other specimens were rejected on pathological grounds, as unsuitable. One group comprised of combination histology (adenosquamous), lymphoma and tumours in which the pathology on review suggested a lower oesophageal rather than gastric primary site.

Section i: Descriptive data of population

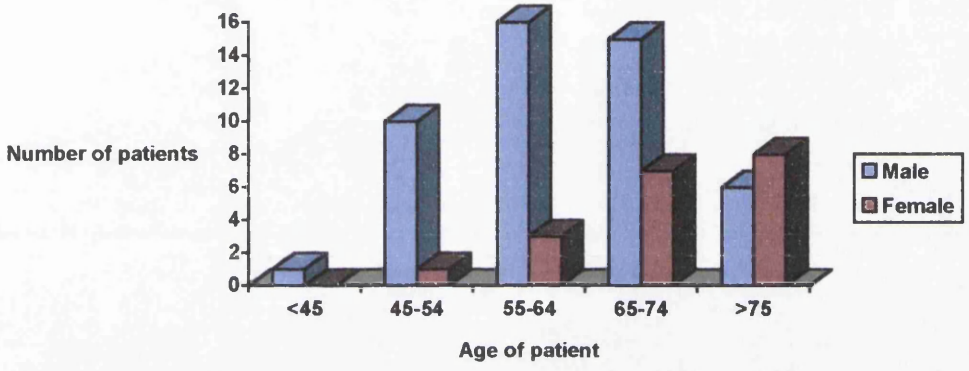
In predicting the outcome for patients with gastric cancer there are a number of clinico-pathological factors which have been used in clinical studies (Harrison 1995). Within the sample population, information on several of these indicators is available and a summary of the incidence of these is shown in Table 12 and the details in Appendix B (Table 3).

Age of patients

Age distribution is shown in Figure 18. The commonest age at presentation was within the 65-74 age group in agreement with the peak age of the hospital and population based studies previously described. The total ages range from 44 to 83 years with a median of 68 (IQ=59-72) and a male to female ratio of 2.5:1.

Males generally present at a younger age with a range of 44 to 83 years but a median of 64 (IQ=55-69) compared to females a range (52-83 years) with a median of 72 (IQ=68-80).

Figure 18 **Age and sex distribution of patients**



Location of tumour

The site of origin for each tumour was determined from the operative and pathology notes with 34%(23) of the samples from tumours of the cardia and fundus, 43%(29) from the body of the stomach and 22%(15) antral tumours. Although the antrum is still considered to be the most common site of presentation, the recent rise in proximal gastric tumours is reflected in this distribution. The tertiary referral practice of the hospital also accounts for higher proximal tumour numbers.

Differentiation of tumours

Tumours were classified using the standard description of well, moderately well and poorly differentiated adenocarcinomas (Broders 1926 classification). Although many of the tumours were also classified using the Lauren (1965) classification into intestinal,

diffuse or mixed; this was not universal and therefore unsuitable for use in subsequent analyses. There were 7.5%(6) well differentiated tumours, 25%(17) moderately well differentiated (total of well/moderately well=33%) and 67%(45) poorly differentiated.

Serosal Involvement

Of the tumours studied 92.5%(62) involved the serosa of the stomach and 7.5%(5) did not invade as far through the gastric wall.

Nodal involvement

In the majority of specimens, nodal involvement was determined as the number of nodes present and those which were positive. During the period of study the majority of surgeons did not practice extended lymph node dissections or marked nodal stations as is the more common practice now and certainly within Japanese studies. In the pathological descriptions, therefore, the position of positive nodes cannot be determined (as in the Japanese research classification) but in many cases the percentage of positive nodes is available and the level of nodal involvement by tumour is shown both as a percentage of nodes and simply the number with positive nodes. Of the 67 tumours 25%(17) had no nodes involved and 75%(50) with positive nodes of which 33%(22) had less than 50% and 34%(23) with more than 50%.In 7.5%(5) cases the percentage was unknown.

Liver metastases

Of 11 patients with metastatic disease, 3 had liver metastases at presentation and a further 8 patients developed liver metastases in the period of follow-up.

Clinico-pathological prognostic factors in gastric cancer

Table 12

		No.	(%)
Serosal involvement	positive	62	(92.5)
n=67	negative	5	(7.5)
nodal involvement	positive	51	(77.3)
n=66	negative	15	(22.7)
% nodal involvement	0	17	(27.4)
n=62	<50	24	(38.7)
	>50	21	(33.8)
histological grade	well	5	(7.5)
n=67	moderate	17	(25.4)
	poor	45	(67.2)
	well/moderate	22	(32.8)
tumour site	cardia	24	(35.8)
n=67	body	29	(43.3)
	antrum	14	(20.9)
liver metastases	positive	11	(16.4)
n=67	negative	56	(83.6)

Section ii: Calculation of receptor density

EGFr and C-erbB-2 receptor density estimations were made on these tumours using radioimmunohistochemistry and compared with conventional immunohistochemical scoring.

Quantitative estimation of c-erbB-2 receptor density by radioimmunohistochemistry

In RIHC the radiolabelled antibody binds in competition with unlabelled antibody to the receptor. Overnight autoradiography is carried out to determine the activity level in each section which is demonstrated on X-ray films with the darkest exposure correlating with the highest activity. This information determines the length of time that each slide should by exposed to photographic emulsion in order to produce a density of single silver grains optimal for image analysis. Using the information from exposure of cell pellets with varying receptor and therefore silver grain densities, the activity in tumour slides is compared to cell pellet slides and the time calculated.

When all the slides have been examined and counted there was a grain count for two hot slides and one control slide for each tumour section and cell pellet. The area which has been counted in each slide was calculated by the computer assisted counting system whilst counting the silver grains in a particular area. In addition, the length of time of exposure and a number corresponding to the RUN or group of cell pellets examined with that batch of tumour was recorded. It was possible to calculate the grain density for each section by taking the average count in the hot slides and subtracting the grain count from the control slide to exclude background activity. Thereafter, the count was converted to number of grains per area counted and per hour as determined by exposure time.

Standardisation of results for each set of test sections is achieved by concomitant running of sections of cell pellets. These are formed from cell lines with known receptor

densities determined by ligand binding studies (Figures 19 and Table 13). Using RIHC, a grain count per area per unit time was calculated for each cell line and for each tumour. When one excludes the highest and lowest expressing pellets in the set of cell pellets which were run with each tumour batch, the ratio of receptor number over grain count per unit area by RIHC produces a linear graphical relationship. By incorporating this scale into each tumour batch and with the receptor number in each cell line known, it is possible to determine the number of receptors in terms of silver grains per unit area per hour of emulsion exposure. A conversion factor for receptor number per unit area is calculated by averaging the ratios for cell pellets. This is applied to grain counts for the tumour biopsies in the same batch and allows calculation of receptor numbers.

In Appendix B (Table 1) the grain counts for each of the tumours is shown with the corresponding control slide and the area of each tumour section. The exposure time is also shown to calculate the grain count per area per hour. The group or run in which each tumour was incubated is identified in order to match the cell pellet counts used to convert the raw counts to receptor number. In each of the three runs of staining by RIHC, the five cell pellets were assayed simultaneously with the tumours.

Table 13 **Results of c-erbB-2 ligand binding analysis on sections**

	receptors / cell ($x10^3$)	receptor /ug protein ($x10^6$)	**receptors/mm2 ($x10^6$)**
N 87	4390	9480	**4880**
BT474	725	1036	**581**
MDA-MB-361	35.7	160	**131**
ZR 75	7.48	16.9	**10.9**
MCF 7	1.33	3.5	**2.48**

(Reeves 1996)

The receptor density in this table has been calculated from ligand binding studies for the cell lines which have been used in the RIHC experiments. Figure 19 shows the linear relationship of the receptor density for the cell lines particularly BT474, MDA-MB-361 and ZR 75 which are used to calculate the converting factor.

Figure 19 **Receptor numbers from ligand binding studies**

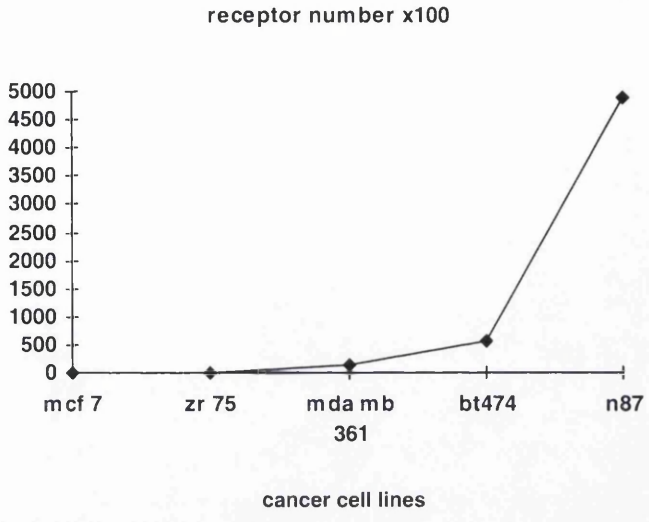


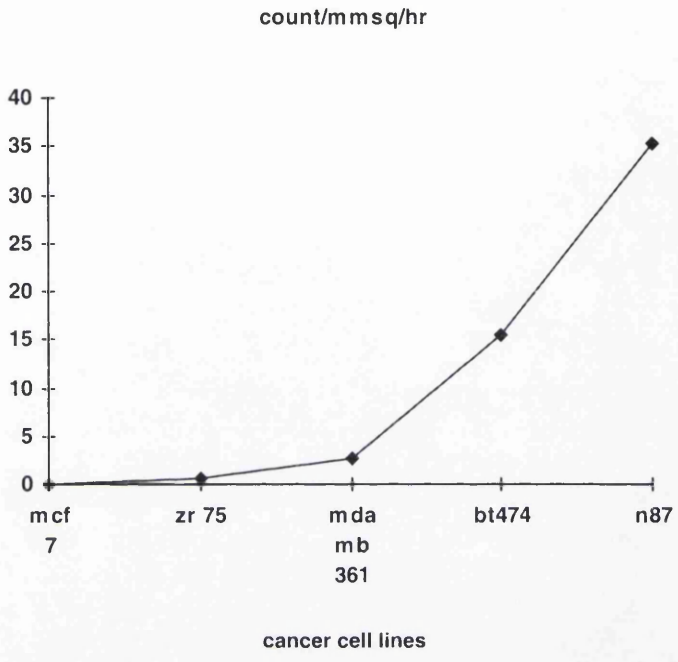
Table 14 **Grain count per mm^2 per hour for cell pellets in each run**

Cell lines	Count per mm^2 per hour			Mean	Variance Standard Deviation	95% CI
	RUN I	**RUN 2**	**RUN 3**			
N87	35.17	45.9	37.94	39.7	5.57	25.8-53.5
BT 474	15.53	27.4	21.8	21.6	5.94	6.8-36.3
MDA MB 361	2.65	2.73	1.59	2.3	0.63	0.7-3.9
ZR 75	0.63	0.22	0.104	0.32	0.28	-0.37-1
MCF 7	0.04	0.02	0.035	0.03	0.0104	0.00581-0.0575

The grain count per mm^2 per hour for the cell lines is shown in Table 14 for the three batches of tumour specimens which were used. Each tumour specimen is numbered to correspond to a 'RUN' of cell lines.

In Figure 20 the linear relationship of the grain count for each cell line is similar to the receptor density count from the ligand binding studies and is consistently found in Runs 2 and 3 (detailed in Appendix B, Figures 1 and 2,Table 4).

Figure 20 **Grain count (x10^2)/mm^2/hour for each of the cell lines in RUN 1**



Calculation of converting factor

The ratio of receptor number in each cell line to grain count /area/time for the three middle points was calculated as shown. The mean of the three ratios was used to convert the grain count for each tumour sample to a receptor number. The calculation for RUN 1 is shown (the calculations of the converting factors for the runs 2 and 3 are found in the Appendix B.

RUN1

BT474 receptor number / (grains/$mm^{2/}$hour) = 5.81 x 10^8 / 1553 = **374115**

MDA MB 361 receptor number / (grains/$mm^{2/}$hour) = 1.309 x 10^8 / 265 = **493963**

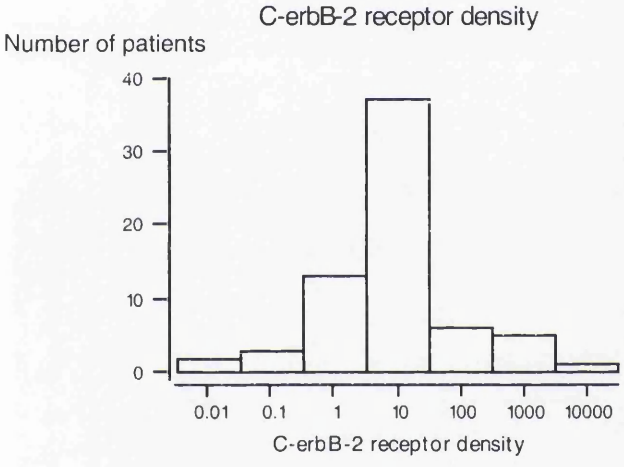
ZR 75 receptor number / (grains/$mm^{2/}$hour) = 1.09 x 10^7 / 63 = **173016**

MEAN = **347031**

Quantitative estimation of c-erbB-2 receptor density by radioimmunohistochemistry

The distribution of receptor numbers for c-erbB-2 is shown in Figure 21. The range of values is from 0 to 3.641 x 10^9 with a median of 8.5 x 10^6 (IQ = 2.9 to 23.5 x 10^6) and for ease of interpretation, the distribution has been shown graphically as a logarithmic transformation of the results.

Figure 21 **Log_{10} c-erbB-2 receptor number (x10^6)**



The histogram shows number of patients on the y-axis and number of receptors (x10^6) per cell on the x-axis

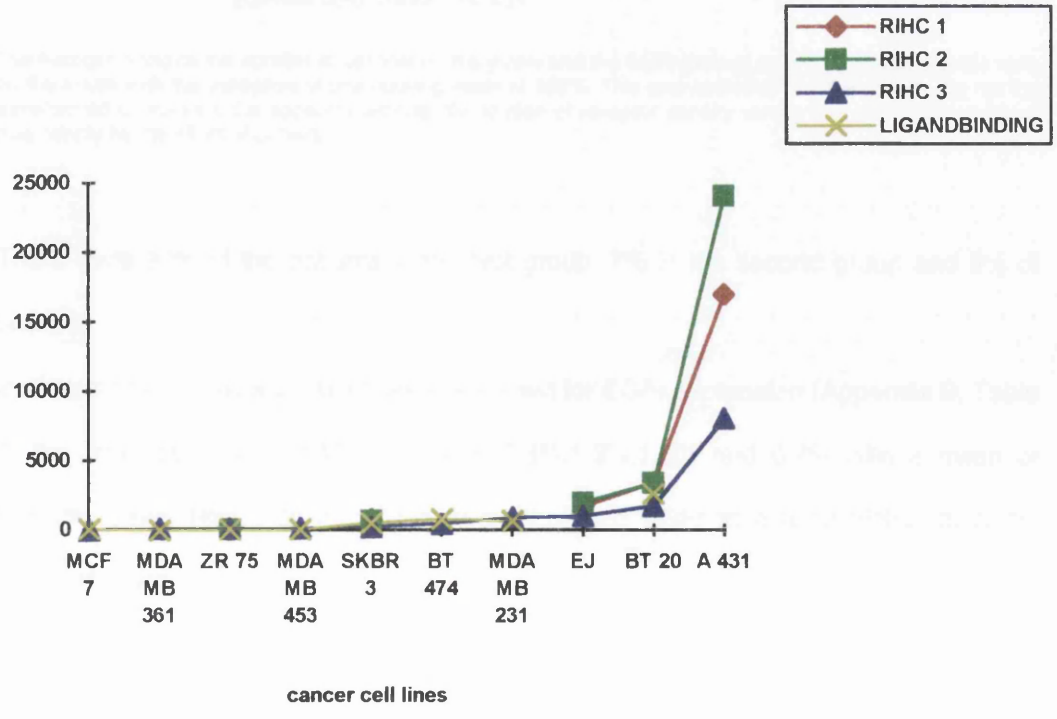
The mean of the receptor number was calculated for the samples of normal mucosa which were analysed. The grain counts are found in Appendix B (Table 4). The specimens had grain counts of 0,7.9,15.7 and 21.6 from a selection including those in which the tumours expressed the highest count and a sample of intermediate and low counts. Using the converting factor from that run (712620) the receptor numbers were calculated and the results were 0,5.63 x10^6,11.2 x10^6 and 15.4 x10^6.The mean was 8.03 x10^6. In this group of tumours 15/67 (22.4%) of tumours expressed c-erbB-2 at a level higher than the range of mucosal values.

Estimation of EGFr expression

In studies using immunohistochemistry to analyse the expression of EGFr, early experiments demonstrated the high density of receptor expression in the vulval epidermoid cell line A 431 and EGFr expression has tended to be shown in many subsequent studies as a percentage of the expression in samples of A 431 cell pellets run with the tumour samples.

In the preceding studies of receptor density of c-erbB-2 a quantitative assessment of expression was made using grain counts in a series of tumour cell line pellets correlated with receptor density from ligand binding studies. In the expression of EGFr there is a narrow spread of distribution of receptor density in the tumour population and tumour cell lines. Ligand binding studies to determine EGFr expression in a series of cell pellets were performed in conjunction with the radioimmunohistochemistry (Figure 22). The RIHC results are detailed in Table 2 in Appendix B and ligand binding data in Table 5.

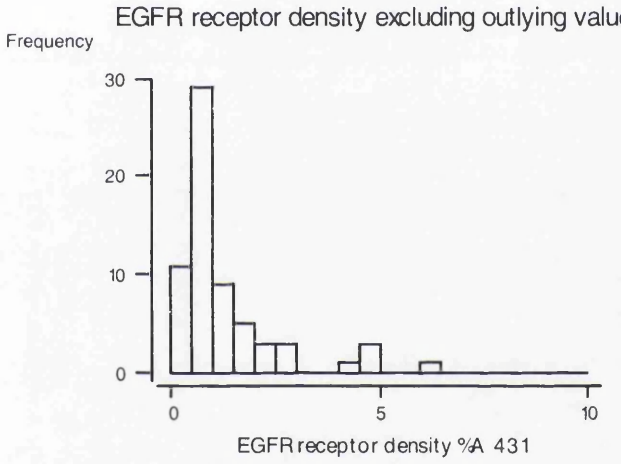
Figure 22 **Graph of grain count / mm^2 / hour for runs 1,2 and 3 displayed with the results of ligand binding studies**



These results show that the technique of radioimmunohistochemistry gives a quantitative measure of receptor density of EGFr corroborated by ligand binding studies. However, in view of the narrow distribution of cell line density we elected to express the receptor density of EGFr in the conventional method, as a percentage of the EGFr density found in A 431 cell pellets.

The distribution of EGFr is demonstrated (Figure 23) with a median of 0.9% (IQ = 0.5 to 1.6%) and a range of 0 to 106%.

Figure 23 **EGFr receptor density as a % of A431 cell line**



The histogram shows the number of patients on the y-axis and the EGFr density as a percentage of A431 cells on the x-axis with the exception of one outlying value at 106%. This was excluded and the distribution not log transformed to illustrate the apparent bimodal distribution of receptor density values but with these numbers may simply be the effect of outliers.

There were 90% of the patients in the first group, 7% in the second group and 3% of outliers.

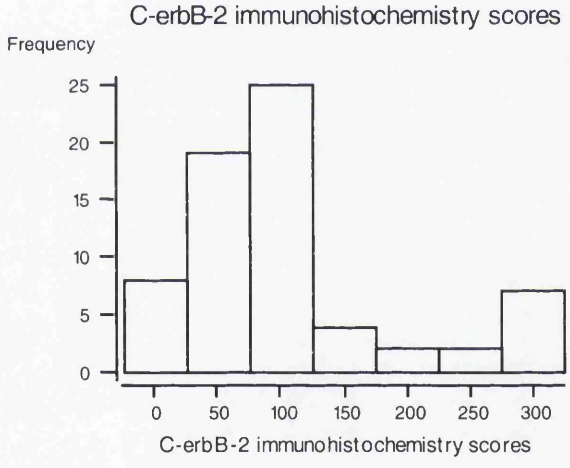
In the samples of mucosa which were analysed for EGFr expression (Appendix B, Table 4) the level as a % of A431 cells was 0.3%,1.3%,1.6% and 0.7% with a mean of 0.975%. Using RIHC, 22.4% of tumours expressed EGFr at a level higher than the mucosal range.

Comparison of c-erbB-2 and EGFr expression using radioimmunohistochemistry and immunohistochemistry

When the receptor number determined by radioimmunohistochemistry was compared to the scoring of slides using conventional immunohistochemistry the spread of results appears similar but some variable areas will be examined.

The expression of c-erbB-2 using immunohistochemistry and the scoring system described (Figure 24) shows a median level of 90 (IQ = 60 - 120) and a mucosal range of 0 -90 with a mean of 52.5.

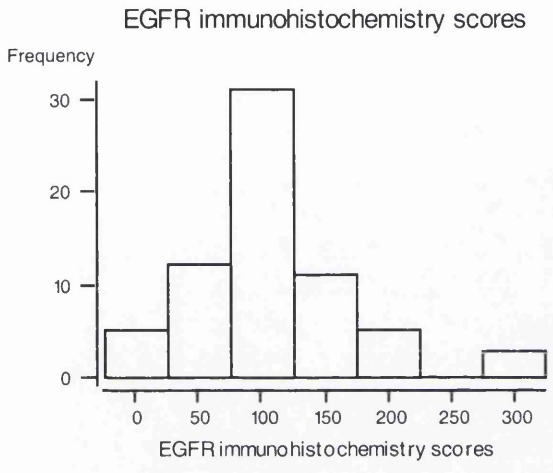
Figure 24 **Distribution of C-erbB-2 expression using immunohistochemistry**



The histoscore on the x-axis is derived from the intensity of staining (grade 1-3) multiplied by the area as a percentage (0-100%)

The expression of EGFr using immunohistochemistry (Figure 25) shows a median of 100 (IQ = 70 - 125) and a mucosal range of 40-105 with a mean of 76.25

Figure 25 **Distribution of EGFr expression using immunohistochemistry**



Those tumours which express intermediate to high levels of receptor in particular in the case of c-erbB-2 are gathered together at the same IHC score 300 rather than the wide range exposed on RIHC (1.25×10^8 to 3.61×10^9)

Using immunohistochemistry to examine EGFr the highest scores were of 300, 295 and 290 but using RIHC the same tumours differed in level of expression from 106%, 10.1% and 4.6%.

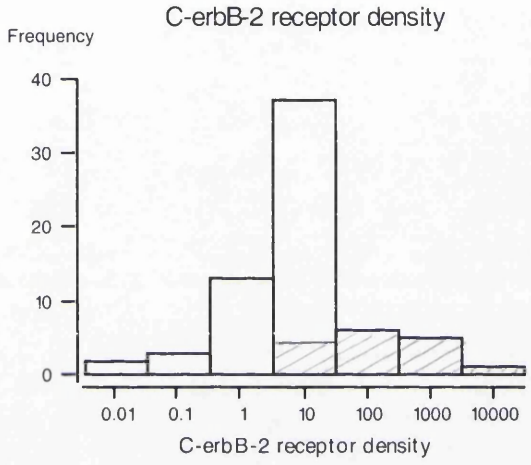
Using IHC in c-erbB-2, 29/67 (43.3%) of tumours expressed levels of receptor more than the range of the mucosal samples.

In samples tested for EGFr, 28/67 (41.8%) expressed levels above that of the mucosal sample range.

C-erbB-2 gene amplification using FISH

The tumours which showed c-erbB-2 amplification (Appendix B, Table 3) are displayed as the shaded area on Figure 26 displaying the relationship with c-erbB-2 expression using RIHC.

Figure 26 **C-erbB-2 amplification as compared with receptor density (x 10^6)**



The histogram shows the log transformed distribution of c-erbB-2 expression from RIHC with the shaded areas representing those tumours in which c-erbB-2 gene amplification has been detected by FISH.

Section iii: Relationship between c-erbB-2, EGFr expression and clinico-pathological factors

Statistical analysis of C-erbB-2, EGFr expression and pathological prognostic factors was performed using the Minitab for Windows 9.2 software © 1993 Minitab Inc. with the Kruskal-Wallis and the Mann-Whitney analysis for non-parametric data.
Life tables and Kaplan-Meier analysis were used to examine the relationship between these factors and survival using SPSS for Windows 6.1.3.©SPSS Inc. 1989-1995.
(Tables 15 and 16).

Tumour grade was expressed as well differentiated, moderately well differentiated and poorly differentiated according to Broders classification (1934). The well and moderately well group of tumours were further analysed as a group to provide comparable analysis to the differentiated and undifferentiated description of tumours in some studies.

For nodal status, receptor density is related to the presence of any positive nodes with a separate analysis of nodal status as a proportion of the number of nodes sampled. In the introduction to this thesis, data from an audit of the patients from our institution showed a statistically significant relationship to survival with more than 50% of nodes involved. Recent changes to the TNM classification of Gastric Cancer also include this information.

Table 15 **C-erbB-2 expression**

	number of patients	median c-erbB-2 expression	
Tumour differentiation			
well	5	40	
moderately well	17	17.4	h=6.99,d.f.=2 **p=0.031**
poor	45	7.48	
well/moderately well	22	19.35	h=6.99,d.f.=1 **p=0.008**
poor	45	7.48	
Serosal involvement			
negative	5	26.2	
positive	62	7.91	h=3.38,d.f.=1 **p=0.066**
Location of tumour			
cardia	23	13.3	
body	29	7.28	
antrum	15	10.1	h=2.31,d.f.=2 **p=0.316**

C-erbB-2

	number of patients	median c-erbB-2 expression	
Nodal involvement (%)			
0	17	11.8	
<50 positive nodes	22	7.69	
>50 positive nodes	23	7.64	
unknown	5		
			h=0.11,d.f.=2 **p=0.945**
Nodal involvement			
positive	17	11.8	
negative	50	7.91	
			h=0.06,d.f.=1 **p=0.807**
Liver metastases			
At presentation			
no	64	8.245	
yes	3	10.1	
			h=0.37,d.f.=1 **p=0.544**
Liver metastases			
At any time			
no	52	7.735	
yes	12	18.4	
no follow-up	3		
			h=1.37,d.f.=1 **p=0.242**

Table 16 **EGFr expression**

	number of patients	median EGFr expression (%A 431)	
Tumour differentiation			
well	5	0.9	h=4.06,d.f.=2 p=0.132
moderately well	17	1.5	h=4.08,d.f.=2 **p=0.130**
poor	45	0.7	(adjusted for ties)
well/moderately well	22	1.0	h=3.08,d.f.=1p=0.07
poor	45	0.7	h=3.10,d.f.=1 **p=0.079**
			(adjusted for ties)
Serosal involvement			
negative	5	0.9	h=0.16,d.f.=1 p=0.683
positive	62	0.85	h=0.17,d.f.=1 **p=0.684**
			(adjusted for ties)
Location of tumour			
cardia	23	0.9	
body	29	0.7	
antrum	15	1.0	h=2.05,d.f.=2 p=0.3
			h=2.06,d.f.=2 **p=0.358**
			(adjusted for ties)

EGFr expression

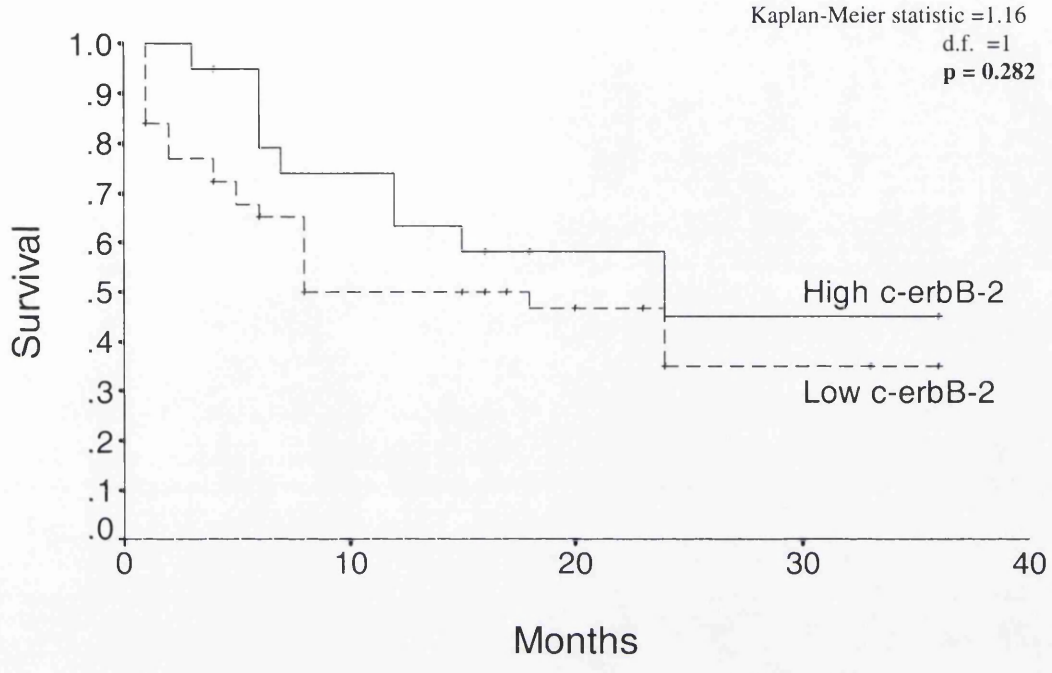
	number of patients	median EGFr expression (%A 431)	
Nodal involvement			
negative	17	0.9	
positive	50	0.8	h=0.03,d.f.=1 **p=0.857**
Nodal involvement(%)			
0	17	0.9	
<50 positive nodes	22	1.0	
>50 positive nodes	23	0.7	h=1.76,d.f.=2 p=0.415
unknown			h=1.77,d.f.=2 **p=0.413** (adjusted for ties)
Liver metastases			
At presentation			
no	64	0.9	
yes	3	0.7	h=0.14,d.f.=1p=0.39 h=0.14,d.f.=1 **p=0.704** (adjusted for ties)
At any time			
no	52	0.9	
yes	12	0.7	h=0.74,d.f.=1 p=0.39
no follow-up	3		h=0.74,d.f.=1 **p=0.388** (adjusted for ties)

Relationship between c-erbB-2 and survival

The patients in this study were followed for a period of 36 months. Survival in months and the status regarding death is detailed in Table 3, Appendix B. Life table analysis of the relationship between survival and c-erbB-2, EGFr and c-erbB-2 amplification is shown.

The level of expression of c-erbB-2 and EGFr have been grouped as low and high. This relates to the level of density (determined by radioimmunohistochemistry in this series) and grouped according to published series which suggest expression is high in 20 to 30% of tumours. Survival in those tumours expressing each receptor in the upper third of the range is compared to survival in the remainder of the tumours.

Figure 27 **Kaplan-Meier analysis of the relationship between c-erbB-2 and survival**

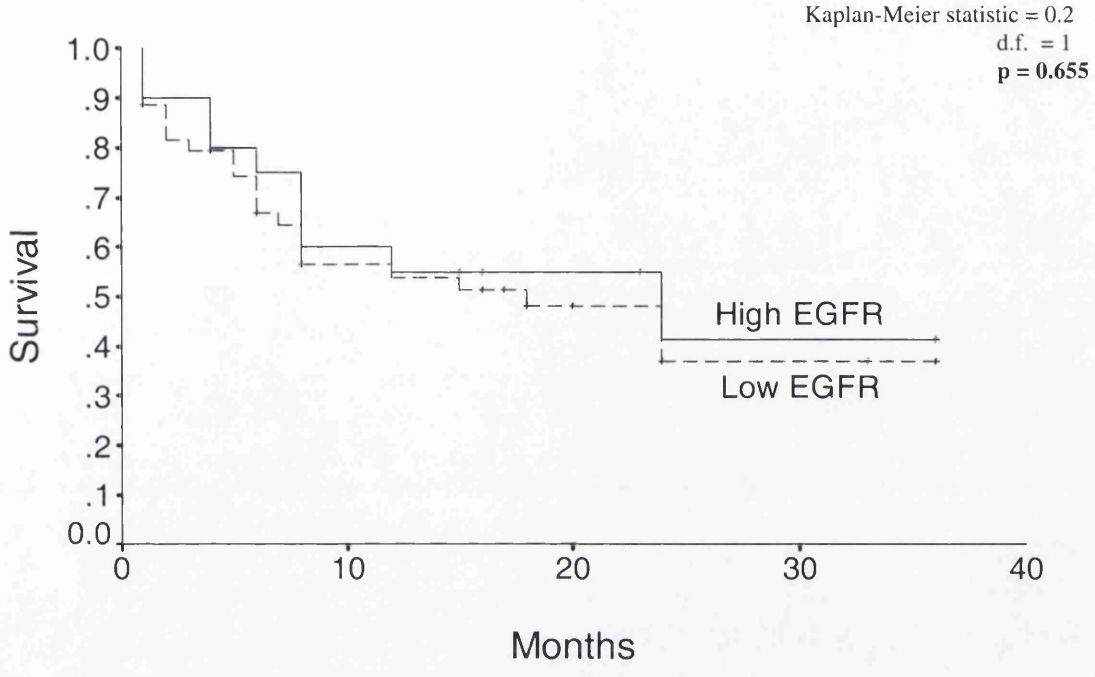


Survival is indicated on the y-axis, the scale shows the proportion of patients remaining alive at the follow-up time indicated, in months on the x-axis. The c-erbB-2 level is divided into 2 groups determined by c-erbB-2 expression.

There is a trend towards better survival in the group of patients expressing the highest level of c-erbB-2. This does not reach statistical significance.

Survival in this and subsequent analyses were not analysed for disease specific and non-specific deaths. Only 3% of deaths were considered to be unrelated to gastric cancer all of which expressed levels of EGFr and c-erbB-2 in the moderate range.

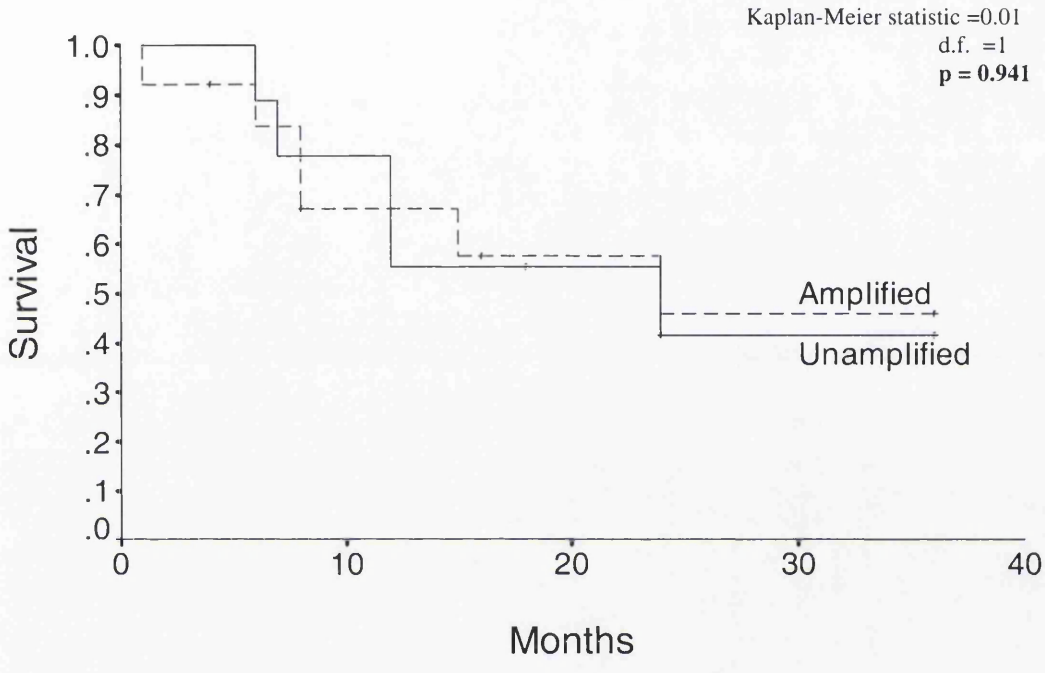
Figure 28 **Kaplan-Meier analysis of the relationship between EGFr and survival**



Survival is indicated on the y-axis, the scale shows the proportion of patients remaining alive at the follow-up time indicated, in months on the x-axis. The EGFr level is divided into 2 groups determined by EGFr expression.

Using two groups there was no statistically significant separation of the survival curves.

Figure 29 **Kaplan-Meier analysis of the relationship between c-erbB-2 gene amplification and survival**



Survival is indicated on the y-axis, the scale shows the proportion of patients remaining alive at the follow-up time indicated, in months on the x-axis. The patients are divided into groups determined by the presence of c-erbB-2 amplification.

There was no statistically significant separation of the survival curves between amplified and unamplified cases.

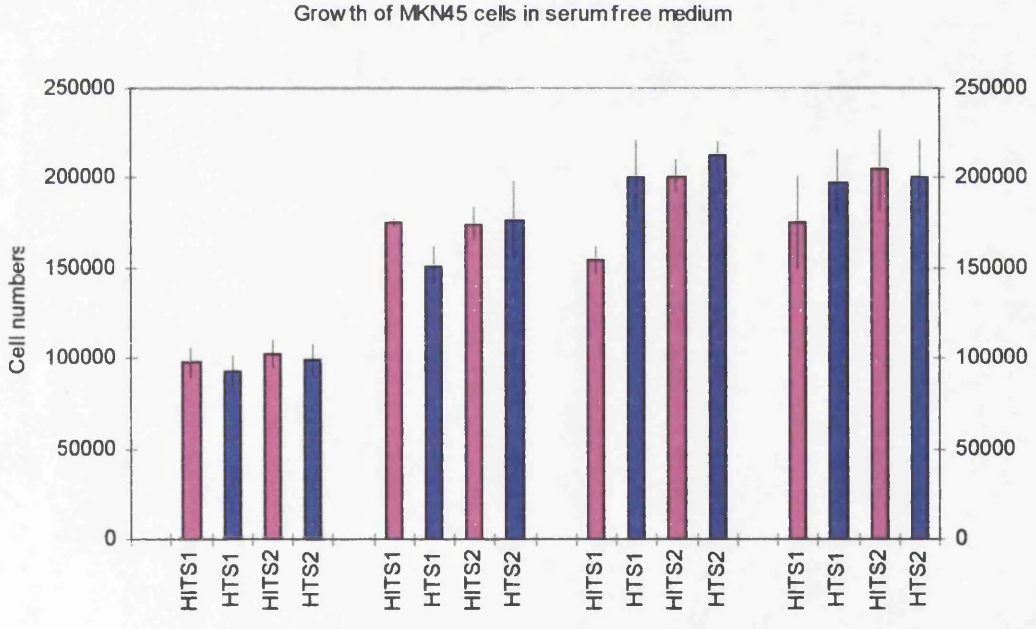
Gastric cancer cell stimulation with IGF-I and the effect of the tyrophostin RG13022.

Section i: Cell growth in serum free medium

In the experiments using IGF stimulation it was vital that the cells were observed to be growing adequately in serum-free and in particular, in insulin free medium, in order to detect stimulation with exogenous IGF-I. Serum-free medium was also required to remove the influence of IGF binding proteins contained in serum.

The results in Figures 30 and 31 and data sheet 1 in the Appendix C demonstrate that there was adequate sustained growth in both cell lines in serum free medium.

Figure 30

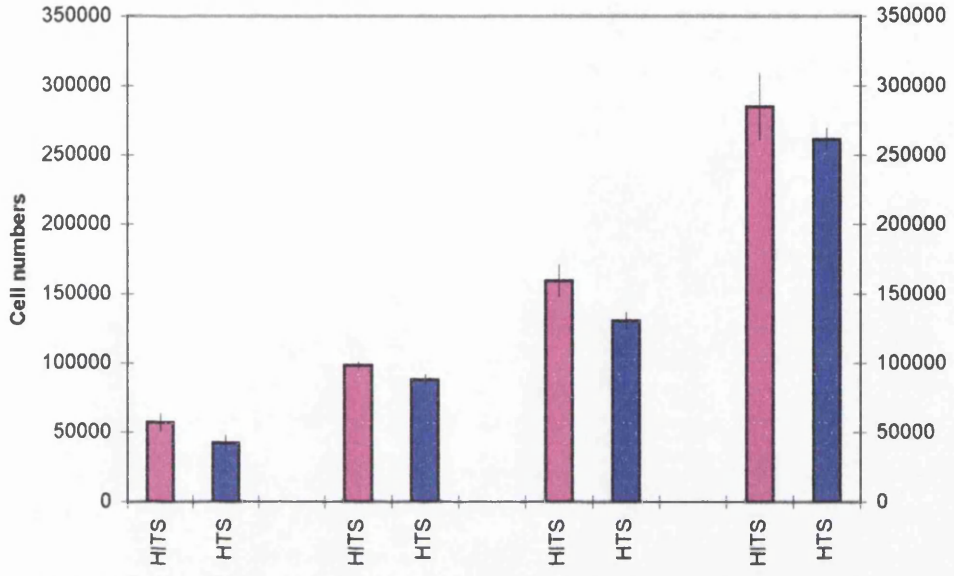


Cell doubling time
HITS= 3.36 days
HTS= 3.58 days

(50,000 cells per well)

Figure 31

Growth of N87 cells in serum free medium



DAY 0 3 6 10

Cell doubling time (40,000 cells per well)

HITS= 3.04 days

HTS= 3.4 days

In the MKN45 cells there is also growth relatively independent of culture medium but the rate of growth is slowed considerably after day 3 due to high cell numbers causing inhibition of further growth. The doubling time initially is calculated as 3.36 days and 3.58 days respectively for HITS and HTS. The high initial plating number also causes the additional cells to be shed into the medium which is discarded. Therefore, the initial plating numbers were reduced accordingly in subsequent experiments.

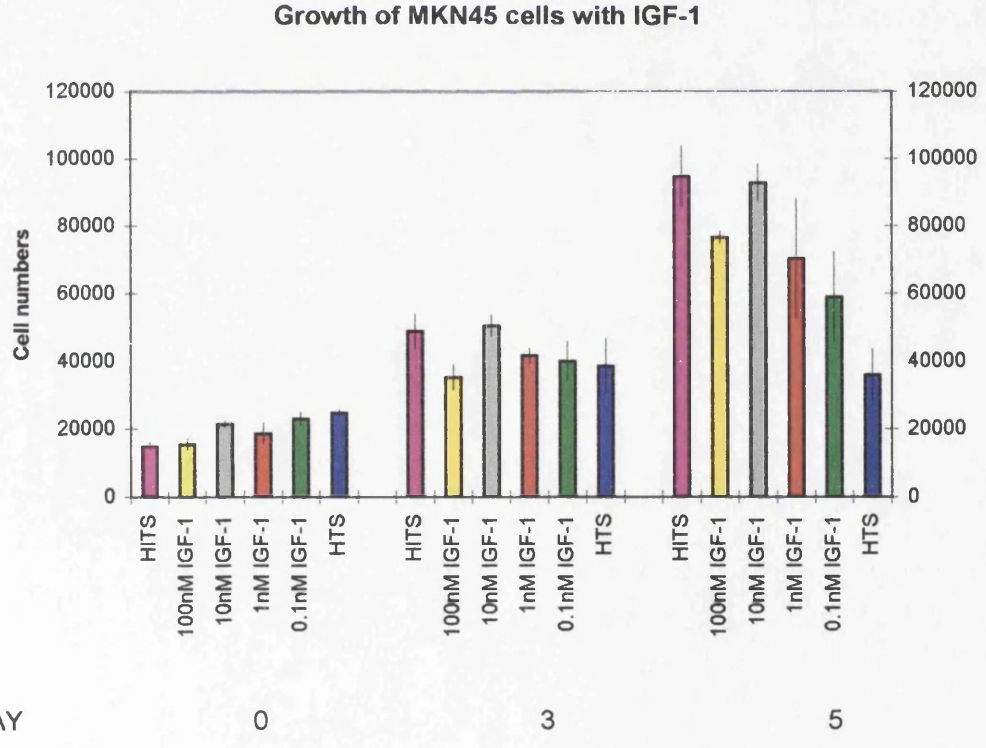
The growth of N87 was observed to be less at the periphery of the plates due to incubator conditions (Data sheet 3 in Appendix C) and this was rectified with frequent rotation of the plates and careful positioning in the incubator. There was a decrease in growth of N87 cell at day 10 in HTS alone showing that to a degree in the longer term these cells were more serum dependent. The doubling time for N87 cells in HITS and HTS was 3.04 days and 3.4 days respectively (Figure 31 and Data sheet 2 in Appendix C).

Section ii: Cell stimulation with IGF- I

To determine the most appropriate dose of IGF-I to stimulate drug treated cells, they were incubated with IGF-I in doses from 0.1nM to 100nM.

In the proliferation studies, there was little difference in the growth stimulation between cells treated with 100,10 and 1nM IGF-I. Higher growth was observed than for cells incubated in HTS alone and in some cases the cells incubated with 10nM IGF- I showed the highest cell numbers in both MKN45 (Figure 32 and Data sheet 4 in Appendix C) and in N87 cells (Figure 33 and Data sheet 5 in Appendix C). The growth in 10nM IGF- I was comparable to growth in insulin supplemented medium. Therefore, in view of the satisfactory growth with 10nM IGF- I this dose was chosen as most appropriate in all the drug treatment experiments.

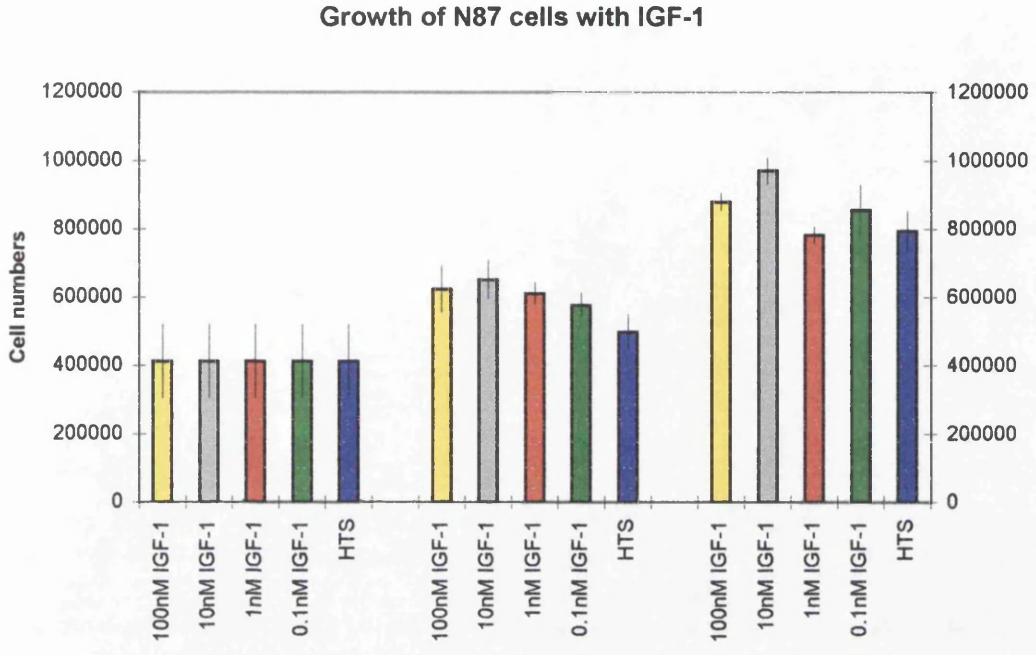
Figure 32



Cell doubling time (40,000 cells per well)

100nM=2.66 days	0.1nM= 3.1 days
10nM= 2.45 days	HTS= 3.21 days
1nM= 2.97 days	

Figure 33

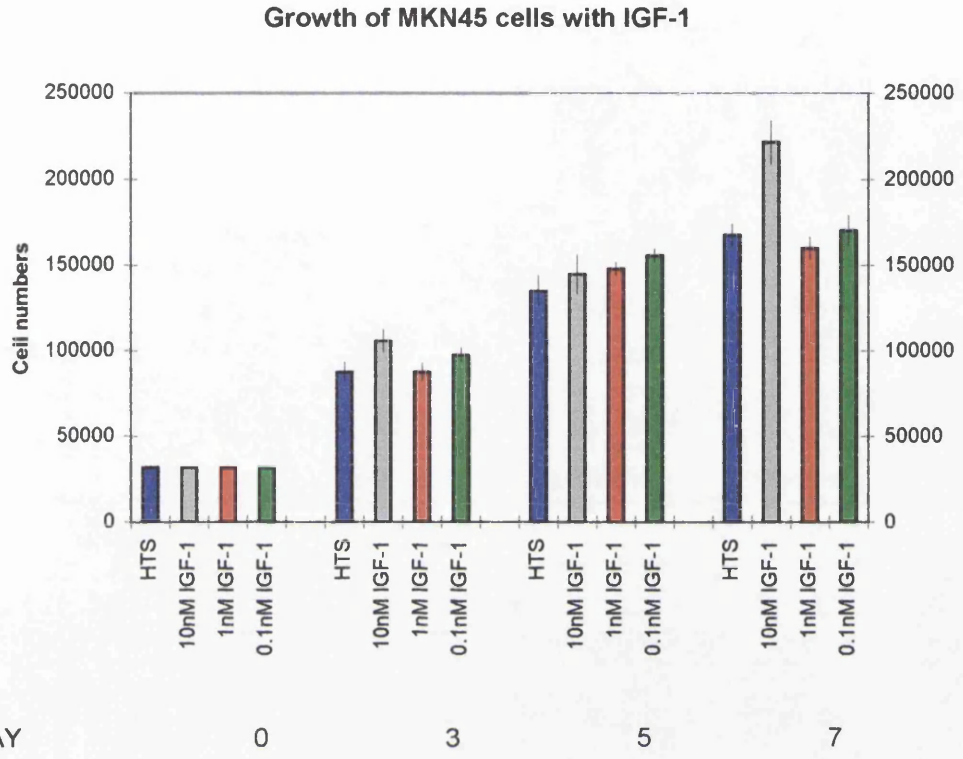


Cell doubling time (40,000 cells per well)

100nM=3.96 days
10nM= 3.79 days
1nM= 4 days
0.1nM= 4.28 days
HTS= 4.95 days

With MKN45 cells in which the mean doubling time (each experiment performed in triplicate) was calculated in HTS to be 2.24 days, the doubling times for treatment with IGF- I were 1.84 days with 10nM IGF- I and 2.1 days with 1nM IGF- I (Figure 34 and Data sheet 6 in Appendix C). The mean was calculated using doubling times from data sheets 4.6 and 8.

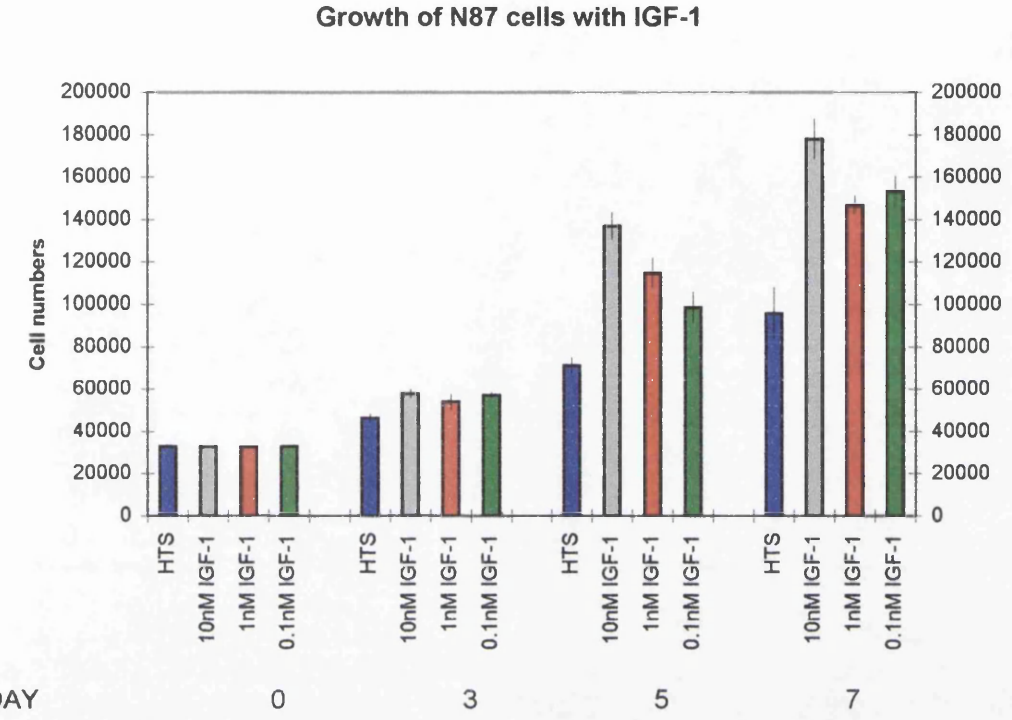
Figure 34



Cell doubling time (30,000 cells per well)

10nM= 1.8 days
1nM= 2.18 days
0.1nM= 1.96 days
HTS= 2.18 days

Figure 35



Cell doubling time (40,000 cells per well)

10nM= 3.4 days
1nM= 3.65 days
0.1nM= 3.45 days
HTS= 4.26 days

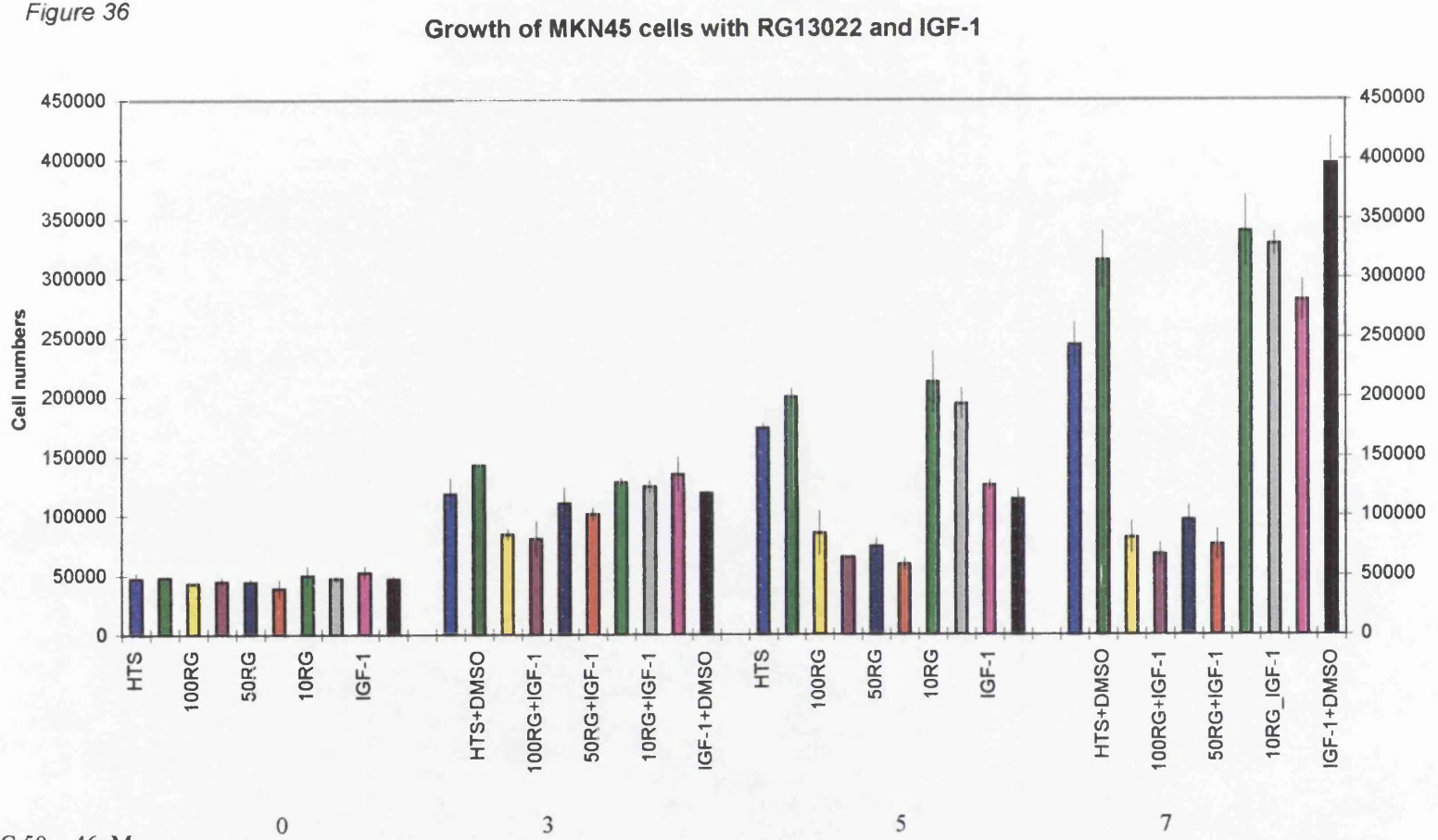
The proliferation of N87 cells in the presence of IGF- I is shown in Figure 35 (Data sheet 7 in Appendix C) and the doubling times are calculated where the mean doubling time for cells in HTS is 4.43 days and the stimulation by IGF- I at 10nM produces cell doubling in 3.17 days. With 1nM IGF- I the doubling time is 3.5 days. The mean is calculated from the doubling times in data sheets 5,7 and 9 in Appendix C).

Section iii: Dose response of MKN45 and N87 to RG13022 inhibition

Cell proliferation studies were undertaken using RG13022 at concentrations of 5 to 100uM in DMSO and compared to the growth of cells incubated in HTS and DMSO or with IGF- I at concentration of 10nM in a medium of HTS and DMSO.

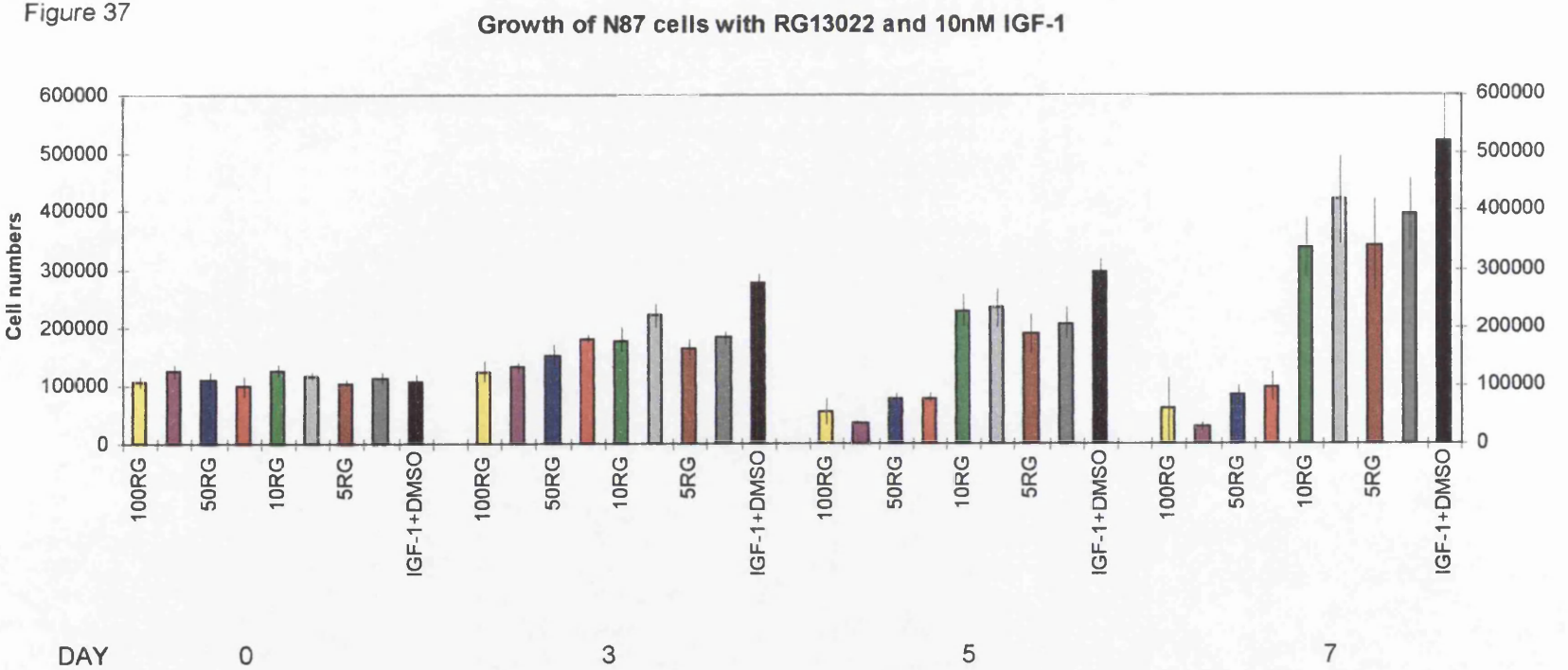
In the initial experiments the cells were incubated with drug with and without IGF- I stimulation. The effect of adding DMSO to the medium was also displayed and showed an apparent potentiation of growth in cells incubated in HTS alone with addition of DMSO in MKN45 cells (Figure 36 and Data sheet 10) and N87 cells (Figure 37 and Data sheet 11) This was not easily explained but since all the cells would have addition of DMSO to the medium in some form in the dose response series this was not relevant to results.

Figure 36



IC 50 = 46uM
%inhibition= 83%
%cell death= 16%

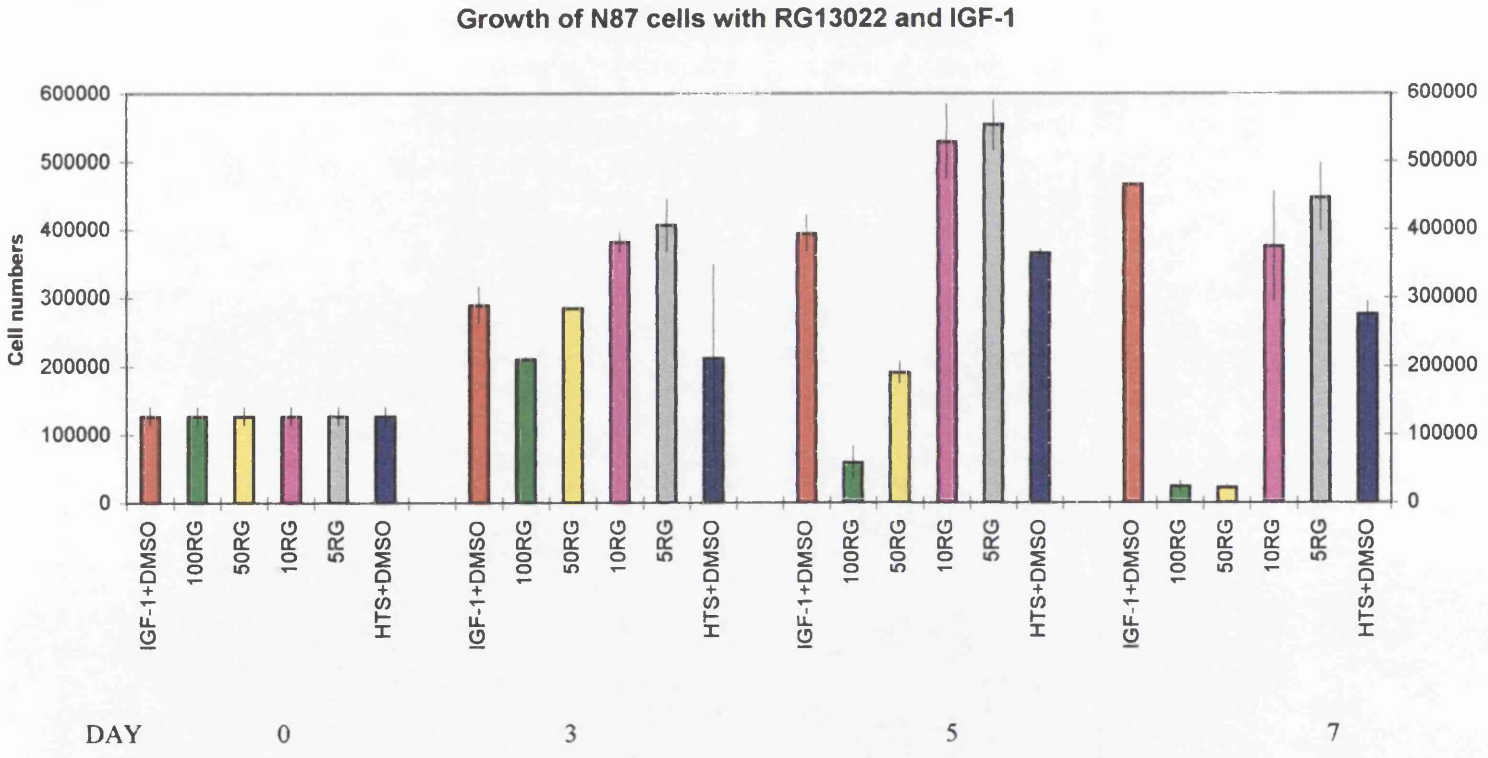
Figure 37



IC 50= 39uM
% inhibition= 88%
% cell death= 80%

(40,000 cells per well)

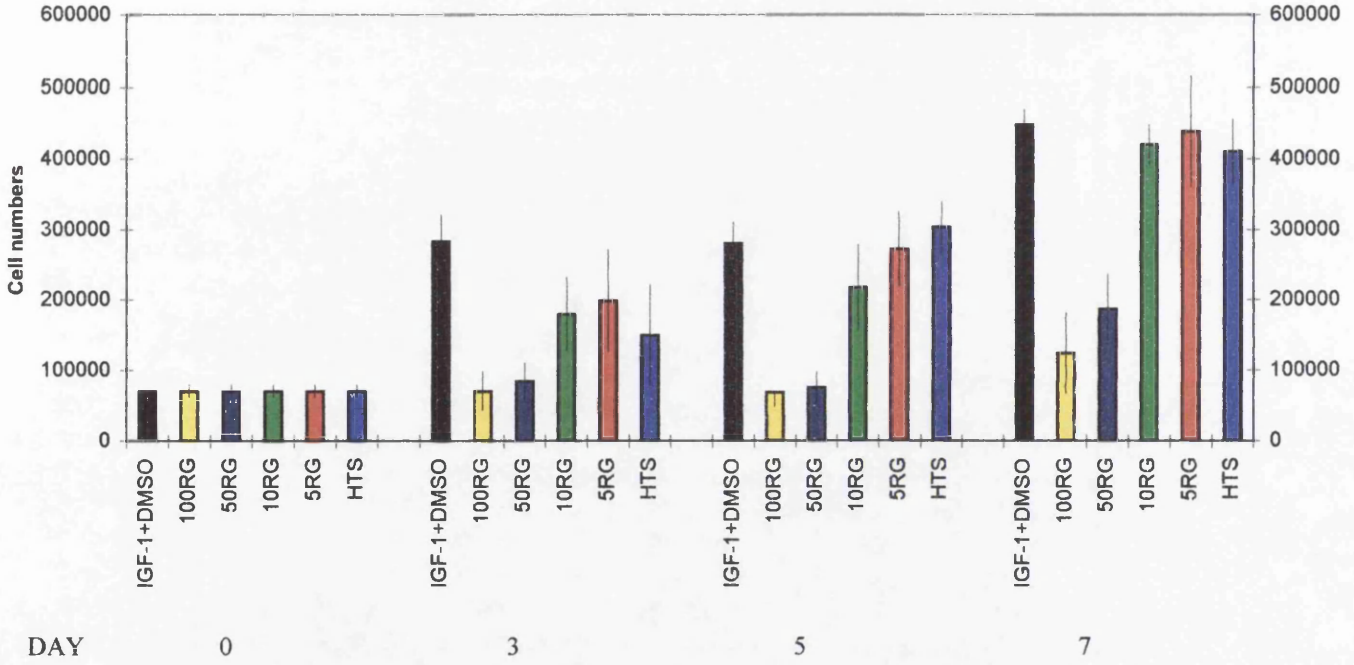
Figure 38



IC 50 = 40uM
% inhibition = 95%
% cell death = 88.6%

Figure 39

Growth of MKN45 cells with RG13022 and IGF-1



IC 50 = 61uM
% inhibition = 72%
% cell death = 0

The N87 cells incubated with RG13022 alone and with IGF- I show that there was a dose dependent inhibition of proliferation (Figure 38, Data sheet 12 in Appendix C). At high concentrations of RG13022 (100uM) there was a mean over three experiments of 92% inhibition of cellular proliferation between the cell number from the RG and IGF- I treated cells and the cells treated with IGF- I alone. At a concentration of 100uM, RG13022 was not only cytostatic but there was considerable mean cell loss of 72% from the day 3 to day 7 counts. Using serum free medium and concentrations of RG13022 from 5uM to 100uM the mean IC 50 was found to be 38.6uM for N87 cells. From 5uM to 50uM there was inhibition of cell growth but at 5 to 10uM there appeared to be a competitive reversible effect with the addition of IGF- I. The mean was calculated using data sheets 11,12 and 13 (Appendix C). Growth inhibition of MKN45 cells (Figure 39, Data sheet 14) at high concentration of RG13022 showed mean inhibition to be 71.8% and the effect was mainly cytostatic with a mean percentage of cell loss of 5%. The mean IC 50 over three experiments was found to be 64.7uM.In contrast to the effect of IGF- I on the drug treated cells, in many cases the cells treated with IGF- I and drug showed more growth inhibition than drug alone. The mean results were calculated using data sheets 14,15 (Appendix C) and 16.

Chapter 10
Discussion of c-erbB-2 and EGFr expression

Section i: Expression of C-erbB-2

In this study of sixty-seven gastric adenocarcinomas, c-erbB-2 expression by immunohistochemistry demonstrated a median score of 90 (IQ=60-120) and 43.3% of tumours expressed a level of receptor in excess of the range found in samples of normal mucosa. This level of expression is high when compared to the majority of published studies. The proportion of tumours expressing a higher level of c-erbB-2 than corresponding normal mucosa was found to be 55% by Kameda et al (1990). The majority of studies reviewed above suggest a range of increased expression between 9% and 28% (Hilton 1992, Motojima 1994) using a number of methods to determine overexpression, mainly expression in tumour sections relative to lack of staining in normal mucosa and comparison to control tissue.

There are a number of possible explanations for these findings.

Increased expression in tumour specimens and the presence of expression in mucosa are likely to be related to better preservation of antigenicity in frozen sections.

Studies to date have used a fairly standard technique of immunohistochemistry in paraffin embedded tissue sections following fixation with a variety of fixatives. In our laboratory, experiments on a series of breast tumour specimens comparing immunohistochemistry on frozen and paraffin-embedded sections have found the level of expression of c-erbB-2 was 92% and 22% respectively (Reeves 1994). Slamon (1989) suggested from his results in breast cancer specimens, that low levels of c-erbB-2 immunoreactivity may not survive the process of fixation which might explain the higher level of expression in frozen sections. However, Lemoine (1991) and Porter (1991) found no difference between the level observed in frozen or paraffin sections in gastric cancer and breast cancer respectively.

The staining intensity of sections has been demonstrated to differ with the use of cryostat or paraffin embedded sections and with the fixatives used in each case. Chiu et

al (1994) determined the fixatives for both preparations which produced optimum membrane staining and found that 8 out of 16 paraffin embedded gastric tumours expressed c-erbB-2, of which 7 expressed a higher level than adjacent non-neoplastic mucosa. In contrast the frozen sections showed c-erbB-2 expression in all 16 cases with 13 of the 16 (78%) having higher levels than adjacent non-neoplastic mucosa. Of the fixatives used, optimum staining was found in paraffin embedded sections with periodate-lysine-paraformaldehyde and in frozen sections with neutral buffered formalin. Although good membrane staining was achieved this was found that in the case of acetone fixation to be at the expense of a degree of loss of tissue morphology which is crucial for accurate histological examination. With other fixatives (Carnoy's and periodate-lysine-paraformaldehyde-dichromate) there was cytoplasmic but no significant membrane staining.

In both methods focal membrane staining was found in normal gastric epithelium. In frozen tissue there was a more uniform staining but in paraffin fixed specimens the staining was heterogeneous and demonstrated intracytoplasmic staining.

Falck and Gullick (1989) had also suggested variation in fixatives to be a possible factor. In an immunohistochemical study of c-erbB-2 in gastric cancer, they found that in comparing positive cases, the South African group had twice those of London. Although they felt that the use of a mercury-based fixative in the London tumours studied did not significantly influence the overall results, it highlights the ongoing difficulties of producing reliable, comparable data with small but possibly significant differences in methodology.

In determining the level of expression of c-erbB-2 a semi-quantitative scoring system was used in our own study and in several others (Jain 1991, Ohguri 1993). It is important to accommodate the well-recognised focal and patchy nature of the membrane staining, particularly in comparison to breast tumours where the staining is more uniform. It is also important to have explicit criteria for scoring membrane and cytoplasmic staining.

The study by Ohguri (1993) demonstrated the difficulty in making comparisons because this group considered the total expression to consist of both membrane and cytoplasmic staining, of which 45% to 50% was cytoplasmic. Furthermore, the studies by Jain (1991) and Lemoine (1991) from the same group have achieved expression of 30% and 26% respectively but in the former study both cytoplasmic and membrane staining were considered positive.

Immunohistochemistry is specific and reliable for tumour sections in the group of tumours with high expression of c-erbB-2 but less so in mucosa and lesser expressing tumours.

In many studies the level of expression is considered to be positive or negative without comparison to mucosal tissue but sometimes comparison to control tissues known to express high levels of c-erbB-2. If tumour sections are compared to a tissue expressing high levels of receptor, then tumours of comparable expression are considered positive. The control tissues, therefore, determine the level of positivity in a series of tumour sections.

To give a more objective quantification the method of radioimmunohistochemistry was adapted to study gastric tumours. A comparison of the results from the two methods shows several advantages of a quantitative analysis. Although the immunohistochemistry scoring system provides a satisfactory separation of results it is within a confined range which has a predetermined minimum and maximum value. Those tumours expressing high levels of c-erbB-2 are found to have maximum histoscores but with receptor estimations using the RIHC method there is a wide separation of these tumours. Within the maximum histoscore group the receptor densities using radioimmunohistochemistry range from 150 to 3641 ($x10^6$) and includes 9% of patients.

Using the scoring system where the level of expression is between 0 and 300, the range of values for the normal mucosa was 0 to 90. and 56.7% of the scores were less than or equal to the maximum in this range. The receptor numbers determined by

radioimmunohistochemistry had a range of 0 to 15.4 8 x 10^6 with 77.6% of receptor values less than or equal to the maximum normal value

The variation between the percentage expressing more than the range of mucosal samples in the methods examined is most likely to be due to the scoring of the intensity of immunohistochemistry. With both methods there is staining of the cellular membrane and cytoplasm in tumours and in mucosa. The area of staining is calculated with a grid system in each field but the degree of intensity of staining is the most subjective factor. The silver grain counts of receptor binding determine density and this incorporates scoring of intensity and area.

Using both methods, there is a wide range of c-erbB-2 expression in tumour specimens and this study has also demonstrated a measurable level of expression in non-neoplastic mucosa. It may appear that an arbitrary level of 'normal' expression has been placed to determine more easily the higher expressing tumours in order to make a comparison with published immunohistochemical studies. In addition, a small sample of mucosal sections were analysed in comparison to the number of tumour sections. If a section of mucosal tissue corresponding to each tumour section had been analysed this may have changed the range of values and consequently the number of tumours expressing more than this range.

The main advantages of RIHC compared to IHC are two-fold. Using a computer assisted analysis of silver grains corresponding to receptors diminishes subjectivity in determining staining intensity. More information is available than simply whether a tumour is 'positive' or 'negative' in this series using both RIHC and the more rigorous protocols for IHC but in RIHC there is even more scope for conformity between laboratories. Comparison of receptor level with prognostic factors and survival is, therefore, improved.

The difficulties with RIHC are related to the complicity of the methods. This is a time-consuming process where a number of standards must be maintained. The cell pellets used to calculate receptor number by ligand binding and those sections of pellets which are run with each group of tumours must be from the same passage of cells in culture to

ensure conformity in receptor number. Just as fixed tissue can be variable in loss of antigen preservation, frozen sections and blocks require careful handling. The strict conditions in which the photographic emulsion is applied cannot be varied in time, temperature, light or humidity. Finally, reproducible calibration of the image analysis system is essential.

It is particularly important to highlight those tumours which express the highest levels of expression in this study and show gene amplification. Tumours in which there was amplification had significantly increased expression of c-erbB-2 and conversely those with single copy genes had low levels of c-erbB-2 expression. Fifteen percent of the total group demonstrated gene amplification. This is consistent with 13% observed by Lemoine (1991) by Southern Blotting but reported amplification in other studies ranged from 5% to 40% (Yoshida 1989, Yokota 1989).

There are, however, a number of tumours which show moderately increased expression without amplification. The mechanism and importance of this finding require further investigation. Transcriptional regulation of c-erbB-2 expression has been demonstrated as a likely mechanism of increased expression without gene amplification. Studies using binding proteins to the individual elements in the promoter region of c-erbB-2 have shown increased activity and a degree of correlation with expression (Kameda 1990).

Radioimmunohistochemistry has the potential to produce a quantitative level of receptor density which may be more reliable in reproducing results within a group and more easily comparable information between centres. In addition to determining those tumours expressing increased levels of c-erbB-2 or those tumours with dramatic levels of c-erbB-2 and gene amplification, RIHC also outlines a population of tumours with moderately high expression but with a single gene copy. In the future, the main value of c-erbB-2 assessment is likely to be prediction of response to therapy targeted to the c-erbB-2 gene or protein. An analysis using IHC or FISH may exclude this potential target group which has been revealed by RIHC. Ultimately, the development of a c-erbB-2 assay which may be integrated into routine testing is important and despite the

invaluable information which RIHC may bring to the understanding of the role of c-erbB-2 in gastric cancer, its place may remain that of a research tool.

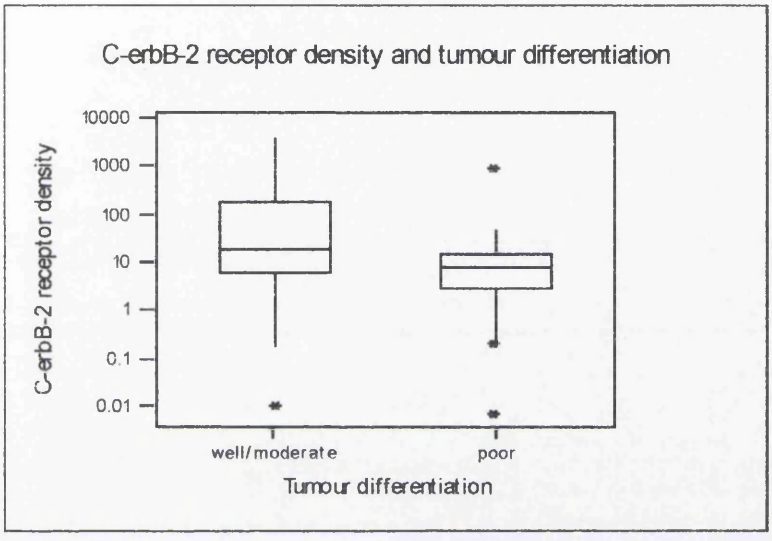
Section ii: Comparison of c-erbB-2 expression with clinical and pathological information

There are a number of pathological factors which are used in the description of gastric tumours (Yu et al 1997). The clinical relevance of these in determining the behaviour of the tumour and prognosis for the patients has been established over many years. Their correlation with the expression of c-erbB-2 may help illustrate its biological significance. Histology of gastric cancers is described using one or more of the recognised classifications, most commonly Broders' (1926) classification which divides the tumour group in to well, moderately well and poorly differentiated tumours. The majority of tumours are poorly differentiated tumours. Difficulties arise in gastric cancer because of tumour heterogenicity and grading can be highly subjective. Also, when examining specimens for immunohistochemistry the tissue must be representative of the overall histological type described.

Several of the tumours were also classified using the Lauren (1964) method which describes the histological growth pattern as intestinal or diffuse. Intestinal type tumours tend to be common in areas of high risk of gastric cancer and are often found as part of a field change in gastric mucosa with chronic atrophic gastritis and intestinal metaplasia. In general the patients are older, more likely to be male and have a better prognosis. The intestinal type consists of moderately differentiated glands whose cells have intestinal type cell features where as the diffuse type displays only small groups or single cells which infiltrate diffusely. There are a number of tumours which do not fit into either type and for routine histological reporting, Broders' classification is used with the additional information from the Lauren typing. The WHO (Watanabe 1990) and Ming (1977) classifications are also used to present additional information and combinations of these have been employed to overcome difficulties in analysing gastric cancer due to the heterogeneous nature of the tumour. Within the literature there is a tendency to group well differentiated, intestinal and tubular tumours when describing the relationship

relationship of histology to c-erbB-2. In this study there is a significant correlation between well to moderately differentiated tumours and high levels of expression of c-erbB-2 (p=0.008);Figure 40.

Figure 40 **Correlation between tumour differentiation and c-erbB-2**



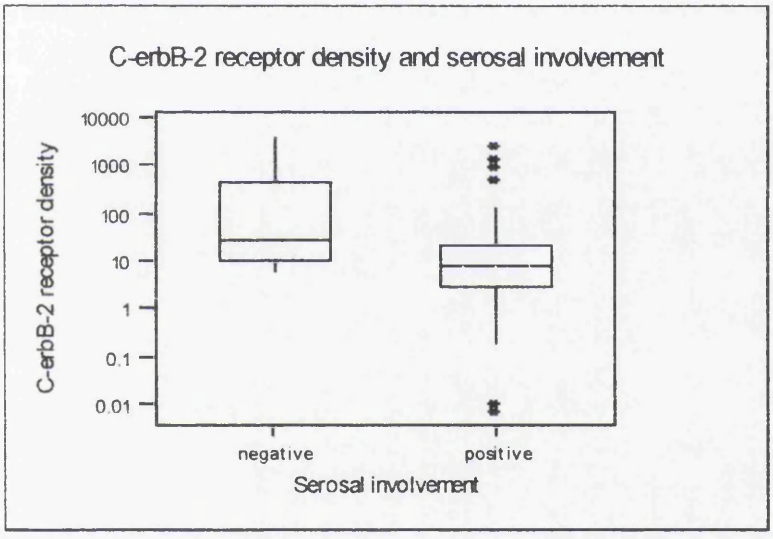
The box plot demonstrates the level of expression of c-erbB-2 as the number of receptors x 10^6 on the y axis on a log transformed scale with tumour differentiation expressed as well to moderately well differentiated tumours compared to poorly differentiated tumours.

There did not appear to be any relationship between the level of expression of c-erbB-2 and the site of the primary tumour. The proportion of antral tumours is lower than found in population studies reflecting more the pattern of hospital referral rather than the exaggerated effect of the recent increased incidence of proximal lesions. Albino et al (1995), however, in a study of 80 patients found a higher level of expression in antral tumours of which all were intestinal type. Although the prognosis of antral tumours tend to be better the survival time of these advanced lesions was less than the other distal tumours but not statistically significant. There are a number of authors, however, who have shown no correlation with the site of the tumour (Motojima 1994, Lemoine 1991). The pathological staging in many countries in particular in the Far East have described the presence of lymph node metastases as particularly important in predicting outcome (Maruyama 1987). However, as previously described in the patients in the West of

Scotland and in particular in the Centre from which the majority of tumour samples were taken, an extensive audit has demonstrated there is more predictive value attached to serosal involvement by the tumour. The advanced nature of the majority of gastric tumours which present in this group and the more conservative lymph node dissection may have a role in these findings.

Correlation of the level of expression of c-erbB-2 receptors with serosal involvement shows a significantly higher level of c-erbB-2 expression in tumours not involving the serosa ($p = 0.068$);(Figure 41).

Figure 41 **Correlation between serosal involvement and c-erbB-2**



The box plot demonstrates the level of expression of c-erbB-2 as the number of receptors x 10^6 on the y axis on a log transformed scale with serosal involvement expressed negative or positive on histology.

There were only 8% of the tumours (5) in the serosa negative group. Three were moderately differentiated tumours of which one was node positive and 2 poorly differentiated tumours both node negative. Of the group, 4 patients survived beyond three years and were disease free and the remaining patient died of cardiovascular disease after 15 months with no evidence of recurrent disease. In Glasgow patients, serosal involvement is a more reliable predictor of prognosis with lymph node status only important for those without serosal involvement. Increased expression of c-erbB-2

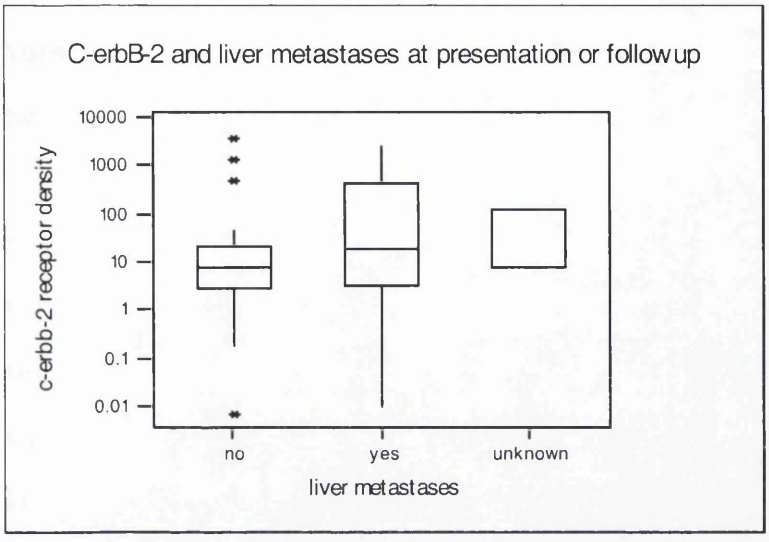
in serosa negative patients may suggest a possible association with better prognosis but the advanced nature of disease in the patients results in a limited number of patients available for analysis.

There was no correlation between the number of positive lymph nodes retrieved at operation and the level of c-erbB-2 expression and no significant correlation when the number of positive lymph nodes was taken as more than or less than 50%. This may reflect surgical factors, particularly the number of nodes retrieved. However, the literature has described both Japanese and British studies reporting no correlation of lymph node status with c-erbB-2 expression.(Uchino 1993, Lemoine 1991).

Although we were unable to study the level of expression in lymph node metastases there have been a number of groups who have shown a high level of expression in the nodes, in some cases higher than the primary tumour (Ohguri 1993) and occasionally with a c-erbB-2 negative primary tumour. This gives some insight to the potential effect of increased expression of c-erbB-2 in gastric cancer. The results of many studies have suggested that tumours with higher levels of expression of c-erbB-2 may exhibit a more aggressive growth pattern and an increased potential to metastasise.

Gastric cancer more commonly presents as a locally advanced lesion and the presence of liver metastases is often a late finding. In the box plot in Figure 42 there is a tendency to exhibit higher levels of c-erbB-2 in the tumours of patients presenting or subsequently developing liver metastases although this does not reach statistical significance (Figure 42).

Figure 42 **Relationship between liver metastases and c-erbB-2 expression**



The box plot demonstrates the level of expression of c-erbB-2 as the number of receptors x 10^6 on the y axis on a log transformed scale with liver metastases at any time recorded for a patient expressed as yes, no or unknown.

There was no statistical relationship between the presence of liver metastases and tumour differentiation. 33% of the total number of tumours were well to moderately differentiated and 36% of the liver metastases group were of this tumour type.

Tahara (1995) acknowledges the increased incidence of c-erbB-2 expression in patients with liver metastases and suggests that there may be differences in the pattern of metastases dependant on the differentiation of the tumour. He describes differing patterns of the cell adhesion molecule CD 44 variant expression with differentiation of the tumour (Matsumara 1992). A likely suppressor gene of metastases, nm23, which encodes nucleotide diphosphate kinase and c-myc transcription factor, is overexpressed in primary gastric carcinoma but reduced in metastatic gastric cancer (Nakayama 1993). A 31 kDa lactose-binding lectin (extracellular matrix binding protein) which is frequently overexpressed in well differentiated tumours may be associated with an increased incidence of liver metastases in this type and in poorly differentiated tumours an association with lymph node spread (Lotan 1994). Interestingly, Livingstone (1995) described a statistically significant reduction in nm23 in British tumours compared to

those from Japanese patients in addition to the higher level of c-erbB-2 expression previously described.

Yonemura (1993), while observing that the serum levels of c-erbB-2 correlated with the tissue immunoreactivity showed that the incidence of positive serum erbB-2 levels was significantly higher in patients with liver metastases. Sasaki (1992) has reported a higher incidence of peritoneal metastases in c-erbB-2 positive tumours. Peritoneal metastases tend to be associated with serosal involvement and would not support the findings of this study that c-erbB-2 levels are higher in patients without serosal involvement.

In this study there appears to be correlation of high c-erbB-2 expression with factors which in general predict a better outcome, in particular differentiated tumours and those with no serosal involvement. However, a Kaplan-Meier analysis of the level of c-erbB-2 expression and patient survival shows no relationship. There was also no statistically significant relationship between c-erbB-2 amplification and survival. With the exception of Jain (1991) who has shown better survival in tumours with high c-erbB-2 and well differentiation, the majority of studies show poorer survival or no significant relationship between these factors.

Section iii: EGFr expression

Using immunohistochemistry to analyse expression of EGFr there is a much tighter distribution of expression of EGFr when compared to c-erbB-2. The majority of published studies on EGFr have used a comparison to tissue with known elevated levels of immunoreactivity and most commonly the vulval epidermoid carcinoma-derived cell line A 431 (Ozanne 1986). By using radioimmunohistochemistry a quantitative assessment of EGFr expression was made in our study. Comparison was made with receptor expression determined by ligand binding studies in a number of cell lines. Limited ligand binding studies to determine EGFr expression in a large group of cell lines were carried out and demonstrated close correlation with grain count.

Standard immunohistochemistry detects EGFr expression in neoplastic and non-neoplastic tissue. The median value of EGFr expression in the tumours was 100 (IQ=70-125) and 41.8% of tumours expressed levels above the mucosal range. Expression of EGFr using RIHC when expressed as % of A431 cells ranged from 0 to 106% with a median of 0.9% (IQ= 0.5 to 1.6%). In published series the level of overexpression was considered to be between 18% and 43% (Lemoine 1991, Lee 1994). These studies used immunohistochemistry and the problems associated with this have been discussed fully above in relation to c-erbB-2. Studies like those of Filipe et al (1995) and Lee et al have determined EGFr immunostaining in neoplastic and non-neoplastic tissue with the former showing increasing intensity with worsening dysplasia. Some studies such as those of Hirona (1995) claim that non-tumour cells were negative. In our studies the non-neoplastic tissue stained positively in all tumours and in the small number of mucosal samples analysed there was a range of staining up to a score of 105 encompassing the scores of 58% of the tumours.

There are some difficulties in defining the criteria used to determine positive staining. In this study positive tumours had membrane staining primarily but in addition may have had cytolasmic immunoreactivity as almost an incidental finding. This contrasts with the

study of Sugiyama et al (1989) where both levels of staining were taken as positive independently. The level of staining in terms of intensity and extent was determined by a scoring system rather than simply presence or absence of staining as shown by Yasui et al. (1988). However, it is evident from the substantial difference in percentage overexpression between IHC and RIHC that there are difficulties with any subjective scoring.

Both methods show extensive expression but the comparative mucosal expression is overemphasised with IHC. The overall correlation of the expression of EGFr by the two methods is good (Spearman rank correlation $r^2 = 0.3$, $p<0.0005$). In the distribution of percentage expression of EGFr there is a suggestion of a bimodal distribution. Although the level of overexpression is 39% and 20.9% of tumours have expression above the interquartile range, the 7% of tumours in the second peak may be those associated with significant effect. Alternatively, these may simply be outliers and to clarify this, a study of substantially increased numbers and statistical power would be required. The level of gene amplification in EGFr is not well studied but ranges from 3% (Yoshida 1989) to 7% (Lemoine 1991) and would be an interesting area of additional study.

These differences in the analysis of immunohistochemistry slides make the results difficult to compare between studies and to compare the results of analysis using comparison of receptor expression with clinico-pathological factors or tumour markers.

Using radioimmunohistochemistry the difficulties of subjectivity in scoring slides are removed, there is better spread of results and comparison to other factors is more reliable, whether by using the grain count or expressed as a percentage of the grain count for A 431 cells.

There appears to be no relationship between the expression of C-erbB-2 and EGFr when analysed using the results of expression from RIHC ($r^2 = 0.004$, $p = 0.594$) and IHC ($r^2 = 0.087$, $p = 0.015$) using Spearman rank correlation.

Section iv: Comparison of EGFr expression with clinical and pathological factors.

Although there have been a few studies in which the presence of EGFr expression has been associated with poor prognosis, the majority of tumour series have insufficient follow-up and tumour numbers to determine a significant relationship. However, it is possible to compare the level of EGFr expression with clinico-pathological indicators of prognosis and tumour aggressiveness. In comparative studies of early and advanced tumours there have been significant differences in the expression of EGF and EGFr and Yasui (1988), Sugiyama (1989) and Tokunaga (1994) have suggested the overexpression of EGFr is related to a biological aggressiveness and therefore higher levels occur in advanced lesions.

In this study, pathological indicators of advanced lesions have not shown a statistically significant relationship with EGFr expression. Comparing the level of EGFr expression both as grain count and as a percentage of A 431 cells there is no correlation with serosal involvement (p=0.694;p=0.489). There were only five patients with no serosal involvement and a significant difference between early and advanced lesions would be difficult to determine with small numbers but of particular note is the patient with EGFr receptor density/%A431 of 4.6% (count 1108). This tumour was found to be serosal and node negative but the level of expression was in the higher second peak in the bimodal distribution. In the analysis of nodal involvement there was no correlation between the presence of positive nodes (p=0.863) or the proportion found to be positive (p=0.413).

The relationship between the expression of EGFr and the differentiation of the tumour tends towards significance (p=0.130;p=0.015) and the group of well and moderately well differentiated tumours have higher levels of expression (p=0.08;p=0.005). Yasui (1988), despite finding an increased level of expression of EGFr in advanced lesions also found correlation with well differentiated tumours. Sugiyama (1989) and Yoshida (1989) in contrast found higher expression in poorly differentiated tumours. In Japanese studies of

primary tumour and metastatic nodal tissue there have been high levels of expression in the involved nodes (Yasui 1988) but there was no relationship to distant, peritoneal or hepatic metastatic spread. We also have shown no relationship between EGFr expression and the presence of liver metastases either at presentation (p=0.716) or at any time during follow-up (p=0.394).

No association with site of tumour (p=0.358) was observed. In those tumours of oesophageal origin which were excluded from the final analysis it was interesting to note that there was an intense staining for EGFr in the tumour and mucosal specimens despite the fact these tumours were adenocarcinomas arising in Barretts epithelium. This requires further study but may indicate that the initiation of the tumour process is different at this site.

This must also be considered with the findings of Filipe (1995) where an increase in intensity of staining was found in intestinal metaplasia and dysplastic epithelium in gastric cancer fields when compared to these changes in mucosa from non-cancer fields.

This may suggest that overexpression of EGFr is a relatively early event in the progression to invasiveness in gastric cancer and it may be the promoting effect of EGF (Sugiyama 1989) which is important in tumour development. Synchronous expression is shown to produce poorer prognosis in gastric cancer.

Using a Kaplan-Meier analysis of the relationship between EGFr expression and survival there is no significant correlation between level of receptor and overall survival (p=0.655). No association between expression of EGFr and c-erbB-2 determined by any method was found despite a correlation of both with tumour differentiation. Others have suggested that EGFr expression is more associated with poorly differentiated tumours (Tahara 1995) and indicates progression to invasiveness, with a tendency to nodal involvement.

In summary, this study has shown correlation of EGFr expression with better tumour differentiation but no correlation with involvement of serosa, lymph nodes, site of tumour or patient survival.

Section v: Discussion of IGF-I studies

The tyrophostin RG13022 is one of a number of compounds developed to inhibit the autophosphorylation of tyrosine kinase receptors in particular EGFr. It has previously been shown to inhibit cellular growth in human breast cancer cell lines (Reddy 1992), pancreatic cancer cells (Gillespie 1993) and gastric cancer cells (Piontek 1993). The latter study investigated the effect of this compound on the AGS human gastric cancer cell line with the addition of exogenous EGF. RG13022 produced a dose dependent growth inhibition of the gastric cells.

In collaboration with Miss V. Morrison and under the supervision of Dr. J. M. S. Bartlett, a series of parallel experiments were undertaken to examine the inhibitory activity of the tyrophostin RG13022 on the gastric cancer cell line MKN45 and N87 and the modulatory effect of stimulation with EGF. Using the methods described but plating the cells at a density of 4×10^4 per well for MKN45 and 6×10^4 per well for N87 the cells were incubated in 1%,2%,5% FCS or in HITS media and subsequently treated with DMSO and RG13022 at concentrations of 1 to 100uM. I will discuss the results of the effect of RG13022 on the gastric cell lines co-stimulated with IGF-1 in comparison to cells influenced by EGF.

In establishing the optimal conditions for culture in this way the growth of MKN45 cells were found to be relatively independent of culture medium. However, the rate of proliferation of N87 was lower in 1% FCS and in serum free medium. In both serum containing (5% FCS) and serum free (HITS) medium, RG13022 produced a dose dependent inhibition of cell growth with both MKN 45 and N 87 cells. For N 87 cells the IC 50 in 5% FCS was 45uM and 31uM in HITS. In MKN45 cells the IC 50 in 5% FCS was 58uM and 32uM in HITS.

At high concentration (100uM) RG13022 was cytostatic and growth inhibition in both cell lines was approximately 90% under all conditions. When drug treatment was removed

from the cell cultures at day 5, in MKN45 cells, there was little effect on proliferation but the N87 cells recovered to resume further cell growth.

Although RG13022 was developed more specifically for the inhibition of the EGF receptor the inhibition of cellular proliferation previously seen in serum free medium supplemented with IGF- I shows that there is also some inhibitory activity in the IGF- I receptor pathway.

The tyrophostin inhibited both cell lines in a dose dependent manner but there were considerable differences between the cell lines and when investigating the effect on the IGF receptor and EGF receptor. The IC 50 for inhibition of growth stimulated by EGF in both cell lines was around 30uM but inhibition in the IGF experiments showed an IC 50 for N87 of around 40uM and for MKN45 a consistent IC 50 of 70uM.

Experiments on gastric and pancreatic cells have suggested that the effect of RG13022 is cytostatic with minimal cell loss and reversible on removal of the drug. This was found to be the case in MKN45 cells with around 5% cell loss at 7 days after drug treatment. However, the N87 cells showed cell loss of 72%. This can in part be explained by the observation that the growth of N 87 cells decreased in serum-free medium at 7 to 10 days without drug treatment but the cell loss is certainly accelerated. The concentration of RG13022 required for inhibition of MKN45 cells was greater and addition of IGF- I often caused some potentiation of the inhibitory effect. This suggested that the RG13022 was causing inhibition of the IGF receptor pathway at different areas in signal transduction.

Immunoblotting techniques were used to examine signal transduction in the EGF receptor and IGF receptor pathways. In the EGF experiments the immunoblotting methods were comparable and monoclonal antibodies to EGFr were used in addition to antiphophotyrosine antibody and antibodies to MAP2 kinase. The effect of tyrophostin treatment on EGF stimulated cells had previously shown that RG13022 reduced phosphorylation of a predominant 170kDa protein co-migrating with EGFr in MKN45

cells but not N 87 cells and modulation of phosphorylation of p185 (c-erbB-2), p85 (p13 kinase) and p42 (MAP2 kinase). In the N87 cells there was an apparent mobility shift of MAP2 kinase which did not occur in the MKN 45 cells and suggested that the antiproliferative effect of RG13022 differed in the two cell lines.

In IGF- I stimulated MKN45 cells using the antiphosphotyrosine antibody (Py 54) the 95kDa subunit of the IGF receptor could be demonstrated in the stimulated and unstimulated state with the inhibitory effect of RG13022 on phosphorylation. The inhibitory effect was also evident in the intensity of the MAP2 kinase band but there was no mobility shift demonstrated (Figure 43). Using the MAP2 kinase antibody there was no obvious difference between IGF stimulated and drug treated cells (Figure 44). With N87 cells there was evidence of a modulatory effect at the 95kDa band but no evidence of changes at the MAP2 kinase or other sites with Py 54 (Figure 45) or the MAP2 kinase antibody (Figure 46).

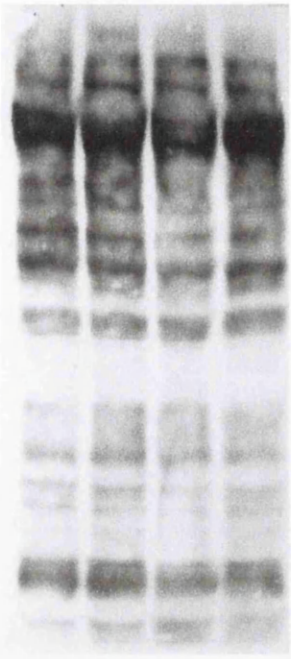
There appeared to be some changes apparent at the 185kDa site of IRS-1 during stimulation of MKN45 cells with some degree of mobility shift but this was inconsistent and the antibody in our hands was unreliable (Figure 47).

In the gastric cancer cell lines MKN45 and N87 the tyrophostin RG13022 originally developed as an inhibitor of the EGF receptor has been shown to have an antiproliferative effect on IGF stimulated cells suggesting activity against the IGF- I receptor. Using immunoblotting techniques it was possible to demonstrate some modulation of the intracellular signalling with IGF- I stimulation and drug treatment with RG13022.

Observation of drug induced inhibition of gastric cancer cells in this way suggests that further advances in the treatment of gastric cancer could be possible. The demonstration of the level of expression of IGF- I receptors in gastric tumours would indicate whether this area would be a worth-while target for growth inhibition. The difficulties with inhibition at this level would need to be addressed and in particular the

systemic effects of inhibition and the need for localised application to the target tissue. In the case of IGF- I the added difficulties of the modulating effect on the receptor number and circulating IGF of binding proteins. Finally, the effect on insulin activity in the patient would require investigation. Novel growth factor inhibitors in conjunction with tissue targeting techniques and conventional chemotherapy may be of some benefit in the future particularly in the treatment of advanced gastric cancer.

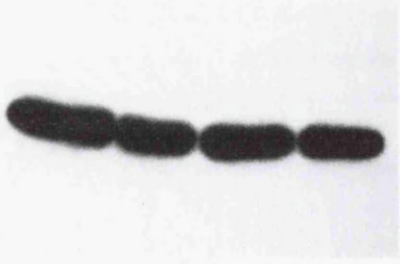
Figure 43 The effect of RG13022 on MKN45 cells co-stimulated with IGF-1



A B C D

The immunoblot uses the antiphosphotyrosine antibody and demonstrates the stimulation of MKN 45 cells with IGF-1 (A=control, B=IGF-1 stimulation) and the inhibitory effect of the RG13022 (C=RG13022 and D=RG13022 and co-stimulation with IGF-1). The stimulatory effect is seen as dark bands at the 95kDa subunit of IGF-1 and the 42kDa site of MAP kinase with corresponding loss of this effect on addition of RG13022.

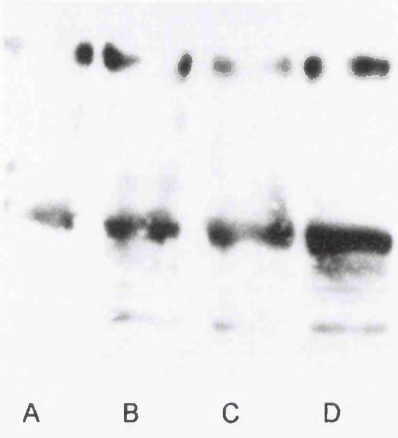
Figure 44 The effect of RG13022 and IGF-1 on MKN 45 cells at the MAP kinase band



A B C D

Using the MAP kinase antibody there was obvious stimulation of MKN 45 cells at the 42kDa site but no distinct difference between stimulated and unstimulated cells. There was no evidence of a mobility shift at this site.

Figure 45 The effect of RG13022 on N 87 cells co-stimulated with IGF-1



With N 87 cells there is some evidence of stimulation at the 95kDa site (A= control compared to B= stimulation with IGF-1). There is little difference in the density of the bands using the antiphosphotyrosine antibody in the cells treated with RG13022 suggesting little effect in the signalling pathway of this cell population.

Figure 46 The effect of RG13022 and IGF-1 on N 87 cells at the MAP kinase band

Stimulation of N 87 cells is evident at the MAP kinase site with the MAP2 kinase antibody but there is no difference between the bands and no mobility shift at this site.

Figure 47 Modulation at the IRS-1 185kDA site on stimulation of MKN 45 cells with IGF-1

There is a mobility shift between the unstimulated cells (A and C) and cells stimulated with IGF-1 (B and D).

GENERAL DISCUSSION

For the most part, the aims of this thesis have been fulfilled. Radioimmunohistochemistry has been demonstrated as a useful technique in determining the level of expression of EGFr and c-erbB-2 in gastric cancer. Comparison with standard immunohistochemistry has highlighted advantages of the former technique, in particular exposing the wide distribution of receptor density in the highest expressing tumours for c-erbB-2 and demonstrating expression in non-neoplastic tissue. Subsequent investigation of a relationship between EGFr and c-erbB-2 and clinico-pathological factors has identified some interesting areas. EGFr expression is estimated at 40% above that of gastric mucosa but there is no significant correlation between EGFr and prognostic factors or survival. As a molecular marker EGFr may provide valuable information in tumour biology especially with regard to expression in pre-malignant tissue. However, additional prognostic information is unlikely and in gastric cancer there is little indication for a use as a therapeutic target.

In contrast, c-erbB-2 which demonstrates higher expression than mucosal samples in 22% of cases and 15% amplification has a significant correlation between increased expression and both lack of serosal involvement and differentiated tumours. This may indicate a subset of tumours in which tumour biology and behaviour are linked to c-erbB-2 expression and indicates some potential as a tumour marker.

It is unlikely that growth factors influence the proliferation and behaviour of cancer cells independently. This thesis has therefore also sought to investigate IGF-1 in gastric cancer and has demonstrated IGF-1 induced cellular proliferation with a dose dependent inhibition by the tyrophostin RG13022. In finding IGF-1 a less potent stimulator of the MAP kinase pathway than EGF in gastric cancer cells and RG13022 less able to inhibit this pathway, our understanding of tumour biology improves despite lack of advances in therapeutics.

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APPENDIX A

Table 1 **Survival by operative procedure**

Type of operation	No.	% surviving at 1 (years)	2	3	4	5 (months)	median survival
Curative resection	129	63	46	35	27	22	19m
	54	78	65	62	57	41	55m
Palliative resection	32	25	13	3	3	0	6m
	115	52	31	18	10	8	12m
Bypass	33	6	0	0	0	0	4m
	23	6	0	0	0	0	3m
Intubation	26	8	4	4	4	0	2m
	4	0	0	0	0	0	0.8m
Laparotomy only	58	9	0	0	0	0	2m
	28	8	0	0	0	0	5.2m
No surgery	51	12	6	3	3	3	1m
	84	4	2	0	0	0	1m
Laser	--	--	--	--	--	--	--
	59	23	9	5	5	2	3.6m
All resection	161	55	39	29	22	18	14m
	170	56	37	30	24	16	15m

Table 2 **Survival by degree of involvement (Curative resections)**

	No.of patients 1986-1994 (**1974-1984**)	% survival 1986-1994 (**1974-1984**)			
		1	2	3	4	5
serosa						
+ve	31 (90)	69(54)	49(35)	45(26)	45(18)	23(13)
-ve	23(33)	90(85)	90(78)	90(59)	74(59)	74(47)
RM						
+ve	4(31)	75(41)	25(7)	0(7)	0(0)	0(0)
-ve	52(95)	78(69)	69(58)	69(43)	63(36)	46(28)
nodes						
+ve	21(65)	69(55)	39(37)	39(27)	39(22)	23(17)
-ve	33(63)	83(71)	83(57)	78(45)	69(37)	53(31)

Table 3

30 DAY MORTALITY	**% 1974-1984 (% 1986-1994)**			
	CARDIA	BODY	ANTRUM	TOTAL
LAPAROTOMY ONLY	33% (25%)	18% (20%)	50% (50%)	29% (32%)
NO SURGERY	50% (53%)	67% (38%)	41% (60%)	51% (49%)
CURATIVE	18% (17%)	11% (9%)	7% (4%)	12% (7%)
PALLIATIVE RESECTION	14% (19%)	12% (8%)	6% (10%)	9% (11%)

APPENDIX B

Table 1: Results for c-erbB-2 expression - radioimmunohistochemistry and immunohistochemistry

Patient	Path.No.	Age	C-erbB-2 gr	C-erbB-2	Run	B-2 grain count			B-2 area			Exposure	B2 IHC
						1	2	Control	1	2	Control		
1	2889/94	59	5.41	1.88	1	248	225	132	7	6.7	4.5	96	70
2	9934/94	58	0.77	0.27	1	124	120	89	3.83	4.09	2.96	96	30
3	8747/90	53	102	40	2	750	714	117	5.6	5.8	3.84	96	235
4	7278/90	83	42	29.9	3	191	327	45	5.21	4.58	3.57	96	100
5	6334/92	82	38.7	15.3	2	354	458	114	6.19	6.05	3.91	96	170
6	11820/90	52	26.8	10.6	2	209	232	118	2.5	3.58	2.52	96	120
7	11569/90	53	7.23	2.86	2	216	297	196	4.77	4.75	4.18	96	10
8	11962/91	55	318	126	2	581	582	101	5.88	5.37	3.73	24	300
9	8819/95	71	1.5	1.07	3	195	206	145	5.59	5.14	4.04	96	80
10	10333/95	59	30	21.4	3	301	387	117	5.44	6.92	4.3	96	100
11	2944/94	66	7.34	2.9	2	110	112	72	2.18	2.76	1.9	96	10
12	11951/90	70	14.1	5.58	2	246	192	104	6.36	5.84	4.66	96	10
13	6905/95	59	34.6	24.6	3	366	366	120	5.15	5.15	3.17	96	100
14	7622/91	48	0.7	0.5	3	114	156	114	3.23	4.5	3.33	96	5
15	5453/94	82	3.44	1.19	1	142	111	69	5.26	5.57	3.44	96	85
16	1445/90	78	11.9	8.5	3	197	272	96	6.18	6.15	3.61	96	70
17	9771/90	68	0.46	0.18	2	249	215	142	5.84	5.92	3.64	96	10
18	8756/91	56	1267	903	3	328	371	53	5.13	5.64	3.73	4	300
19	61283/92	70	6113	2419	2	1340	1430	60	5.4	5.24	3.79	4	300
20	8063/94	71	29.8	11.8	2	349	392	159	4.38	4.97	3.14	96	160

Table 1 (contd)

Patient	Path.No.	Age	C-erbB-2 gr	C-erbB-2	Run	B-2 grain count			B-2 area			Exposure	B2 IHC
						1	2	Control	1	2	Control		
21	921/91	82	119.8	41.6	1	849	657	83	4.55	5.78	2.7	96	270
22	6795/92	49	18.8	7.44	2	208	348	164	4.43	4.88	3.94	96	90
23	7787/94	83	9202	3641	2	1600	2920	66	4.85	6.82	3.43	4	300
24	10732/93	65	3815	1324	1	1090	993	82	6.37	5.69	4.08	4	300
25	8417/95	64	29.4	21	3	302	240	84	6.16	5.18	4.29	96	80
26	9896/94	77	75.5	26.2	1	615	614	102	6.1	5.09	3.31	96	185
27	4104/94	52	19.3	7.64	1	183	207	89	4.41	4.99	3.87	96	90
28	1044/91	68	19.8	7.83	2	381	290	136	6.9	7.97	5.21	96	85
29	1085/91	79	2.93	1.02	1	148	132	94	6.01	5.27	4.27	96	10
30	4036/95	64	10.5	7.48	3	167	103	64	4.32	3.87	2.8	96	90
31	169046/92	69	5831	2307	2	1250	1450	65	5.42	5.38	3.88	4	280
32	1037/92	68	1.19	0.41	1	270	172	149	6.93	6.03	4.52	96	85
33	7852/91	68	20	14.2	3	165	130	25	5.51	6	3.96	96	80
34	9349/90	63	33.7	13.3	2	660	583	224	7.35	6.68	3.98	96	90
35	1659/94	77	41.3	14.3	1	399	435	36	5.47	5.96	1.08	96	195
36	8281/91	64	20.2	7.99	2	282	257	93	4.21	4.67	2.25	96	100
37	413/92	69	47.6	33.9	3	576	576	171	6.64	6.64	4.17	96	70
38	11622/90	53	15	10.7	3	208	169	80	5.19	5.56	3.89	96	60
39	2514/94	44	12.3	4.27	1	224	165	99	5.8	5.44	4.35	96	60
40	1369/92	72	0.01	0.01	3	253	254	212	7.83	6.34	5.29	96	100

Table 1 (contd)

Patient	Path.No.	Age	C-erbB-2 gr	C-erbB-2	Run	B-2 grain count			B-2 area			Exposure	B2 IHC
						1	2	Control	1	2	Control		
41	7459/92	70	38.8	27.6	3	375	400	99	5.77	5.58	3.19	96	40
42	1375/93	69	63.8	45.5	3	569	432	97	6.31	5.42	4.02	96	105
43	8970/91	62	10.6	7.55	3	101	157	61	4.41	5.25	3.69	96	50
44	10050/90	69	49	19.4	2	539	362	137	6.11	5.02	4.04	96	100
45	6747/94	72	7.95	2.76	1	234	185	106	5.28	5.72	3.48	96	30
46	10536/93	80	59.4	23.5	2	343	358	55	5.55	4.31	3.81	96	120
47	1252/92	66	1337	464	1	295	301	44	5.05	3.8	3.17	4	300
48	10578/95	68	64	45.6	3	375	386	30	5.16	5.63	3.33	96	70
49	8104/91	65	18.4	7.28	2	290	305	135	6.2	5.42	4.03	96	60
50	7306/92	53	6.93	2.4	1	303	377	199	9.28	9.17	6.59	96	95
51	3729/95	82	14.2	10.1	3	277	242	124	5.77	5.09	3.63	96	60
52	808871/94	48	11.1	3.85	1	169	228	97	4.02	5.54	3.14	96	65
53	10283/95	50	24.5	17.4	3	319	348	110	6.18	6.53	3.8	96	60
54	10456/93	74	0.64	0.22	1	157	159	122	3.83	4	3.07	96	70
55	11126/92	70	11.5	4.55	2	315	315	204	5.14	5.14	4.06	96	105
56	12678/94	63	16.1	5.59	1	209	177	109	3.93	3.35	2.9	96	50
57	895/95	59	8.54	3.38	2	175	249	139	4.03	4.03	3.13	96	60
58	4451/92	46	39.9	15.8	2	438	349	159	5.65	4.98	4.45	96	135
59	3918/94	76	18.2	6.35	1	257	280	97	5.6	5.87	3.3	96	120
60	9649/94	61	3.95	1.37	1	161	252	111	5.99	6.67	3.85	96	90

Table 1 (contd)

Patient	Path.No.	Age	C-erbB-2 gr	C-erbB-2	Run	B-2 grain count			B-2 area			Exposure	B2 IHC
						1	2	Control	1	2	Control		
61	12966/94	55	9.06	3.14	1	174	180	109	4.65	4.49	3.63	96	30
62	8284/91	62	28	9.72	1	422	360	148	6.7	6.83	4.79	96	110
63	5036/91	75	21.9	7.6	1	519	525	234	9.27	9.26	6.63	96	170
64	462930/92	74	53.8	21.3	2	291	344	109	3.56	3.56	2.9	96	100
65	8482/90	69	22	15.7	3	207	246	77	4.46	6.48	3.78	96	10
66	8235/91	83	0.02	0.01	1	200	193	155	7.99	8.55	6.53	96	0
67	519/95	62	6.2	2.15	1	122	162	86	4.82	5.74	4.11	96	30

Key to table:

Path.No:	pathology specimen number for that tumour
Age:	patient age
C-erbB-2 gr:	C-erbB-2 silver grain count (grain count / mm^2 /hour)
C-erbB-2:	C-erbB-2 receptor density (x 10^6)
Run:	Batch number for tumour specimens and cell pellets
B-2 grain count:	Raw c-erbB-2 silver grain counts (hot 1&2, control)
B-2 area:	Area corresponding to grain count for each section)x 10^{-2} mm^2
Exposure:	Length of exposure time in hours
B2 IHC:	C-erbB-2 immunohistochemistry score

Table 2: Results for EGFr expression - radioimmunohistochemistry and immunohistochemistry

Patient	Path.No	EGFr gr	EGFr	EGFr grain count			EGFr area			Exposure	EGFr IHC
				1	2	Control	1	2	Control		
1	2889/94	250	1.5	359	293	58	4.18	3.48	2.3	24	110
2	9934/94	164	1	1350	907	281	3.17	3.13	3.12	164	120
3	8747/90	234	11	1840	2910	274	4.82	5.54	3.67	164	190
4	7278/90	142	1.8	1280	1610	562	4.71	5.14	3.59	96	100
5	6334/92	1108	4.6	1580	1750	111	5	6.16	3.42	24	290
6	11820/90	115	0.5	945	957	169	3.34	3.81	2.2	164	75
7	11569/90	77	0.3	980	1000	357	4.87	3.6	3.34	164	60
8	11962/91	81	0.4	1070	1320	386	3.82	4.64	2.57	164	60
9	8819/95	79	1	778	1250	428	5.25	5.57	3.85	96	40
10	10333/95	80	1	1940	1980	463	13.5	13.5	6.75	96	90
11	2944/94	153	0.9	2000	2040	243	5.87	5.83	2.58	164	120
12	11951/90	25600	106	4310	4940	170	4.36	4.15	2.7	4	300
13	6905/95	226	2.8	1320	1230	151	4.88	4.41	2.62	96	105
14	7622/91	57	0.7	1090	764	755	4.23	3.53	4.11	96	70
15	5453/94	86	0.5	1580	1580	754	4.4	4.32	3.41	164	90
16	1445/90	32	0.4	1410	771	677	5.55	5.49	4.15	96	20
17	9771/90	217	0.9	2410	1990	370	4.87	4.49	3.23	164	80
18	8756/91	1	0.001	753	775	572	6.43	6.27	4.52	96	95
19	61283/92	424	2.5	765	664	79	5.47	5.07	2.33	24	205
20	8063/94	215	0.9	2400	1710	190	5.38	3.66	1.99	164	100

Table 2 (contd)

Patient	Path.No	EGFr gr	EGFr	EGFr grain count			EGFr area			Exposure	EGFr IHC
				1	2	Control	1	2	Control		
21	921/91	205	1.2	2580	1930	282	5.63	5.01	3.21	164	100
22	6795/92	49	0.2	667	780	314	4.18	4.72	3.8	164	30
23	7787/94	210	0.9	2260	2560	442	5.11	5.09	3.45	164	130
24	10732/93	296	1.7	533	480	112	4.64	4.8	3.09	24	210
25	8417/95	49	0.6	584	965	407	4.12	5.58	3.62	96	20
26	9896/94	84	0.5	1440	1370	389	5.31	4.9	2.84	164	100
27	4104/94	54	0.2	1220	1270	536	4.07	3.72	2.32	164	110
28	1044/91	2429	10.1	837	483	136	5.48	4.95	4.63	4	295
29	1085/91	152	0.9	1700	1940	367	5.07	4.75	3.04	164	80
30	4036/95	25	0.3	752	874	412	4.82	5.13	2.96	96	100
31	169046/92	223	0.9	2280	2590	374	5.19	5.4	3.98	164	125
32	1037/92	101	0.6	1050	1790	302	4.82	4.89	2.38	164	60
33	7852/91	111	1.4	899	1400	159	8.49	9.11	6.62	96	100
34	9349/90	134	0.6	1500	1820	632	3.72	4.49	3.43	164	90
35	1659/94	142	0.8	1860	1580	573	4.32	4.83	4.02	164	120
36	8281/91	102	0.4	1030	1160	36	3.9	3.53	1.08	164	80
37	413/92	40	0.5	774	870	423	6.41	6.16	4.6	96	5
38	11622/90	207	2.6	1710	933	321	4.9	4.06	3.33	96	100
39	2514/94	105	0.6	2240	1480	564	6.74	5.4	4.2	164	100
40	1369/92	497	6.2	3620	4050	655	5.95	6.91	5.49	96	140

Table 2 (contd)

Patient	Path.No	EGFr gr	EGFr	EGFr grain count			EGFr area			Exposure	EGFr IHC
				1	2	Control	1	2	Control		
41	7459/92	320	4	2460	1370	251	5.64	4.52	3.58	96	170
42	1375/93	36	0.5	1060	1240	608	6.11	6.69	4.19	96	75
43	8970/91	169	2.1	1350	1140	294	5.17	5.49	4.15	96	30
44	10050/90	140	0.6	1620	1620	359	4.61	4.47	2.81	164	90
45	6747/94	85	0.5	1090	1520	576	3.22	4.75	3.06	164	50
46	10536/93	165	0.7	2310	1330	398	4.58	4.99	3.62	164	130
47	1252/92	80	0.5	861	1060	295	3.68	4.03	2.51	164	70
48	10578/95	165	2	1420	1840	654	5.64	5.18	4.57	96	110
49	8104/91	109	1.4	1500	1130	419	4.51	4	3.22	164	60
50	7306/92	89	0.5	1930	1640	749	5.73	5.21	4.15	164	130
51	3729/95	55	0.7	749	947	438	5.81	5.19	4.33	96	100
52	808871/94	53	0.3	1260	1260	731	4.06	4.06	3.26	164	125
53	10283/95	78	1	927	1110	386	5.3	5.16	3.22	96	60
54	10456/93	284	1.7	694	308	124	4.88	4.34	3.06	24	110
55	11126/92	134	0.6	2350	2560	1170	4.84	5.26	4.38	164	125
56	12678/94	119	0.7	1460	1690	523	4.64	4.42	3.43	164	95
57	895/95	129	0.5	866	1400	322	3.1	3.96	2.96	164	10
58	4451/92	46	0.2	801	579	330	3.3	2.96	2.27	164	100
59	3918/94	341	2	3140	3560	292	4.56	5.51	2.76	164	210
60	9649/94	775	4.6	1060	485	86	3.64	3.5	2.82	24	190

Table 2 (contd)

Patient	Path.No	EGFr gr	EGFr	EGFr grain count			EGFr area			Exposure	EGFr IHC
				1	2	Control	1	2	Control		
61	12966/94	265	1.6	2260	2180	402	3.59	3.55	2.14	164	140
62	8284/91	113	0.7	775	737	190	2.8	2.87	2.34	164	85
63	5036/91	159	0.9	1420	1320	247	4.06	3.63	2.62	164	130
64	462930/92	189	0.8	1510	1380	359	3.61	2.79	2.41	164	115
65	8482/90	381	4.7	2220	1670	76	4.94	5.1	3.58	96	60
66	8235/91	1	0.001	398	325	234	4.5	4.77	2.92	164	5
67	519/95	172	1	1630	1160	181	4.99	3.12	2.93	164	130

Key to table:

Path.No:	pathology specimen number for that tumour
EGFr gr:	EGFr silver grain count (grain count / mm^2 /hour)
EGFr:	EGFR expression as a % of A431 cells
Run:	Batch number for tumour specimens and cell pellets
EGFr grain count:	Raw EGFr silver grain counts (hot 1&2, control)
EGFr area:	Area corresponding to grain count for each section)x 10^{-2} mm^2
Exposure:	Length of exposure time in hours
EGFr IHC:	EGFr immunohistochemistry score

Table 3: Clinico-pathological information, FISH results and grouping for EGFr and c-erbB-2 in survival curves

Patient	Site	Serosa	Nodes	Nodes%	Type	Liver metastases presentation	Liver metastases any	Survival	Status	FISH	egfr2	egfr3	B2 gp2	B2 gp3	Sex
1	2	2	2	3	2	1	1	4	2	*	2	3	1	1	1
2	2	2	2	2	3	1	1	36	1	*	1	2	1	1	1
3	3	2	2	2	1	1	1	15	2	1	1	2	2	3	1
4	3	2	2	*	3	1	1	36	1	1	2	3	2	3	2
5	2	1	1	1	2	1	1	36	1	*	2	3	1	2	2
6	1	2	2	*	2	1	1	8	2	*	1	1	1	2	2
7	2	2	2	2	3	1	1	36	1	*	1	1	1	1	1
8	3	2	2	3	2	1	*	*	1	2	1	1	2	3	1
9	3	2	2	3	3	1	1	8	1	1	1	2	1	1	1
10	2	2	2	3	3	1	1	4	1	1	1	2	2	3	2
11	3	2	2	2	3	1	1	33	1	*	1	2	1	1	1
12	2	2	2	3	3	1	1	1	2	*	2	3	1	2	2
13	2	2	2	2	3	1	1	36	1	*	2	3	2	3	2
14	1	2	2	3	3	1	2	24	2	*	1	2	1	1	1
15	1	2	2	3	3	1	1	1	2	*	1	1	1	1	1
16	1	2	2	3	3	1	1	1	2	1	1	1	1	2	1
17	2	2	1	1	1	1	1	36	1	*	1	2	1	1	1
18	1	2	2	*	3	1	2	7	2	2	1	1	2	3	1
19	1	2	2	*	2	1	2	12	2	2	2	3	2	3	2
20	3	2	1	1	3	1	1	1	2	*	1	2	1	2	2

Table 3: Clinico-pathological information, FISH results and grouping for EGFr and c-erbB-2 in survival curves

Patient	Site	Serosa	Nodes	Nodes%	Type	Liver metastases		Survival	Status	FISH	egfr2	egfr3	B2 gp2	B2 gp3	Sex
						presentation	any								
21	3	2	2	2	3	1	2	24	2	2	2	3	2	3	2
22	2	2	2	2	3	1	1	36	1	*	1	1	1	2	1
23	1	1	1	1	2	1	1	18	1	2	1	2	2	3	1
24	1	2	1	1	2	1	1	36	1	2	2	3	2	3	1
25	2	2	2	3	3	1	2	16	1	1	1	2	2	3	1
26	2	1	2	2	2	1	1	36	1	2	1	1	2	3	1
27	3	2	2	3	3	1	1	5	2	*	1	1	1	2	1
28	3	2	2	2	3	1	1	4	2	*	2	3	1	2	1
29	2	2	1	1	3	1	1	1	2	*	1	2	1	1	2
30	3	2	1	1	3	1	1	36	1	1	1	1	1	2	1
31	2	2	2	2	2	2	2	12	2	2	1	2	2	3	1
32	2	2	2	3	3	1	1	8	2	*	1	2	1	1	1
33	3	2	1	1	3	1	1	24	2	*	2	3	1	2	1
34	1	2	1	1	3	1	1	2	2	*	1	2	1	2	1
35	1	2	1	1	3	1	1	24	1	2	1	2	1	2	2
36	2	2	2	2	1	1	*	*	1	*	1	1	1	2	1
37	2	2	2	3	3	1	1	6	2	1	1	1	2	3	2
38	1	2	2	3	2	1	1	8	2	*	2	3	1	1	1
39	2	2	2	2	3	2	2	17	1	*	1	2	1	1	1
40	1	2	1	1	2	1	2	8	2	1	2	3	1	1	1

Table 3: Clinico-pathological information, FISH results and grouping for EGFr and c-erbB-2 in survival curves

Patient	Site	Serosa	Nodes	Nodes%	Type	Liver metastases presentation	Liver metastases any	Survival	Status	FISH	egfr2	egfr3	B2 gp2	B2 gp3	Sex
41	1	2	2	3	2	1	1	6	2	*	2	3	2	3	1
42	1	1	1	1	3	1	1	36	1	1	1	1	2	3	1
43	1	2	2	2	3	1	1	36	1	1	2	3	1	2	1
44	2	2	2	3	3	1	1	3	2	*	1	2	2	3	1
45	2	2	2	2	3	1	2	20	1	*	1	1	1	1	2
46	2	2	2	3	3	1	2	24	2	1	1	2	2	3	2
47	2	2	2	3	1	1	1	6	2	2	1	1	2	3	1
48	1	2	2	2	1	1	1	36	1	*	2	3	2	3	2
49	2	2	2	2	3	1	*	*	1	*	2	3	1	2	1
50	1	2	1	1	3	1	1	18	2	*	1	1	1	1	1
51	3	2	1	1	3	2	2	5	2	*	1	2	1	2	2
52	1	2	2	*	3	1	1	6	1	*	1	1	1	1	1
53	1	2	2	2	2	1	1	17	1	*	1	2	1	2	1
54	1	2	2	3	3	1	1	1	2	*	2	3	1	1	1
55	2	2	2	3	3	1	1	2	2	*	1	2	1	1	1
56	2	2	2	3	3	1	1	1	2	*	1	2	1	2	1
57	2	2	2	3	3	1	1	4	1	*	1	1	1	1	1
58	1	2	2	2	2	1	2	24	2	*	1	1	1	2	1
59	3	1	1	1	3	1	1	15	1	*	2	3	1	2	1
60	1	2	2	2	2	1	1	16	1	*	2	3	1	1	1

Table 3: Clinico-pathological information, FISH results and grouping for EGFr and c-erbB-2 in survival curves

Patient	Site	Serosa	Nodes	Nodes%	Type	Liver metastases presentation	Liver metastases any	Survival	Status	FISH	egfr2	egfr3	B2 gp2	B2 gp3	Sex
61	3	2	2	2	2	1	1	23	1	*	2	3	1	1	1
62	2	2	2	3	3	1	1	6	2	*	1	2	1	2	2
63	2	2	2	3	3	1	1	2	2	*	1	2	1	2	2
64	3	2	1	1	2	1	1	36	1	*	1	2	2	3	2
65	1	2	2	2	3	1	1	8	2	*	2	3	1	2	1
66	2	2	1	1	3	1	1	8	2	1	1	1	1	1	1
67	2	2	2	2	3	1	1	1	1	*	1	2	1	1	1

Key to table:

Patient:	patient number
Site:	location of tumour (1=cardia, 2=body, 3=antrum)
Serosa:	serosal involvement (1=no, 2=yes)
Nodes:	nodal involvement (1=negative, 2=positive)
Nodes%:	Percentage nodal involvement (1=0, 2=<50%, 3=>50%,*=unknown
Type:	Tumour differentiation (1=well, 2=moderate, 3=poor)
Liver metastases:	liver metastases at presentation or at any time (1=no, 2=yes)
Survival:	length of survival in months to end of period of study
Status:	alive=1,dead=2
FISH:	amplification of c-erbB-2)1=no, 2=yes, *=unknown)
egfr2:	EGFr measurement grouped 1=lower 70%,2=highest 30%
egfr3:	EGFr measurement 1=lowest 30%,2=intermediate group,3=highest 30%
B2 gp2:	c-erbB-2 measurement grouped 1=lower 70%,2=highest 30%
B2 gp3:	c-erbB-2 measurement 1=lowest 30%,2=intermediate group,3=highest 30%
Sex:	male =1, female =2

Table 4: Results for control tissue (gastric mucosa) and cancer cell lines - RIHC and IHC

Control tissue	EGFr grain count			Area			Exposure	EGFr IHC
	1	2	Control	1	2	Control		
9934/94 mucosa	3610	2270	789	13.5	13.5	6.75	96	60
7787/94 mucosa	4080	3520	1060				96	105
9896/94 mucosa	1800	1950	574				96	100
4104/94 mucosa	1260	1780	598				96	40
RUN 1								
A431	11400	7700	194	13.5	13.5	6.75	4	
BT20	2550	1650	124				4	
EJ	6180	5860	225				24	
SKBR3	3000	2510	222				24	
ZR75	1930	2340	682				164	
RUN 2								
A431	13900	13300	300				4	
BT20	2160	2240	188				4	
EJ	7860	5650	187				24	
SKBR3	2380	3140	242				24	
ZR75	2220	1970	375				164	
RUN 3								
A431	3550	5510	95				4	
BT20	912	1190	52				4	
EJ	547	712	44				24	
SKBR3	910	1020	108				24	
ZR75	2350	1590	313				96	
BT474	6910	5930	130				96	
MDA-MB-453	1240	1980	68				96	
MCF7	1290	1370	926				96	
MDA-MB-231	13600	10300	135				96	
MDA-MB-361	1240	130	213				96	

Key to table:

EGFr grain count:	Raw EGFr silver grain counts (hot 1&2, control)
Run:	Batch number for tumour specimens and cell pellets
Area:	Area corresponding to grain count for each section)x 10^{-2} mm^2
Exposure:	Length of exposure time in hours
EGFR IHC:	EGFr immunohistochemistry score

Table 4: Results for control tissue (gastric mucosa) and cancer cell lines - RIHC and IHC

Control tissue	**C-erbB-2 grain count**			**Area**			**Exposure**	**B2 IHC**
	1	2	Control	1	2	Control		
9934/94 MUCOSA	141	245	104	5.82	5.92	4.11	96	70
7787/94 MUCOSA	301	95	79	5.5	5.45	3.75	96	90
9896/94 MUCOSA	291	187	81	5.8	5.99	4.09	96	50
4104/94 MUCOSA	93	43	54	3.58	3.51	2.58	96	0
RUN 1								
N87	2030	2340	143	13.5	13.5	6.75	4	
BT474	1050	1000	93				4	
MDA-MB-361	1070	1070	125				24	
ZR75	1060	1150	146				96	
MCF7	537	564	248				96	
RUN 2								
N87	2460	2750	64				4	
BT474	1620	1780	111				4	
MDA-MB-361	930	1280	111				24	
ZR75	595	512	131				96	
MCF7	476	257	169				96	
RUN 3								
N87	2390	2040	83				4	
BT474	1340	1290	69				4	
MDA-MB-361	647	782	100				24	
ZR75	625	378	183				96	
MCF7	397	326	158				96	

Key to table:

C-erbB-2 grain count:	Raw c-erbB-2 silver grain counts (hot 1&2, control)
Run:	Batch number for tumour specimens and cell pellets
Area:	Area corresponding to grain count for each section)x 10^{-2} mm^2
Exposure:	Length of exposure time in hours
B2 IHC:	C-erbB-2 immunohistochemistry score

Data sheet 1 Growth of MKN 45 cells in serum free medium 50,000 cells per well

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN COUNT	MEAN OF REPLICATES	
DAY 0	10%FCS	2771	2846	2933	71250		
DAY 0	10%FCS	4840	4934	4904	122316.67		
DAY 0	10%FCS	3406	3335	3394	84458.333	97963	
DAY 0	10%FCS	4500	4405	4491	111633.33	18364	
DAY 0	10%FCS	3881	3963	3804	97066.667	2	
DAY 0	10%FCS	4093	3989	4044	101050	8213	
DAY 0	10%FCS	2953	3085	2936	74783.333		
DAY 0	10%FCS	3982	4038	3911	99425		
DAY 0	10%FCS	3174	3176	3211	79675	93004	
DAY 0	10%FCS	5141	5240	5082	128858.33	19616	
DAY 0	10%FCS	3630	3637	3715	91516.667	2	
DAY 0	10%FCS	3422	3334	3296	83766.667	8773	
DAY 0	10%FCS	4626	4632	4666	116033.33		
DAY 0	10%FCS	4325	4374	4331	108583.33		
DAY 0	10%FCS	3654	3297	3528	87325	102197	
DAY 0	10%FCS	4447	4546	4439	111933.33	16842	
DAY 0	10%FCS	4555	4578	4559	114100	2	
DAY 0	10%FCS	2991	2979	3055	75208.333	7532	
DAY 0	10%FCS	2539	2470	2379	61566.667		
DAY 0	10%FCS	3804	3855	3885	96200		
DAY 0	10%FCS	4778	4740	4737	118791.67	99154	98080
DAY 0	10%FCS	3776	3891	3745	95100	20791	18001
DAY 0	10%FCS	4296	4375	4291	108016.67	2	5
DAY 0	10%FCS	4657	4688	4485	115250	9298	3754
DAY 3	HITS						
DAY 3	HITS	6916	7009	7221	176216.67	175539	
DAY 3	HITS	6859	7000	6818	172308.33	2951	
DAY 3	HITS	7151	6948	7272	178091.67	1	
DAY 3	HITS					2086	
DAY 3	HITS						
DAY 3	HTS	5441	5401	5171	133441.67	151417	
DAY 3	HTS	7262	7312	7365	182825	21316	
DAY 3	HTS	6623	6485	6547	163791.67	2	
DAY 3	HTS	5674	5650	5693	141808.33	10658	
DAY 3	HTS						
DAY 3	HTS	5513	5343	5370	135216.67		
DAY 3	HITS	6094	6189	6127	153416.67	174382	
DAY 3	HITS	6223	6346	6198	156391.67	18584	
DAY 3	HITS					2	
DAY 3	HITS	7321	7393	7231	182875	9292	
DAY 3	HITS	7342	7396	7246	183200		
DAY 3	HITS	7938	7854	7731	196025		
DAY 3	HTS	4301	4352	4129	106516.67	176668	
DAY 3	HTS	7473	7671	7407	187925	42590	
DAY 3	HTS	6701	6785	6932	170150	2	
DAY 3	HTS	8333	8115	8243	205758.33	21295	
DAY 3	HTS	8965	8074		212987.5		
DAY 3	HTS						
DAY 6	HITS	5872	6111	6218	151675		
DAY 6	HITS	4784	5331	5079	126616.67		
DAY 6	HITS	6044	6086	5832	149683.33	153646	

DAY 6	HITS	6013	6030	6044	150725	17233
DAY 6	HITS	7071	7249	7041	178008.33	2
DAY 6	HITS	6605	6684	6531	165166.67	7707
DAY 6	HTS	7316	7826	7951	192441.67	
DAY 6	HTS					200896
DAY 6	HTS	6339	6212	6468	158491.67	35183
DAY 6	HTS	8471	8327	8370	209733.33	2
DAY 6	HTS	9834	9612	9704	242916.67	20313
DAY 6	HTS					
DAY 6	HITS					200922
DAY 6	HITS	6800	6704	6789	169108.33	19630
DAY 6	HITS	8502	8338	8222	208850	2
DAY 6	HITS	8922	8904	8779	221708.33	9815
DAY 6	HITS	7862	7837	8136	198625	
DAY 6	HITS	8156	8407	8195	206316.67	
DAY 6	HTS	8872	8792	8777	220341.67	
DAY 6	HTS	7298	7524	8082	190866.67	
DAY 6	HTS	8149	8111	8062	202683.33	212969
DAY 6	HTS	9022	9185	10037	235366.67	15611
DAY 6	HTS	9216	8719	8461	219966.67	2
DAY 6	HTS	8397	8472	8162	208591.67	6981
DAY10	HITS					
DAY10	HITS	4592	4462	4368	111850	
DAY10	HITS	5224	5429	5433	134050	175172
DAY10	HITS	7383	7177	7299	182158.33	52545
DAY10	HITS	8171	8257	8458	207383.33	2
DAY10	HITS	9526	9695	9629	240416.67	26273
DAY10	HTS	6665	6493	6529	164058.33	
DAY10	HTS	6189	6139	6146	153950	
DAY10	HTS	7072	7181	7015	177233.33	197279
DAY10	HTS	7977	8193	7886	200466.67	41520
DAY10	HTS	8825	8898	9061	223200	2
DAY10	HTS	10668	10627	10477	264766.67	18568
DAY10	HITS	7516	7544	7721	189841.67	
DAY10	HITS	6691	6842	6757	169083.33	204373
DAY10	HITS					39764
DAY10	HITS					2
DAY10	HITS	7987	7824	7865	197300	22958
DAY10	HITS	10365	10383	10604	261266.67	
DAY10	HTS	6710	6635	6507	165433.33	
DAY10	HTS	7000	6842	6862	172533.33	
DAY10	HTS	6176	6989	6092	160475	200769
DAY10	HTS	7766	7665	7752	193191.67	47170
DAY10	HTS	9334	9316	9204	232116.67	2
DAY10	HTS	11041	11170	11493	280866.67	21095

Data sheet 2 Growth of N 87 cells in serum free medium 40,000 cells per well

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN COUNT	MEAN OF REPLICATES
DAY 0	10% FCS	1940	1997	1913	48750	
DAY 0	10% FCS	1729	1791	1721	43675	
DAY 0	10% FCS	2887	2916	2823	71883.333	57379
DAY 0	10% FCS	1581	1607	1711	40825	14649
DAY 0	10% FCS	2526	2761	2604	65758.333	2
DAY 0	10% FCS	2977	2898	2931	73383.333	6551
DAY 0	10% FCS	1247	1222	1179	30400	
DAY 0	10% FCS	2379	2492	2307	59816.667	
DAY 0	10% FCS	1864	1813	1829	45883.333	42561
DAY 0	10% FCS	1238	1222	1115	29791.667	12882
DAY 0	10% FCS	1423	1294	1450	34725	2
DAY 0	10% FCS	2207	2257	2106	54750	5761
DAY 0	10% FCS	773	787	765	19375	
DAY 0	10% FCS	1102	1073	1065	27000	
DAY 0	10% FCS	2089	2047	1992	51066.667	30946
DAY 0	10% FCS	735	697	725	17975	14611
DAY 0	10% FCS	1919	1893	1897	47575	2
DAY 0	10% FCS	950	890	882	22683.333	6534
DAY 0	10% FCS	740	687	637	17200	
DAY 0	10% FCS	860	900	929	22408.333	
DAY 0	10% FCS	798	756	822	19800	19676
DAY 0	10% FCS	845	901	918	22200	3970
DAY 0	10% FCS	920	931	963	23450	2
DAY 0	10% FCS	580	466	514	13000	1775
DAY 3	HITS	3708	3698	3788	93283.333	
DAY 3	HITS	3869	3755		95300	98664
DAY 3	HITS	3894	4002		98700	5814
DAY 3	HITS	4383	4392		109687.5	2
DAY 3	HITS	3937	3984		99012.5	2600
DAY 3	HITS	3923	3757		96000	
DAY 3	HTS	3146	3111		78212.5	88181
DAY 3	HTS	3530	3574		88800	8268
DAY 3	HTS	3331	3366		83712.5	2
DAY 3	HTS	3388	3323		83887.5	3698
DAY 3	HTS	3699	3724		92787.5	
DAY 3	HTS	4037	4098		101687.5	
DAY 3	HITS	3154	3191		79312.5	84188
DAY 3	HITS	3120	2967		76087.5	8848
DAY 3	HITS	3304	3323		82837.5	2
DAY 3	HITS	3429	3241		83375	3957
DAY 3	HITS	3269	3302		82137.5	
DAY 3	HITS	4128	3982		101375	
DAY 3	HTS	2833	2817		70625	48115
DAY 3	HTS	1821	1737		44475	16574
DAY 3	HTS	2703	2568		65887.5	2
DAY 3	HTS	1698	1720		42725	7412
DAY 3	HTS	1438	1415		35662.5	
DAY 3	HTS	1144	1201		29312.5	
DAY 6	HITS	4761	4716	4853	119416.67	
DAY 6	HITS	5858	5815	5737	145083.33	
DAY 6	HITS	6025	5952	5877	148783.33	159453

DAY 6	HITS	6816	6811	6862	170741.67	26447
DAY 6	HITS	7380	7381	7565	186050	2
DAY 6	HITS	7415	7547	7435	186641.67	11828
DAY 6	HTS	4442	4348	4514	110866.67	
DAY 6	HTS	5176	5175	5098	128741.67	130546
DAY 6	HTS	5704	5845	5566	142625	13695
DAY 6	HTS	4826	4821	4810	120475	2
DAY 6	HTS	5295	5288	5360	132858.33	6125
DAY 6	HTS	5929	5974	5822	147708.33	
DAY 6	HITS	4984	4805	4942	122758.33	141149
DAY 6	HITS	5064	5072	5046	126516.67	23906
DAY 6	HITS	5486	5438	5464	136566.67	2
DAY 6	HITS	5519	5461	5449	136908.33	10691
DAY 6	HITS	5390	5413	5481	135700	
DAY 6	HITS	7100	7444	8069	188441.67	
DAY 6	HTS	3836	3875	3689	95000	
DAY 6	HTS	3822	3839	3801	95516.667	
DAY 6	HTS	2796	2788	2716	69166.667	75453
DAY 6	HTS	2584	2557	2454	63291.667	15733
DAY 6	HTS	2841	2703	2788	69433.333	2
DAY 6	HTS	2455	2337	2445	60308.333	7036
DAY10	HITS	9244	9329	9657	235250	
DAY10	HITS	10884	10483	10582	266241.67	
DAY10	HITS	9265	9305	9050	230166.67	284876
DAY10	HITS	13508	13608	13527	338691.67	54242
DAY10	HITS	11060	11168	11001	276908.33	2
DAY10	HITS	14323	14627	14490	362000	24258
DAY10	HTS	9742	9409	9401	237933.33	
DAY10	HTS	10782	10942	10865	271575	
DAY10	HTS	10532	10546	10610	264066.67	261364
DAY10	HTS	9464	9564	9665	239108.33	18440
DAY10	HTS	10713	10886	11959	279650	2
DAY10	HTS	12108	11758	9236	275850	8246
DAY10	HITS	9519	9524	9918	241341.67	
DAY10	HITS	10557	10772	10745	267283.33	294168
DAY10	HITS	10500	10561	10221	260683.33	44669
DAY10	HITS	12113	12371	12302	306550	2
DAY10	HITS	13475	13597	13399	337258.33	19977
DAY10	HITS	14132	14063	14032	351891.67	
DAY10	HTS	9850	9736	10053	246991.67	
DAY10	HTS	8731	8654	8637	216850	
DAY10	HTS	7921	7976	7982	198991.67	188094
DAY10	HTS	6094	6201	6057	152933.33	43748
DAY10	HTS	7531	7514	7431	187300	2
DAY10	HTS	5039	5144	4877	125500	19565

Data sheet 3 Growth of N 87 cells in serum free medium 40,000 cells per well

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES
DAY O	HITS	430	408	435	10608	12478
DAY O	HITS	573	546	599	14317	1880
DAY O	HITS	515	489	486	12417	2
DAY O	HITS	474	455	433	11350	841
DAY O	HITS	452	458	409	10992	
DAY O	HITS	596	600	626	15183	
DAY O	HITS	440	478	462	11500	11339
DAY O	HITS	359	406	415	9833	1404
DAY O	HITS	450	465	469	11533	2
DAY O	HITS	375	365	416	9633	628
DAY O	HITS	500	476	494	12250	
DAY O	HITS	506	522	566	13283	
DAY O	HITS	293	219	256	6400	7206
DAY O	HITS	286	296	277	7158	904
DAY O	HITS	349	337	344	8583	2
DAY O	HITS	291	325	321	7808	404
DAY O	HITS	230	271	234	6125	
DAY O	HITS	294	281	284	7158	
DAY O	HITS	312	272	301	7375	7569
DAY O	HITS	275	334	277	7383	821
DAY O	HITS	235	241	276	6267	2
DAY O	HITS	301	304	314	7658	367
DAY O	HITS	318	327	311	7967	
DAY O	HITS	365	346	341	8767	
DAY 3	HITS	1068	1070	1061	26658	30336
DAY 3	HITS	1077	1087	1213	28142	4745
DAY 3	HITS	1046	1017	1005	25567	2
DAY 3	HITS	1282	1167	1230	30658	2122
DAY 3	HITS	1232	1344	1322	32483	
DAY 3	HITS	1481	1593	1547	38508	
DAY 3	HTS	1054	1049	1046	26242	26268
DAY 3	HTS	1014	1001	1077	25767	2570
DAY 3	HTS	1176	1146	1255	29808	2
DAY 3	HTS	1133	1130	1175	28650	1150
DAY 3	HTS	909	945	961	23458	
DAY 3	HTS	926	944	972	23683	
DAY 3	HITS	680	690	711	17342	25463
DAY 3	HITS	1266	1263	1342	32258	6102
DAY 3	HITS	1040	1009	1079	26067	2
DAY 3	HITS	1263	1245	1224	31100	2729
DAY 3	HITS	1021	1081	1122	26867	
DAY 3	HITS	725	777	795	19142	
DAY 3	HTS	582	583	611	14800	13196
DAY 3	HTS	472	710	350	12767	1362
DAY 3	HTS	496	483	497	12300	2
DAY 3	HTS	570	589	577	14467	609
DAY 3	HTS	536	551	548	13625	
DAY 3	HTS	436	472	438	11217	
DAY 5	HITS	1631	1667	1681	41492	54756
DAY 5	HITS	2624	2610	2588	65183	8899
DAY 5	HITS	1982	1964	1907	48775	2

DAY 5	HITS	2157	2090	1999	52050	3980
DAY 5	HITS	2341	2346	2461	59567	
DAY 5	HITS	2493	2386	2497	61467	
DAY 5	HTS	1764	1724	1751	43658	37446
DAY 5	HTS	1100	1048	988	26133	12169
DAY 5	HTS	2189	2189	2235	55108	2
DAY 5	HTS	1604	1552	1520	38967	5442
DAY 5	HTS	1572	1571	1580	39358	
DAY 5	HTS	882	862	830	21450	
DAY 5	HITS	1479	1568	1478	37708	33972
DAY 5	HITS	704	736	699	17825	20203
DAY 5	HITS	2658	2546	2686	65750	2
DAY 5	HITS	348	311	272	7758	9035
DAY 5	HITS	1730	1657	1674	42175	
DAY 5	HITS	1297	1281	1336	32617	
DAY 5	HTS	844	781	799	20200	15664
DAY 5	HTS	632	551	611	14950	4907
DAY 5	HTS	808	788	841	20308	2
DAY 5	HTS	310	328	332	8083	2195
DAY 5	HTS	735	728	742	18375	
DAY 5	HTS	521	429	498	12067	
DAY 7	HITS	3939	4180	4314	103608	115904
DAY 7	HITS	5199	5309	5102	130083	22259
DAY 7	HITS	5983	5876	5973	148600	2
DAY 7	HITS	5023	4903	4857	123192	9955
DAY 7	HITS	3362	3502	3578	87017	
DAY 7	HITS	4115	4114	4122	102925	
DAY 7	HTS	4454	4479	4472	111708	101350
DAY 7	HTS	4416	4528	4455	111658	14061
DAY 7	HTS	3126	3154	3140	78500	2
DAY 7	HTS	3864	3887	3925	97300	6288
DAY 7	HTS	3790	3728	3768	94050	
DAY 7	HTS	4571	4489	4726	114883	
DAY 7	HITS	5655	5756	5670	142342	105551
DAY 7	HITS	4881	4816	4732	120242	45363
DAY 7	HITS	2648	2537	2555	64500	2
DAY 7	HITS	4818	5015	4942	123125	20287
DAY 7	HITS	5788	6004	5947	147825	
DAY 7	HITS	1390	1418	1425	35275	
DAY 7	HTS	2861	2964	3034	73825	42556
DAY 7	HTS	2339	2288	2261	57400	34754
DAY 7	HTS	3618	3495	3411	87700	2
DAY 7	HTS	655	671	631	16308	15542
DAY 7	HTS	407	455	417	10658	
DAY 7	HTS	384	383	366	9442	

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES
DAY O	HITS	522	511	491	12700	14910
DAY O	HITS	605	623	624	15433.3	2084
DAY O	HITS	561	539	575	13958.3	2
DAY O	HITS	665	746	695	17550	1203
DAY O	HTS	604	581	579	14700	15469
DAY O	HTS	707	763	727	18308.3	2893
DAY O	HTS	483	480	448	11758.3	2
DAY O	HTS	690	671	692	17108.3	1670
DAY O	HTS	767	803	817	19891.7	21431
DAY O	HTS	847	836	810	20775	1524
DAY O	HTS	899	933	984	23466.7	2
DAY O	HTS	874	888	829	21591.7	880
DAY O	HTS	758	689	761	18400	18663
DAY O	HTS	791	821	811	20191.7	5464
DAY O	HTS	952	1026	974	24600	2
DAY O	HTS	452	464	459	11458.3	3155
DAY O	HTS	961	931	993	24041.7	22975
DAY O	HTS	1099	1095	1038	26933.3	3729
DAY O	HTS	909	912	932	22941.7	2
DAY O	HTS	751	718	689	17983.3	2153
DAY O	HTS	961	934	960	23791.7	24708
DAY O	HTS	1066	980	1014	25500	1564
DAY O	HTS	1076	1083	1019	26483.3	2
DAY O	HTS	936	901	930	23058.3	903
DAY 3	HITS	1433	1431	1412	35633.3	48871
DAY 3	HITS	2100	2040	2133	52275	9057
DAY 3	HITS	2076	2058	2040	51450	2
DAY 3	HITS	2278	2208	2249	56125	5229
DAY 3	100nM IGF1	1256	1227	1278	31341.7	35308
DAY 3	100nM IGF1	1253	1282	1256	31591.7	6569
DAY 3	100nM IGF1	1779	1840	1791	45083.3	2
DAY 3	100nM IGF1	1317	1329	1340	33216.7	3793
DAY 3	10nM IGF1	1825	1814	1773	45100	50481
DAY 3	10nM IGF1	1858	1832	1837	46058.3	5754
DAY 3	10nM IGF1	2215	2136	2154	54208.3	2
DAY 3	10nM IGF1	2241	2241	2305	56558.3	3322
DAY 3	1nM IGF1	1771	1738	1660	43075	41615
DAY 3	1nM IGF1	1841	1904	1830	46458.3	4215
DAY 3	1nM IGF1	1593	1578	1682	40441.7	2
DAY 3	1nM IGF1	1471	1504	1403	36483.3	2434
DAY 3	0.1nM IGF1	1369	1376	1439	34866.7	39933
DAY 3	0.1nM IGF1	2053	2096	2066	51791.7	10436
DAY 3	0.1nM IGF1	1861	1736	1782	44825	2
DAY 3	0.1nM IGF1	1139	1120	1131	28250	6025
DAY 3	HTS	1791	1694	1811	44133.3	38492
DAY 3	HTS	2037	2046	2096	51491.7	14174
DAY 3	HTS	1605	1585	1592	39850	2
DAY 3	HTS	821	718	680	18491.7	8183
DAY 5	HITS	3016	3093	3009	75983.3	94577
DAY 5	HITS	3782	3639	3009	86916.7	15980
DAY 5	HITS	4071	4242	4261	104783	2

DAY 5	HITS	4514	4411	4350	110625	9226
DAY 5	100nM	3117	3259	3157	79441.7	76646
DAY 5	100nM	3012	2946	3103	75508.3	3291
DAY 5	100nM	2898	2922	2880	72500	2
DAY 5	100nM	3270	3145	3081	79133.3	1900
DAY 5	10nM	4045	4022	4133	101667	92781
DAY 5	10nM	3409	3460	3364	85275	9733
DAY 5	10nM	3949	4002	4132	100692	2
DAY 5	10nM	3418	3281	3320	83491.7	5619
DAY 5	1nM	2917	2787	2808	70933.3	70363
DAY 5	1nM	3513	3578	3700	89925	30851
DAY 5	1nM	3724	3682	3872	93983.3	2
DAY 5	1nM	1110	1066	1017	26608.3	17812
DAY 5	0.1nM	2056	2177	2129	53016.7	58969
DAY 5	0.1nM	3377	3368	3405	84583.3	23292
DAY 5	0.1nM	2753	2743	2713	68408.3	2
DAY 5	0.1nM	1230	1195	1159	29866.7	13448
DAY 5	HTS	1258	1222	1179	30491.7	35829
DAY 5	HTS	2011	2020	1968	49991.7	13313
DAY 5	HTS	1773	1668	1707	42900	2
DAY 5	HTS	799	783	810	19933.3	7686

DAY	TREATMENT	COUNT 1	COUNT 2	COUNT 3	MEAN	MEAN OF	
		DILUTION	100			REPLICATES	
DAY O	HITS	2288	2437	2336	235367	412322	412322
DAY O	HITS	4985	5052	5055	503067	153265	153265
DAY O	HITS					1	1
DAY O	HITS	4974	5014	4968	498533	108375	108375
DAY O	HTS	4121	4181	4131	414433	429289	
DAY O	HTS	4098	4035	4022	405167	34072	
DAY O	HTS					1	
DAY O	HTS	4652	4712	4684	468267	24093	
DAY O	HTS	4836	4743	4765	478133	459083	
DAY O	HTS	4823	4867	4753	481433	24171	
DAY O	HTS	4451	4423	4400	442467	2	
DAY O	HTS	4365	4305	4359	434300	13955	
DAY O	HTS					471300	
DAY O	HTS	4921	4686	4570	472567	1791	
DAY O	HTS	4694	4687	4720	470033	1	
DAY O	HTS					1791	
DAY O	HTS	4792			479200	395000	
DAY O	HTS	3810	3685	3712	373567	56893	
DAY O	HTS	3740	3700	3762	373400	2	
DAY O	HTS	3475	3650	3490	353833	32847	
DAY O	HTS	3485	3452	3395	344400	378058	421120
DAY O	HTS	4227	4207	4335	425633	34940	67190
DAY O	HTS	3732	3752	3925	380300	2	4
DAY O	HTS	3670	3540	3647	361900	20173	15414
DAY 3	HITS	3467	3564	3527	351933	403500	
DAY 3	HITS	4564	4719	4443	457533	46684	
DAY 3	HITS	3844	3851	3717	380400	2	
DAY 3	HITS	4293	4271	4160	424133	26953	
DAY 3	100nM IGF1	4696	4722	4730	471600	624017	
DAY 3	100nM IGF1	6543	6573	6528	654800	119675	
DAY 3	100nM IGF1	7627	7577	7605	760300	2	
DAY 3	100nM IGF1	6164	6188	5929	609367	69094	
DAY 3	10nM IGF1					651900	
DAY 3	10nM IGF1	6502	6571	6462	651167	80336	
DAY 3	10nM IGF1	7293	7277	7408	732600	1	
DAY 3	10nM IGF1	5749	5653	5756	571933	56806	
DAY 3	1nM IGF1	5600	5658	5638	563200	611250	
DAY 3	1nM IGF1	6344	6507	6358	640300	56265	
DAY 3	1nM IGF1	5778	5543	5636	565233	2	
DAY 3	1nM IGF1	6704	6757	6827	676267	32485	
DAY 3	0.1nM IGF1	5599	5697	5729	567500	654508	
DAY 3	0.1nM IGF1	6644	6698	6662	666800	61862	
DAY 3	0.1nM IGF1	6774	6707	6615	669867	2	
DAY 3	0.1nM IGF1	7216	7051	7149	713867	35716	
DAY 3	HTS	6082	5959	6249	609667	499025	
DAY 3	HTS	4816	4801	4750	478900	87221	
DAY 3	HTS	5057	5152	5058	508900	2	
DAY 3	HTS	3971	4092	3896	398633	50357	

DAY 5	HITS	3764	3920	3788	382400	498958	
DAY 5	HITS	3893	3862	3789	384800	135004	
DAY 5	HITS	5823	5883	5917	587433	2	
DAY 5	HITS	6434	6341	6461	641200	77945	
DAY 5	100nM	8988	9332	9475	926500	879417	
DAY 5	100nM	8488	8173	8229	829667	44956	
DAY 5	100nM	8963	9353	8891	906900	2	
DAY 5	100nM	8418	8530	8690	854600	25955	
DAY 5	10nM	9786	9811	9674	975700	970558	
DAY 5	10nM	9472	9521	9732	957500	66318	
DAY 5	10nM	8838	9117	8865	894000	2	
DAY 5	10nM	10461	10609	10581	1055033	38289	
DAY 5	1nM	7417	7369	7388	739133	782117	
DAY 5	1nM	7712	7720	7739	772367	43826	
DAY 5	1nM	8518	8449	8333	843333	2	
DAY 5	1nM	7760	7757	7692	773633	25303	
DAY 5	0.1nM	7740	7784	7766	776333	915800	
DAY 5	0.1nM	8337	8319	8453	836967	128824	
DAY 5	0.1nM	9864	10119	10402	1012833	2	
DAY 5	0.1nM	10282	10283	10547	1037067	74376	
DAY 5	HTS	8537	8474	8719	857667	794550	855175
DAY 5	HTS	8039	7862	7831	791067	101132	125284
DAY 5	HTS	8636	8723	8931	876333	2	3
DAY 5	HTS	6564	6547	6483	653133	58389	47353

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES	
DAY O	HTS	1193	1139	1180	29266.7	34083	31918
DAY O	HTS	1337	1262	1298	32475	2867	3759
DAY O	HTS	1464	1488	1448	36666.7	2	4
DAY O	HTS	1495	1486	1450	36925	1282	912
DAY O	HTS	1393	1349	1431	34775		
DAY O	HTS	1411	1367	1349	34391.7		
DAY O	HTS	1436	1438	1403	35641.7	33035	
DAY O	HTS	1297	1285	1296	32316.7	3219	
DAY O	HTS	1280	1341	1283	32533.3	2	
DAY O	HTS	1516	1502	1521	37825	1440	
DAY O	HTS	1246	1236	1238	31000		
DAY O	HTS	1152	1203	1112	28891.7		
DAY 0	HTS	1202	1201	1190	29941.7	28636	
DAY 0	HTS	1236	1220	1222	30650	3067	
DAY 0	HTS	1104	1143	1147	28283.3	2	
DAY 0	HTS	1114	1168	1154	28633.3	1372	
DAY 0	HTS	1306	1240	1227	31441.7		
DAY 0	HTS	908	930	906	22866.7		
DAY 0	HTS	1552	1493	1502	37891.7	34375	
DAY 0	HTS	1513	1488	1457	37150	3494	
DAY 0	HTS	1501	1394	1468	36358.3	2	
DAY 0	HTS	1236	1277	1241	31283.3	1563	
DAY 0	HTS	1155	1155	1186	29133.3		
DAY 0	HTS	1407	1395	1330	34433.3		
DAY 3	HTS	3485	3433	3427	86208.3	87793	
DAY 3	HTS	4135	4027	4228	103250	12833	
DAY 3	HTS	3259	3017	3064	77833.3	2	
DAY 3	HTS	4059	4032	4170	102175	5739	
DAY 3	HTS	3435	3517	3370	86016.7		
DAY 3	HTS	2888	2853	2812	71275		
DAY 3	10nM IGF1	5119	4967	4926	125100	105867	
DAY 3	10nM IGF1	4166	4039	4122	102725	14816	
DAY 3	10nM IGF1	4303	4242	4224	106408	2	
DAY 3	10nM IGF1	4018	4051	3903	99766.7	6626	
DAY 3	10nM IGF1	3355	3299	3297	82925		
DAY 3	10nM IGF1	4817	4675	4701	118275		
DAY 3	1nM IGF1	3750	3744	3751	93708.3	87690	
DAY 3	1nM IGF1	3736	3891	3899	96050	11788	
DAY 3	1nM IGF1	3707	3853	3648	93400	2	
DAY 3	1nM IGF1	3703	3742	3680	92708.3	5272	
DAY 3	1nM IGF1	3522	3383	3358	85525		
DAY 3	1nM IGF1	2601	2575	2594	64750		
DAY 3	0.1nM IGF1	3815	3846	3782	95358.3	97700	
DAY 3	0.1nM IGF1	4441	4334	4532	110892	9460	
DAY 3	0.1nM IGF1	3707	3765	3728	93333.3	2	
DAY 3	0.1nM IGF1	3387	3252	3378	83475	4231	
DAY 3	0.1nM IGF1	4228	4164	4142	104450		
DAY 3	0.1nM IGF1	3987	3972	3884	98691.7		
DAY 5	HTS	5024	5015	4914	124608	135013	
DAY 5	HTS	4767	4684	4808	118825	20069	
DAY 5	HTS	4379	4419	4434	110267	2	

DAY 5	HTS	5659	5956	5849	145533	8975
DAY 5	HTS	6321	6421	6702	162033	
DAY 5	HTS	5938	6030	5889	148808	
DAY 5	10nM IGF1	4314	4624	4421	111325	144792
DAY 5	10nM IGF1	5329	5243	5270	132017	23033
DAY 5	10nM IGF1	6225	6073	6153	153758	2
DAY 5	10nM IGF1	6389	6362	6183	157783	11517
DAY 5	10nM IGF1	6774	6789	6726	169075	
DAY 5	10nM IGF1	6375	6297	6559	160258	
DAY 5	1nM IGF1	5963	5987	5803	147942	147804
DAY 5	1nM IGF1	5358	5214	5218	131583	8732
DAY 5	1nM IGF1	6078	6030	6086	151617	2
DAY 5	1nM IGF1	5922	5977	6190	150742	3905
DAY 5	1nM IGF1	6453	6213	6239	157542	
DAY 5	1nM IGF1	6039	5892	5757	147400	
DAY 5	0.1nM IGF1	6049	6132	6243	153533	155513
DAY 5	0.1nM IGF1	6363	6474	6381	160150	8810
DAY 5	0.1nM IGF1	6045	5871	5960	148967	2
DAY 5	0.1nM IGF1	6471	6267	6382	159333	3940
DAY 5	0.1nM IGF1	5821	5687	5675	143192	
DAY 5	0.1nM IGF1	6828	6683	6637	167900	
DAY 7	HTS	5798	5766	5840	145033	167543
DAY 7	HTS	6669	6732	6583	166533	14531
DAY 7	HTS	6977	7143	7140	177167	2
DAY 7	HTS	6477	6339	6359	159792	6499
DAY 7	HTS	6537	6869	6921	169392	
DAY 7	HTS	7458	7478	7545	187342	
DAY 7	10nM IGF1	7374	7561	7460	186625	221497
DAY 7	10nM IGF1	8678	8671	8625	216450	28853
DAY 7	10nM IGF1	8920	8974	8920	223450	2
DAY 7	10nM IGF1	7947	7672	7833	195433	12903
DAY 7	10nM IGF1	9663	9831	9765	243825	
DAY 7	10nM IGF1	10546	10561	10477	263200	
DAY 7	1nM IGF1				#DIV/0!	159845
DAY 7	1nM IGF1	6082	6024	5973	150658	13247
DAY 7	1nM IGF1	7057	6777	7042	173967	2
DAY 7	1nM IGF1	6423	6541	6303	160558	6623
DAY 7	1nM IGF1	6774	6970	6799	171192	
DAY 7	1nM IGF1	5776	5701	5665	142850	
DAY 7	0.1nM IGF1	5867	5831	5721	145158	170168
DAY 7	0.1nM IGF1	6443	6393	6397	160275	19922
DAY 7	0.1nM IGF1	6267	6134	6323	156033	2
DAY 7	0.1nM IGF1	7004	7244	6931	176492	8909
DAY 7	0.1nM IGF1	7364	7392	7341	184142	
DAY 7	0.1nM IGF1	7966	7985	7918	198908	

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES	
DAY O	HTS	1283	1280	1317	32333.3	32943	32791
DAY O	HTS	1333	1311	1274	32650	1465	2040
DAY O	HTS	1382	1349	1370	34175	2	4
DAY O	HTS	1370	1410	1419	34991.7	655	495
DAY O	HTS	1248	1214	1237	30825		
DAY O	HTS	1346	1302	1274	32683.3		
DAY O	HTS	1238	1285	1281	31700	33751	
DAY O	HTS	1340	1377	1338	33791.7	1660	
DAY O	HTS	1495	1452	1466	36775	2	
DAY O	HTS	1308	1321	1363	33266.7	742	
DAY O	HTS	1357	1377	1304	33650		
DAY O	HTS	1369	1305	1325	33325		
DAY O	HTS	1272	1264	1225	31341.7	31678	
DAY O	HTS	1317	1297	1282	32466.7	2573	
DAY O	HTS	1388	1268	1220	32300	2	
DAY O	HTS	1439	1422	1412	35608.3	1151	
DAY O	HTS	1205	1211	1255	30591.7		
DAY O	HTS	1142	1099	1090	27758.3		
DAY O	HTS	1346	1337	1372	33791.7	31433	
DAY O	HTS	1297	1283	1271	32091.7	1323	
DAY O	HTS	1217	1230	1249	30800	2	
DAY O	HTS	1204	1216	1202	30183.3	592	
DAY O	HTS	1258	1209	1204	30591.7		
DAY O	HTS	1215	1272	1250	31141.7		
DAY 2	HTS	1927	1994	1938	48825	46168	
DAY 2	HTS	1958	1990	1986	49450	4602	
DAY 2	HTS	1825	1760	1718	44191.7	2	
DAY 2	HTS	1844	1878	1896	46816.7	2058	
DAY 2	HTS	1972	2034	1983	49908.3		
DAY 2	HTS	1463	1558	1517	37816.7		
DAY 2	10nM IGF1	1942	1966	1967	48958.3	57706	
DAY 2	10nM IGF1	2335	2389	2442	59716.7	4582	
DAY 2	10nM IGF1	2398	2330	2415	59525	2	
DAY 2	10nM IGF1	2273	2358	2290	57675	2049	
DAY 2	10nM IGF1	2517	2459	2498	62283.3		
DAY 2	10nM IGF1	2277	2365	2327	58075		
DAY 2	1nM IGF1	2609	2637	2435	64008.3	53933	
DAY 2	1nM IGF1	2101	2085	2110	52466.7	8222	
DAY 2	1nM IGF1	2284	2356	2243	57358.3	2	
DAY 2	1nM IGF1	2318	2365	1962	55375	3677	
DAY 2	1nM IGF1	2170	2280	2180	55250		
DAY 2	1nM IGF1	1636	1530	1531	39141.7		
DAY 2	0.1nM IGF1	2250	2235	2290	56458.3	57008	
DAY 2	0.1nM IGF1	2439	2405	2493	61141.7	2794	
DAY 2	0.1nM IGF1	2182	2178	2218	54816.7	2	
DAY 2	0.1nM IGF1	2142	2133	2125	53333.3	1250	
DAY 2	0.1nM IGF1	2313	2340	2252	57541.7		
DAY 2	0.1nM IGF1	2353	2430	2268	58758.3		
DAY 5	HTS	2921	3087	2890	74150	71203	
DAY 5	HTS	2898	2948	2930	73133.3	8410	
DAY 5	HTS	2337	2389	2189	57625	2	

DAY 5	HTS	2928	2898	2815	72008.3	3761
DAY 5	HTS	3328	3367	3272	83058.3	
DAY 5	HTS	2707	2713	2649	67241.7	
DAY 5	10nM IGF1	4923	4911	4914	122900	136997
DAY 5	10nM IGF1	5804	5900	5952	147133	13098
DAY 5	10nM IGF1	4901	4838	4975	122617	2
DAY 5	10nM IGF1	5994	5983	5828	148375	6549
DAY 5	10nM IGF1	5760	5707	5808	143958	
DAY 5	10nM IGF1	3573	3484	3370	86891.7	
DAY 5	1nM IGF1	4483	4456	4476	111792	114900
DAY 5	1nM IGF1	5075	5047	4939	125508	15528
DAY 5	1nM IGF1	4724	4758	4619	117508	2
DAY 5	1nM IGF1	5182	5279	5080	129508	6944
DAY 5	1nM IGF1	4905	4622	4782	119242	
DAY 5	1nM IGF1	3210	3750	3341	85841.7	
DAY 5	0.1nM IGF1	3915	3798	3892	96708.3	98503
DAY 5	0.1nM IGF1	4412	4421	4476	110908	16071
DAY 5	0.1nM IGF1	3718	3487	3622	90225	2
DAY 5	0.1nM IGF1	4951	4939	5031	124342	7187
DAY 5	0.1nM IGF1	3449	3402	3377	85233.3	
DAY 5	0.1nM IGF1	3362	3342	3328	83600	
DAY 7	HTS	3456	3201	3224	82341.7	95669
DAY 7	HTS	3385	3354	3404	84525	27520
DAY 7	HTS	2813	2803	2804	70166.7	2
DAY 7	HTS	5348	5026	5101	128958	12307
DAY 7	HTS	5343	5298	5221	132183	
DAY 7	HTS	3037	3062	3002	75841.7	
DAY 7	10nM IGF1	7732	7650	7670	192100	177906
DAY 7	10nM IGF1	8251	8133	8063	203725	21261
DAY 7	10nM IGF1	7724	7613	7618	191292	2
DAY 7	10nM IGF1	5898	6055	6029	149850	9508
DAY 7	10nM IGF1	6375	6255	6330	158000	
DAY 7	10nM IGF1	6831	6882	6983	172467	
DAY 7	1nM IGF1	5801	5834	5710	144542	146819
DAY 7	1nM IGF1	6352	6352	6397	159175	9950
DAY 7	1nM IGF1	5475	5529	5458	137183	2
DAY 7	1nM IGF1	6322	6439	6297	158817	4450
DAY 7	1nM IGF1	5695	5774	5810	143992	
DAY 7	1nM IGF1	5450	5515	5500	137208	
DAY 7	0.1nM IGF1	5873	5882	5960	147625	157518
DAY 7	0.1nM IGF1	6391	6303	6214	157567	16559
DAY 7	0.1nM IGF1	7223	7163	7119	179208	2
DAY 7	0.1nM IGF1	6722	6872	6695	169075	7405
DAY 7	0.1nM IGF1	6202	6302	6681	159875	
DAY 7	0.1nM IGF1	5272	5296	5243	131758	

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES	
DAY O	HTS	914	873	898	22375	23483	21251
DAY O	HTS	993	952	977	24350	1136	2640
DAY O	HTS	986	981	971	24483.3	2	4
DAY O	HTS	850	878	887	21791.7	508	640
DAY O	HTS	936	961	941	23650		
DAY O	HTS	970	952	988	24250		
DAY O	HTS	795	827	774	19966.7	20889	
DAY O	HTS	878	797	881	21300	3198	
DAY O	HTS	1031	1058	1014	25858.3	2	
DAY O	HTS	667	711	675	17108.3	1430	
DAY O	HTS	722	729	735	18216.7		
DAY O	HTS	932	924	890	22883.3		
DAY O	HTS	706	680	714	17500	19382	
DAY O	HTS	743	757	764	18866.7	1359	
DAY O	HTS	858	852	840	21250	2	
DAY O	HTS	825	730	751	19216.7	608	
DAY O	HTS	791	732	735	18816.7		
DAY O	HTS	872	787	818	20641.7		
DAY 3	HTS	4054	4065	4067	101550	104894	
DAY 3	HTS	4144	3974	4028	101217	9992	
DAY 3	HTS	4183	4132	4124	103658	2	
DAY 3	HTS	3805	3741	3669	93458.3	4468	
DAY 3	HTS	4160	4285	4291	106133		
DAY 3	HTS	4919	4919	4964	123350		
DAY 3	10nM IGF1	3675	3685	3818	93150	109290	
DAY 3	10nM IGF1	3887	3716	3823	95216.7	15061	
DAY 3	10nM IGF1	4739	4673	4711	117692	2	
DAY 3	10nM IGF1	4110	3990	3992	100767	6735	
DAY 3	10nM IGF1	4756	4631	4827	118450		
DAY 3	10nM IGF1	5177	5228	5251	130467		
DAY 3	1nM IGF1	4519	4652	4650	115175	122243	
DAY 3	1nM IGF1	4840	4827	4792	120492	10644	
DAY 3	1nM IGF1	4272	4299	4243	106783	2	
DAY 3	1nM IGF1	5080	5012	5192	127367	4760	
DAY 3	1nM IGF1	5482	5559	5460	137508		
DAY 3	1nM IGF1	5051	5032	5053	126133		
DAY 5	HTS	7129	7062	6942	176108	177150	
DAY 5	HTS	7605	7553	7550	189233	12779	
DAY 5	HTS	7075	6910	6950	174458	2	
DAY 5	HTS	6419	6337	6433	159908	5715	
DAY 5	HTS	7808	7751	7763	194350		
DAY 5	HTS	6928	6618	6715	168842		
DAY 5	10nM IGF1	7965	7872	7788	196875	215950	
DAY 5	10nM IGF1	8423	8691	8347	212175	14456	
DAY 5	10nM IGF1	9112	8847	8955	224283	2	
DAY 5	10nM IGF1	8482	8513	8624	213492	6465	
DAY 5	10nM IGF1	9738	9506	9483	239392		
DAY 5	10nM IGF1	8349	8479	8310	209483		
DAY 5	1nM IGF1	7745	7458	7596	189992	182671	
DAY 5	1nM IGF1	8189	7994	7767	199583	13343	
DAY 5	1nM IGF1	6970	7136	6840	174550	2	

DAY 5	1nM IGF1	7672	7694	7487	190442	5967
DAY 5	1nM IGF1	7239	7173	7076	179067	
DAY 5	1nM IGF1	6508	6487	6492	162392	
DAY 7	HTS	11245	10913	11178	277800	256236
DAY 7	HTS	11430	11208	11131	281408	28727
DAY 7	HTS	9296	8995	8985	227300	2
DAY 7	HTS	8492	8664	8524	214000	12847
DAY 7	HTS	10429	10403	10501	261108	
DAY 7	HTS	11067	11107	10922	275800	
DAY 7	10nM IGF1	14452	14529	14073	358783	368574
DAY 7	10nM IGF1	15961	15319	15335	388458	40236
DAY 7	10nM IGF1	17082	16768	16942	423267	2
DAY 7	10nM IGF1	12405	11836	11924	301375	17994
DAY 7	10nM IGF1	15077	15106	15012	376625	
DAY 7	10nM IGF1	14442	14562	14548	362933	
DAY 7	1nM IGF1	13509	13412	13103	333533	324144
DAY 7	1nM IGF1	14121	14021	13992	351117	36036
DAY 7	1nM IGF1	14716	14731	14505	366267	2
DAY 7	1nM IGF1	11062	10881	9604	262892	16116
DAY 7	1nM IGF1	12630	12746	12630	316717	
DAY 7	1nM IGF1	12631	12358	12732	314342	

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES
DAY O	HTS	1864	1913	1842	46825	39258
DAY O	HTS	1592	1507	1553	38766.7	4071
DAY O	HTS	1524	1524	1457	37541.7	2
DAY O	HTS	1609	1572	1664	40375	1821
DAY O	HTS	1422	1401	1460	35691.7	
DAY O	HTS	1469	1428	1465	36350	
DAY O	HTS	1743	1646	1717	42550	39411
DAY O	HTS	1507	1472	1437	36800	2210
DAY O	HTS	1670	1580	1544	39950	2
DAY O	HTS	1536	1476	1431	37025	988
DAY O	HTS	1594	1580	1554	39400	
DAY O	HTS	1660	1597	1632	40741.7	
DAY O	HTS	1695	1681	1729	42541.7	44879
DAY O	HTS	1775	1735	1657	43058.3	1924
DAY O	HTS	1785	1809	1767	44675	2
DAY O	HTS	1892	1906	1882	47333.3	860
DAY O	HTS	1826	1748	1811	44875	
DAY O	HTS	1868	1880	1867	46791.7	
DAY 3	HTS	2572	2535	2481	63233.3	60365
DAY 3	HTS	2577	2462	2500	62825	8506
DAY 3	HTS	1773	1683	1745	43341.7	2
DAY 3	HTS	2460	2477	2478	61791.7	3804
DAY 3	HTS	2561	2639	2515	64291.7	
DAY 3	HTS	2714	2602	2689	66708.3	
DAY 3	10nM IGF1	4220	4336	4329	107375	106201
DAY 3	10nM IGF1	3792	3763	3788	94525	7700
DAY 3	10nM IGF1	4332	4273	4483	109067	2
DAY 3	10nM IGF1	4063	3949	3918	99416.7	3444
DAY 3	10nM IGF1	4501	4533	4636	113917	
DAY 3	10nM IGF1	4501	4543	4505	112908	
DAY 3	1nM IGF1	3645	3710	3625	91500	85375
DAY 3	1nM IGF1	4187	4191	4110	104067	15930
DAY 3	1nM IGF1	3905	3887	4040	98600	2
DAY 3	1nM IGF1	3363	3393	3432	84900	7124
DAY 3	1nM IGF1	2713	2754	2613	67333.3	
DAY 3	1nM IGF1	2587	2682	2633	65850	
DAY 6	HTS	2547	2454	2350	61258.3	71039
DAY 6	HTS	2950	2931	2847	72733.3	8889
DAY 6	HTS	2931	2887	2961	73158.3	2
DAY 6	HTS	2481	2427	2499	61725	3975
DAY 6	HTS	2902	2809	2932	72025	
DAY 6	HTS	3417	3371	3452	85333.3	
DAY 6	10nM IGF1	4885	5029	4942	123800	138814
DAY 6	10nM IGF1	5633	5809	5626	142233	8387
DAY 6	10nM IGF1	5435	5413	5526	136450	2
DAY 6	10nM IGF1	5586	5699	5656	141175	3751
DAY 6	10nM IGF1	5534	5687	5622	140358	
DAY 6	10nM IGF1	5873	5946	6045	148867	
DAY 6	1nM IGF1	4862	5037	4873	123100	122739
DAY 6	1nM IGF1	5458	5331	5301	134083	10496
DAY 6	1nM IGF1	4803	4800	4694	119142	2

DAY 6	1nM IGF1	4916	5021	4907	123700	4694
DAY 6	1nM IGF1	5202	5243	5365	131750	
DAY 6	1nM IGF1	4171	4164	4224	104658	
DAY 8	HTS	3525	3710	3649	90700	83731
DAY 8	HTS	4172	4050	4062	102367	19615
DAY 8	HTS	2122	2036	2068	51883.3	2
DAY 8	HTS	4045	4076	4144	102208	8772
DAY 8	HTS	3440	3361	3350	84591.7	
DAY 8	HTS	2847	2867	2762	70633.3	
DAY 8	10nM IGF1	8475	8399	8357	210258	238393
DAY 8	10nM IGF1	9711	9565	9487	239692	22089
DAY 8	10nM IGF1	9118	9133	9011	227183	2
DAY 8	10nM IGF1	10264	10140	9717	251008	9878
DAY 8	10nM IGF1	11258	11182	10427	273892	
DAY 8	10nM IGF1	9200	9044	9155	228325	
DAY 8	1nM IGF1	6993	7066	6923	174850	148335
DAY 8	1nM IGF1	7796	7888	7834	195983	37122
DAY 8	1nM IGF1	4232	4310	4370	107600	2
DAY 8	1nM IGF1	6764	6779	6704	168725	16601
DAY 8	1nM IGF1	5498	5212	5544	135450	
DAY 8	1nM IGF1	4398	4238	4252	107400	

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES
DAY 0	HTS	2140	2113	2049	52516.7	47492
DAY 0	HTS	1933	1865	1806	46700	4680
DAY 0	HTS	1745	1709	1737	43258.3	1
DAY 0	HTS+DMSO	1985	1994	1982	49675	48261
DAY 0	HTS+DMSO	2022	1896	1921	48658.3	1649
DAY 0	HTS+DMSO	1858	1843	1873	46450	1
DAY 0	100RG	1744	1622	1725	42425	43306
DAY 0	100RG	1722	1651	1752	42708.3	1288
DAY 0	100RG	1865	1743	1766	44783.3	1
DAY 0	100RG+IGF1	1830	1868	1899	46641.7	45064
DAY 0	100RG+IGF1	1931	1855	1894	47333.3	3350
DAY 0	100RG+IGF1	1644	1632	1670	41216.7	1
DAY 0	50RG	1835	1845	1822	45850	44669
DAY 0	50RG	1860	1810	1864	46116.7	2280
DAY 0	50RG	1669	1695	1681	42041.7	1
DAY 0	50RG+IGF1	1835	1834	1801	45583.3	39353
DAY 0	50RG+IGF1	1656	1682	1630	41400	7468
DAY 0	50RG+IGF1	1235	1240	1254	31075	1
DAY 0	10RG	2171	2188	2127	54050	49864
DAY 0	10RG	2149	2225	2213	54891.7	7991
DAY 0	10RG	1601	1633	1644	40650	1
DAY 0	10RG+IGF1	2005	2010	1995	50083.3	47408
DAY 0	10RG+IGF1	1889	1837	1851	46475	2352
DAY 0	10RG+IGF1	1789	1839	1852	45666.7	1
DAY 0	IGF1	2202	2162	2179	54525	52586
DAY 0	IGF1	2258	2263	2240	56341.7	5014
DAY 0	IGF1	1909	1880	1838	46891.7	1
DAY 0	IGF1+DMSO	1897	1982	1956	48625	47058
DAY 0	IGF1+DMSO	1947	1883	1901	47758.3	2010
DAY 0	IGF1+DMSO	1835	1757	1783	44791.7	1
DAY 3	HTS	4200	4213	4218	105258	118353
DAY 3	HTS	4756	4731	4661	117900	13327
DAY 3	HTS	5219	5309	5300	131900	1
DAY 3	HTS+DMSO	5552	5415	5493	137167	142933
DAY 3	HTS+DMSO	5891	5894	5829	146783	5087
DAY 3	HTS+DMSO	5816	5852	5714	144850	1
DAY 3	100RG	3343	3340	3433	84300	84453
DAY 3	100RG	3634	3509	3547	89083.3	4556
DAY 3	100RG	3176	3258	3163	79975	1
DAY 3	100RG+IGF1	3393	3372	3399	84700	80586
DAY 3	100RG+IGF1	3677	3764	3739	93166.7	15065
DAY 3	100RG+IGF1	2611	2536	2520	63891.7	1
DAY 3	50RG	4903	5175	5010	125733	110403
DAY 3	50RG	4202	4165	4345	105933	13656
DAY 3	50RG	4009	4032	3904	99541.7	1
DAY 3	50RG+IGF1	4278	4301	4320	107492	101492
DAY 3	50RG+IGF1	4073	4058	3945	100633	5620
DAY 3	50RG+IGF1	3868	3881	3813	96350	1
DAY 3	10RG	5252	5275	5186	130942	128219
DAY 3	10RG	5240	5161	5165	129717	3705
DAY 3	10RG	4954	5012	4914	124000	1

DAY 3	10RG+IGF1	4798	4860	4863	121008	124569
DAY 3	10RG+IGF1	5171	5240	5275	130717	5346
DAY 3	10RG+IGF1	4838	4898	4902	121983	1
DAY 3	IGF1	5640	5611	5618	140575	134917
DAY 3	IGF1	5950	5919	5620	145742	14507
DAY 3	IGF1	4658	4740	4814	118433	1
DAY 3	IGF1+DMSO	4797	4801	4821	120158	119311
DAY 3	IGF1+DMSO	4800	4650	4702	117933	1204
DAY 3	IGF1+DMSO	4877	4778	4726	119842	1
DAY 5	HTS	6976	6910	6845	172758	173614
DAY 5	HTS	6934	6748	6811	170775	3350
DAY 5	HTS	7018	7149	7110	177308	1
DAY 5	HTS+DMSO	8062	8004	8026	200767	199536
DAY 5	HTS+DMSO	8270	8244	8238	206267	7423
DAY 5	HTS+DMSO	7533	7818	7638	191575	1
DAY 5	100RG	4063	4350	4384	106642	85211
DAY 5	100RG	3019	3095	3091	76708.3	18691
DAY 5	100RG	2884	2928	2862	72283.3	1
DAY 5	100RG+IGF1	2807	2794	2738	69491.7	64867
DAY 5	100RG+IGF1	2859	2824	2843	71050	9393
DAY 5	100RG+IGF1	2153	2158	2176	54058.3	1
DAY 5	50RG	3280	3219	3332	81925	73856
DAY 5	50RG	2811	2835	2513	67991.7	7224
DAY 5	50RG	2885	2908	2805	71650	1
DAY 5	50RG+IGF1	2623	2604	2556	64858.3	59283
DAY 5	50RG+IGF1	2133	2212	2156	54175	5357
DAY 5	50RG+IGF1	2354	2371	2333	58816.7	1
DAY 5	10RG	9146	9015	8849	225083	212564
DAY 5	10RG	9184	9293	9094	229758	25839
DAY 5	10RG	7301	7423	7218	182850	1
DAY 5	10RG+IGF1	7405	7482	7438	186042	193681
DAY 5	10RG+IGF1	7363	7471	7488	186017	13253
DAY 5	10RG+IGF1	8390	8442	8246	208983	1
DAY 5	IGF1	5312	5231	5115	130483	125756
DAY 5	IGF1	4818	4902	4912	121933	4346
DAY 5	IGF1	4869	5089	5024	124850	1
DAY 5	IGF1+DMSO	4646	4651	4702	116658	113853
DAY 5	IGF1+DMSO	4939	4813	4663	120125	8050
DAY 5	IGF1+DMSO	4293	4131	4149	104775	1
DAY 7	HTS	10478	10850	10482	265083	243650
DAY 7	HTS	9304	8999	9255	229650	18850
DAY 7	HTS	9347	9497	9502	236217	1
DAY 7	HTS+DMSO	11771	11872	11674	294308	315236
DAY 7	HTS+DMSO	13730	13667	13651	342067	24420
DAY 7	HTS+DMSO	12103	12583	12434	309333	1
DAY 7	100RG	3986	3840	3785	96758.3	81453
DAY 7	100RG	2874	2798	2775	70391.7	13686
DAY7	100RG	2412	3442	3411	77208.3	1
DAY 7	100RG+IGF1	3011	3001	3020	75266.7	67575
DAY 7	100RG+IGF1	2226	2212	2408	57050	9433
DAY 7	100RG+IGF1	2845	2791	2813	70408.3	1
DAY 7	50RG	4180	4117	4193	104083	96492
DAY 7	50RG	4097	4115	4146	102983	12209

DAY 7	50RG	3296	3304	3289	82408.3	1
DAY 7	50RG+IGF1	3632	3531	3548	89258.3	75589
DAY 7	50RG+IGF1	2967	3006	2849	73516.7	12760
DAY 7	50RG+IGF1	2571	2534	2574	63991.7	1
DAY 7	10RG	14646	14608	14366	363500	339689
DAY 7	10RG	14146	13826	13897	348908	29521
DAY 7	10RG	12278	12196	12325	306658	1
DAY 7	10RG+IGF1	13319	13078	13775	334767	328658
DAY 7	10RG+IGF1	13632	13554	13557	339525	10133
DAY 7	10RG+IGF1	12957	12907	12308	318100	
DAY 7	10RG+IGF1	12978	12818	12873	322242	2
DAY 7	IGF1	10413	10559	10435	261725	281408
DAY 7	IGF1	11801	11304	11361	287217	17517
DAY 7	IGF1	12018	11751	11665	295283	1
DAY 7	IGF1+DMSO	16606	16371	16004	408175	396054
DAY 7	IGF1+DMSO	14527	14725	14656	365900	22404
DAY 7	IGF1+DMSO	15848	15626	15697	393092	2
DAY 7	IGF1+DMSO	16782	16753	16511	417050	12935

Data sheet 11 Growth of N 87 cells with RG 13022 and 10nM IGF-1 40,000 cells per well

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES
DAY 0	IGF-1	2805	2697	2747	68742	110777
DAY 0	IGF-1	3992	4001	3948	99508	54123
DAY 0	IGF-1	3382	3481	3351	85117	2
DAY 0	IGF-1	7587	7580	7602	189742	31248
DAY 0	IGF-1+DMSO	4840	4846	4926	121767	104670
DAY 0	IGF-1+DMSO	4936	4931		123338	102894
DAY 0	IGF-1+DMSO	3375	3378		84413	2
DAY 0	IGF-1+DMSO	3510	3623		89163	59406
DAY 0	100RG	4537	4636		114663	106878
DAY 0	100RG	4607	4638		115563	9539
DAY 0	100RG	3952	4005		99463	2
DAY 0	100RG	3882	3944		97825	5507
DAY 0	100RG+IGF1	5206	5221		130338	123266
DAY 0	100RG+IGF1	5003	5220		127788	10851
DAY 0	100RG+IGF1	4214	4353		107088	2
DAY 0	100RG+IGF1	5035	5193		127850	6265
DAY 0	50RG	4500	4597		113713	107938
DAY 0	50RG	4914	4832		121825	14902
DAY 0	50RG	4310	4427		109213	2
DAY 0	50RG	3606	3354		87000	8604
DAY 0	50RG+IGF1	3918	3976		98675	98119
DAY 0	50RG+IGF1	3224	3333		81963	17673
DAY 0	50RG+IGF1	4870	4935		122563	2
DAY 0	50RG+IGF1	3548	3594		89275	10204
DAY 0	10RG	5664	5262		136575	123863
DAY 0	10RG	5043	5049		126150	9926
DAY 0	10RG	4714	4823		119213	2
DAY 0	10RG	4479	4602		113513	5731
DAY 0	10RG+IGF1	4962	5025		124838	116091
DAY 0	10RG+IGF1	4542	4764		116325	6823
DAY 0	10RG+IGF1	4661	4537		114975	2
DAY 0	10RG+IGF1	4292	4366		108225	3939
DAY 0	5RG				#DIV/0!	104038
DAY 0	5RG	3987	4042		100363	3813
DAY 0	5RG	4165	4137		103775	1
DAY 0	5RG	4402	4236		107975	2696
DAY 0	5RG+IGF1	4999	5077		125950	112731
DAY 0	5RG+IGF1	4270	4406		108450	9580
DAY 0	5RG+IGF1	4482	4547		112863	2
DAY 0	5RG+IGF1	4193	4100		103663	5531
DAY 0	HTS	4553	4437		112375	111584
DAY 0	HTS	4370	4479		110613	2761
DAY 0	HTS	4566	4629		114938	2
DAY 0	HTS	4330	4343		108413	1594
DAY 0	HTS+DMSO	4478	4447		111563	106728
DAY 0	HTS+DMSO	4477	4510		112338	9257
DAY 0	HTS+DMSO	4404	4404		110100	2
DAY 0	HTS+DMSO	3778	3655		92913	5345

DAY 3	IGF-1	6449	7603	7851	182525	194538
DAY 3	IGF-1	5413	9956	10102	212258	16441
DAY 3	IGF-1	3598	10435	10525	204650	2
DAY 3	IGF-1	2903	9113	9430	178717	9492
DAY 3	IGF-1+DMSO	8224	12713	12617	279617	277356
DAY 3	IGF-1+DMSO	8290	13635	13748	297275	14717
DAY 3	IGF-1+DMSO	6170	12963	12937	267250	2
DAY 3	IGF-1+DMSO	3547	14118	14169	265283	8497
DAY 3	100RG	2534	6847	6902	135692	123390
DAY 3	100RG	2272	6717	6816	131708	16396
DAY 3	100RG	1716	6710	6783	126742	2
DAY 3	100RG	928	5527	5475	99417	9466
DAY 3	100RG+IGF1	1734	6990	7113	131975	130046
DAY 3	100RG+IGF1	1850	7359	7487	139133	9339
DAY 3	100RG+IGF1	1466	7023	7364	132108	2
DAY 3	100RG+IGF1	969	6553	6514	116967	5392
DAY 3	50RG	2414	8067	8222	155858	152142
DAY 3	50RG	2547	8521	8570	163650	16919
DAY 3	50RG	2049	8631	8736	161800	2
DAY 3	50RG	1204	7001	7066	127258	9768
DAY 3	50RG+IGF1	953	10007	9845	173375	179306
DAY 3	50RG+IGF1	1263	10353	10427	183692	6293
DAY 3	50RG+IGF1	788	10715	10781	185700	2
DAY 3	50RG+IGF1	899	9992	10044	174458	3633
DAY 3	10RG	1572	8863	9040	162292	177510
DAY 3	10RG	2780	10465	10520	198042	21645
DAY 3	10RG	2006	10574	10708	194067	2
DAY 3	10RG	1415	8601	8661	155642	12497
DAY 3	10RG+IGF1	4025	10340	10029	203283	220292
DAY 3	10RG+IGF1	4705	12645	12614	249700	20266
DAY 3	10RG+IGF1	2367	11779	11633	214825	2
DAY 3	10RG+IGF1	2124	11857	11622	213358	11700
DAY 3	5RG	1451	8131	8091	147275	162094
DAY 3	5RG	2286	9972	9932	184917	16139
DAY 3	5RG	2004	8421	8293	155983	2
DAY 3	5RG	2088	8502	8634	160200	9318
DAY 3	5RG+IGF1	1671	9522	9476	172242	181623
DAY 3	5RG+IGF1	1895	11091	10598	196533	10720
DAY 3	5RG+IGF1	1687	9500	9905	175767	2
DAY 3	5RG+IGF1	1975	9875	9984	181950	6189
DAY 3	HTS	1628	8069	7940	146975	150302
DAY 3	HTS	2253	8591	8538	161517	8071
DAY 3	HTS	2038	7509	7571	142650	2
DAY 3	HTS	2102	7903	8003	150067	4660
DAY 3	HTS+DMSO	2255	7560	7620	145292	156271
DAY 3	HTS+DMSO	2484	7876	7960	152667	13368
DAY 3	HTS+DMSO	2172	8069	7926	151392	2
DAY 3	HTS+DMSO	2672	9170	9246	175733	7718
DAY 5	IGF-1	11721	8335	8151	235058	256319
DAY 5	IGF-1	12960	10121	10164	277042	22886

DAY 5	IGF-1	10799	8836	8927	238017	2
DAY 5	IGF-1	15043	9023	8953	275158	13213
DAY 5	IGF-1+DMSO	14177	9683	9770	280250	296410
DAY 5	IGF-1+DMSO	13393	10411	10889	289108	20485
DAY 5	IGF-1+DMSO	11144	14094	13934	326433	2
DAY 5	IGF-1+DMSO	14152	10241	10389	289850	11827
DAY 5	100RG	4340	3121	3024	87375	53802
DAY 5	100RG	2713	1564	1519	48300	22874
DAY 5	100RG	2072	1514	1548	42783	2
DAY 5	100RG	1614	1419	1377	36750	13206
DAY 5	100RG+IGF1	1951	1206	1197	36283	34052
DAY 5	100RG+IGF1	1552	1219	1208	33158	1492
DAY 5	100RG+IGF1	1460	1288	1262	33417	2
DAY 5	100RG+IGF1	1496	1248	1258	33350	861
DAY 5	50RG	3433	2579	2588	71667	77946
DAY 5	50RG	3004	3109	3204	77642	9144
DAY 5	50RG	2548	4205	4164	90975	2
DAY 5	50RG	1359	3641	3580	71500	5279
DAY 5	50RG+IGF1	1884	3931	3907	81017	78575
DAY 5	50RG+IGF1	1417	3968	4058	78692	7976
DAY 5	50RG+IGF1	1259	4555	4605	86825	2
DAY 5	50RG+IGF1	891	3581	3660	67767	4605
DAY 5	10RG	10744	9660	10005	253408	229244
DAY 5	10RG	7980	10566	10430	241467	28041
DAY 5	10RG	5314	11223	11423	233000	2
DAY 5	10RG	3812	9450	9430	189100	16190
DAY 5	10RG+IGF1	4393	13224	13482	259158	233338
DAY 5	10RG+IGF1	7993	11316	11201	254250	34354
DAY 5	10RG+IGF1	3206	12428	12674	235900	2
DAY 5	10RG+IGF1	2263	10003	9819	184042	19835
DAY 5	5RG	4837	9685	9833	202958	188023
DAY 5	5RG	5718	10293	10726	222808	34109
DAY 5	5RG	2829	9745	9437	183425	2
DAY 5	5RG	1341	7867	7940	142900	19693
DAY 5	5RG+IGF1	3983	12659	12448	242417	205965
DAY 5	5RG+IGF1	3320	10567	10536	203525	27617
DAY 5	5RG+IGF1	4080	10113	10125	202650	2
DAY 5	5RG+IGF1	2297	9359	9376	175267	15945
DAY 5	HTS	4666	8516	8465	180392	189715
DAY 5	HTS	2952	10870	11010	206933	11785
DAY 5	HTS	2699	9733	9737	184742	2
DAY 5	HTS	2643	9816	9956	186792	6804
DAY 5	HTS+DMSO	4107	9119	9348	188117	174435
DAY 5	HTS+DMSO	3108	9257	9289	180450	12571
DAY 5	HTS+DMSO	3209	8732	8449	169917	2
DAY 5	HTS+DMSO	3263	7819	8029	159258	7258
DAY 7	IGF-1	18421	9801	9706	316067	374902
DAY 7	IGF-1	22278	13641	13596	412625	41668
DAY 7	IGF-1	22266	12405	12438	392575	2
DAY 7	IGF-1	22845	11315	11241	378342	24057

DAY 7	IGF-1+DMSO	22115	24707	22790	580100	519592
DAY 7	IGF-1+DMSO	24608	22237	22492	577808	79051
DAY 7	IGF-1+DMSO	22105	19400	19508	508442	2
DAY 7	IGF-1+DMSO	18451	15448	15543	412017	45640
DAY 7	100RG	6995	4713	4716	136867	61948
DAY 7	100RG	3431	1399	1260	50750	51455
DAY 7	100RG	2843	968	920	39425	2
DAY 7	100RG	1346	580	564	20750	29707
DAY 7	100RG+IGF1	3024	689	655	36400	28746
DAY 7	100RG+IGF1	2248	630	615	29108	7285
DAY 7	100RG+IGF1	2062	779	829	30583	2
DAY 7	100RG+IGF1	982	653	632	18892	4206
DAY 7	50RG	4937	2227	2186	77917	81902
DAY 7	50RG	3008	4052	4047	92558	18820
DAY 7	50RG	3292	4422	4275	99908	2
DAY 7	50RG	1711	2488	2668	57225	10866
DAY 7	50RG+IGF1	3130	4871	4890	107425	97621
DAY 7	50RG+IGF1	2460	5759	5831	117083	24540
DAY 7	50RG+IGF1	1925	5259	5325	104242	2
DAY 7	50RG+IGF1	1357	3098	2953	61733	14168
DAY 7	10RG	9868	13778	13316	308017	336996
DAY 7	10RG	13971	17394	17697	408850	51909
DAY 7	10RG	9110	15815	15834	339658	2
DAY 7	10RG	5294	14805	14876	291458	29970
DAY 7	10RG+IGF1	11828	23315	22430	479775	419390
DAY 7	10RG+IGF1	13750	18777	18752	427325	74578
DAY 7	10RG+IGF1	13330	20793	20858	458175	2
DAY 7	10RG+IGF1	3948	16931	16595	312283	43057
DAY 7	5RG	14225	14479	14368	358933	341352
DAY 7	5RG	16749	14948	15104	390008	78264
DAY 7	5RG	12563	17292	16994	390408	2
DAY 7	5RG	4725	11471	10931	226058	45186
DAY 7	5RG+IGF1	10554	18104	17417	383958	393785
DAY 7	5RG+IGF1	11769	22701	22941	478425	63383
DAY 7	5RG+IGF1	8774	18951	18829	387950	2
DAY 7	5RG+IGF1	5010	16622	17345	324808	36594
DAY 7	HTS	9594	15807	16060	345508	340396
DAY 7	HTS	9194	17658	17396	368733	47953
DAY 7	HTS	7618	18607	18914	376158	2
DAY 7	HTS	6165	13379	12998	271183	27686
DAY 7	HTS+DMSO	8550	15198	15320	325567	318004
DAY 7	HTS+DMSO	9663	19001	18878	396183	58357
DAY 7	HTS+DMSO	7175	13705	13772	288767	2
DAY 7	HTS+DMSO	6782	12281	12317	261500	33693

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES	
DAY O	HTS	4093	3865	4040	99983.3	118278	
DAY O	HTS	5172	5121	5114	128392	15873	
DAY O	HTS	4571	4487	4614		1	
DAY O	HTS	5102	5043	5030	126458	11224	
DAY O	HTS	5758	5845	5791	144950	135683	
DAY O	HTS	5576	5269	5504	136242	9558	
DAY O	HTS	5214	5314	5289		1	
DAY O	HTS	5017	5004	5082	125858	6759	
DAY O	HTS	5662	5617	5632	140925	131765	
DAY O	HTS	5312	5344	5203	132158	8267	
DAY O	HTS	5378	5225	5372	133125	2	
DAY O	HTS	4801	4895	4806	120850	4773	
DAY O	HTS	5089	5145	5068		136204	
DAY O	HTS	5418	5385	5387	134917	1821	
DAY O	HTS	5525	5486	5488	137492	1	
DAY O	HTS	5015	4844	5071		1821	
DAY O	HTS	4924	5010	4986	124333	118783	
DAY O	HTS	4555	4622	4601	114817	16910	
DAY O	HTS	5548	5539	5487	138117	2	
DAY O	HTS	3888	3895	3961	97866.7	9763	
DAY O	HTS	4889	5151	4898	124483	117533	126575
DAY O	HTS	5085	5103	5170	127983	13381	13722
DAY O	HTS	4664	4808	4870	119517	2	4
DAY O	HTS	3977	3904	3897	98150	7726	3148
DAY 3	IGF1+dmso	10066	10218	9940	251867	289533	
DAY 3	IGF1+dmso	11806	12149	12067	300183	26848	
DAY 3	IGF1+dmso	11660	11657	11666	291525	2	
DAY 3	IGF1+dmso	12465	12789	12493	314558	15501	
DAY 3	100RG	8480	8440	8659	213158	210058	
DAY 3	100RG	8363	8270	8361	208283	2694	
DAY 3	100RG	8411	8251	8386	208733	1	
DAY 3	100RG					1905	
DAY 3	50RG	10438	10606	10241	260708	284475	
DAY 3	50RG	11612	11924	11586	292683	20908	
DAY 3	50RG	12001	12143	11860	300033	1	
DAY 3	50RG					14784	
DAY 3	10RG	15605	15530	15527	388850	381228	
DAY 3	10RG	14450	14631	14467	362900	15947	
DAY 3	10RG	15945	15699	15388	391933	1	
DAY 3	10RG					11276	
DAY 3	5RG	16835	17322	16897	425450	405888	
DAY 3	5RG	15107	15134	15106	377892	39592	
DAY 3	5RG	17392	17433	17241	433883	1	
DAY 3	5RG					39592	
DAY 3	HTS	11501	11534	11514	287908	211676	
DAY 3	HTS	12225	12412	12350	308225	137246	
DAY 3	HTS	10947	11414	11135	10287	2	
DAY 3	HTS	9750	9476	9608	240283	79239	
DAY 5	IGF1+dmso	14079	14507	14318	357533	393960	
DAY 5	IGF1+dmso	16783	16774	16679	418633	26797	
DAY 5	IGF1+dmso	15539	15857	15547	391192	2	
DAY 5	IGF1+dmso	16437	16353	16228	408483	15471	

DAY 5	100RG	3438	3409	3227	83950	58597
DAY 5	100RG	2337	2155	2174	55550	23975
DAY 5	100RG	1521	1428	1406	36291.7	1
DAY 5	100RG					16953
DAY 5	50RG	6875	6787	6962	171867	190336
DAY 5	50RG	7826	7835	8016	197308	16154
DAY 5	50RG	8063	8033	8124	201833	1
DAY 5	50RG					11423
DAY 5	10RG	20753	20428	21164	519542	528822
DAY 5	10RG	19559	18969	18997	479375	54681
DAY 5	10RG	23556	23596	23354	587550	1
DAY 5	10RG					38666
DAY 5	5RG	20769	20616	21074	520492	553228
DAY 5	5RG	23891	23881	23582	594617	37812
DAY 5	5RG	21699	21887	21763	544575	1
DAY 5	5RG					26737
DAY 5	HTS	14489	14837	14613	366158	364933
DAY 5	HTS	14230	14284	14346	357167	7232
DAY 5	HTS	15120	14648	14809	371475	1
DAY 5	HTS					5114
DAY 7	IGF1+dmso	18018	18370	18218	455050	466027
DAY 7	IGF1+dmso	19292	19522	19239	483775	12791
DAY 7	IGF1+dmso	19020	18295	18685	466667	2
DAY 7	IGF1+dmso	18371	18218	18445	458617	7385
DAY 7	100RG	1460	1046	1011	29308.3	23931
DAY 7	100RG	1086	977	1137	26666.7	7150
DAY 7	100RG	681	622	595	15816.7	1
DAY 7	100RG					5056
DAY 7	50RG	848	779	817	20366.7	22310
DAY 7	50RG	787	698	732	18475	3473
DAY 7	50RG	1067	1043	999	25908.3	2
DAY 7	50RG	986	990	963	24491.7	2005
DAY 7	10RG	11741	11967	11908	296800	375023
DAY 7	10RG	14336	14263	14027	355217	80067
DAY 7	10RG	19582	19490	19362	486950	2
DAY 7	10RG	14557	14254	14524	361125	46226
DAY 7	5RG	19743	19481	19496	489333	446994
DAY 7	5RG	15443	15762	15796	391675	50106
DAY 7	5RG	18266	18524	18407	459975	1
DAY 7	5RG					35431
DAY 7	HTS	11776	11899	11996	297258	275850
DAY 7	HTS	10529	10591	10568	264067	18572
DAY 7	HTS	10854	10656	10437	266225	1
DAY 7	HTS					13132

Data sheet 13 Growth of N 87 cells with RG 13022 and 10nM IGF-1 30,000 cells per well

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES
DAY O	HTS	1331	1319	1338	33233.33	32486
DAY O	HTS	1317	1310	1287	32616.67	820
DAY O	HTS	1276	1251	1266	31608.33	1
DAY O	HTS	1470	1495	1494	37158.33	36675
DAY O	HTS	1598	1590	1544	39433.33	3029
DAY O	HTS	1367	1312	1333	33433.33	1
DAY O	HTS	1374	1367	1436	34808.33	36222
DAY O	HTS	1368	1472	1356	34966.67	2313
DAY O	HTS	1576	1569	1522	38891.67	1
DAY O	HTS	1379	1392	1410	34841.67	35786
DAY O	HTS	1455	1510	1454	36825	995
DAY O	HTS	1406	1413	1464	35691.67	1
DAY O	HTS	1564	1575	1506	38708.33	36942
DAY O	HTS	1372	1372	1286	33583.33	2910
DAY O	HTS	1574	1551	1499	38533.33	1
DAY O	HTS	1581	1592	1582	39625	34825
DAY O	HTS	1380	1423	1487	35750	5323
DAY O	HTS	1171	1178	1143	29100	1
DAY 4	HTS	6291	6306	6251	157066.7	152628
DAY 4	HTS	6112	6052	5979	151191.7	3923
DAY 4	HTS	5953	6032	5970	149625	1
DAY 4	IGF1	6310	6468	6449	160225	174369
DAY 4	IGF1	7516	7466	7310	185766.7	12991
DAY 4	IGF1	7067	7104	7083	177116.7	1
DAY 4	5 RG	6381	6525	6481	161558.3	151175
DAY 4	5 RG	6184	6381	6431	158300	15250
DAY 4	5 RG	5302	5413	5325	133666.7	1
DAY 4	10RG	5456	5570	5520	137883.3	147539
DAY 4	10RG	6130	6147	6128	153375	8423
DAY 4	10RG	6159	5978	6026	151358.3	1
DAY 4	50 RG	5037	5084	4738	123825	102500
DAY 4	50 RG	4139	3999	4087	101875	884
DAY 4	50 RG	4118	4077	4180	103125	1
DAY 4	100 RG	2220	2199	2231	55416.67	55561
DAY 4	100 RG	2232	2180	2197	55075	572
DAY 4	100 RG	2220	2294	2229	56191.67	1
DAY 6	HTS	7415	7493	7493	186675	201636
DAY 6	HTS	8282	8369	8469	209333.3	12959
DAY 6	HTS	8026	8942	8100	208900	1
DAY 6	IGF1	10497	10388	10137	258516.7	269667
DAY 6	IGF1	11503	11638	11522	288858.3	16693
DAY 6	IGF1	10533	10433	10429	261625	1
DAY 6	5 RG	9517	9745	9678	241166.7	257953
DAY 6	5 RG	10538	10722	10561	265175	14584
DAY 6	5 RG	10622	10601	10879	267516.7	1
DAY 6	10 RG	7802	8561	8276	205325	221939
DAY 6	10 RG	9003	9075	9149	226891.7	14774
DAY 6	10 RG	9476	9225	9331	233600	1
DAY 6	50 RG	4968	4830	5007	123375	104950
DAY 6	50 RG	4194	4005	3985	101533.3	16977
DAY 6	50 RG	3636	3547	3610	89941.67	1
DAY 6	100 RG	1260	1202	1294	31300	36897

DAY 6	100 RG	1459	1481	1454	36616.67	5743
DAY 6	100 RG	1743	1665	1725	42775	1
DAY 8	HTS	11766	11874	11681	294341.7	318981
DAY 8	HTS	13311	13166	13075	329600	21406
DAY 8	HTS	13436	13204	13320	333000	1
DAY 8	IGF1	15384	15402	15324	384250	404725
DAY 8	IGF1	17829	17925	17828	446516.7	36195
DAY 8	IGF1	15496	15243	15270	383408.3	1
DAY 8	5 RG	11995	12158	12094	302058.3	319678
DAY 8	5 RG	13091	13075	13330	329133.3	15273
DAY 8	5 RG	13091	13165	13085	327841.7	1
DAY 8	10 RG	10052	10043	9930	250208.3	238550
DAY 8	10 RG	9898	9794	9805	245808.3	16529
DAY 8	10 RG	8858	8795	8703	219633.3	1
DAY 8	50 RG	4340	4152	4179	105591.7	90528
DAY 8	50 RG	3477	3420	3451	86233.33	13441
DAY 8	50 RG	3060	3241	3270	79758.33	1
DAY 8	100 RG	1111	999	1031	26175	27519
DAY 8	100 RG	1049	1033	1001	25691.67	2758
DAY 8	100 RG	1243	1251	1189	30691.67	1

Data sheet 14 Growth of MKN 45 cells with RG 13022 and IGF-1 50,000 cells per well

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES
DAY O	HTS	1907	1952	1919	48150	59165
DAY O	HTS	2379	2456	2430	60541.7	7780
DAY O	HTS	2326	2583	2476	61541.7	2
DAY O	HTS	2676	2681	2614	66425	4492
DAY O	HTS	3018	2965	2933	74300	73015
DAY O	HTS	2980	3047	3024	75425	2470
DAY O	HTS	2930	2795	2985	72583.3	2
DAY O	HTS	2871	2746	2753	69750	1426
DAY O	HTS	2853	2999	2868	72666.7	74700
DAY O	HTS	2875	2778	2692	69541.7	4661
DAY O	HTS	3072	3072	3003	76225	2
DAY O	HTS	3321	3175	3148	80366.7	2691
DAY O	HTS	2947	3005	2903	73791.7	71754
DAY O	HTS	3170	3157	3110	78641.7	7805
DAY O	HTS	2984	2933	2969	74050	2
DAY O	HTS	2405	2428	2431	60533.3	4506
DAY O	HTS	3132	3089	3137	77983.3	66050
DAY O	HTS	3351	3228	3189	81400	16683
DAY O	HTS	2396	2301	2373	58916.7	2
DAY O	HTS	1890	1779	1839	45900	9632
DAY O	HTS	2822	2712	2654	68233.3	65460
DAY O	HTS	2839	2887	2916	72016.7	18327
DAY O	HTS	3370	3262	3228	82166.7	2
DAY O	HTS	1596	1609	1526	39425	10581
DAY 3	IGF1+dmso	9216	9319	9310	232042	283460
DAY 3	IGF1+dmso	12991	12850	12817	322150	38024
DAY 3	IGF1+dmso	11949	11797	11946	297433	2
DAY 3	IGF1+dmso	11404	11283	11179	282217	21953
DAY 3	100RG	4273	4206	4097	104800	70104
DAY 3	100RG	3029	3126	3061	76800	27798
DAY 3	100RG	2493	2368	2306	59725	2
DAY 3	100RG	1612	1523	1556	39091.7	16049
DAY 3	50RG	3758	3786	3670	93450	84058
DAY 3	50RG	3838	3953	3620	95091.7	25887
DAY 3	50RG	4060	4077	4110	102058	2
DAY 3	50RG	1800	1903	1773	45633.3	14946
DAY 3	10RG	7484	7599	7603	189050	179579
DAY 3	10RG	8535	8496	8465	212467	53052
DAY 3	10RG	8664	8473	8646	214858	2
DAY 3	10RG	4228	4096	3909	101942	30629
DAY 3	5RG	9436	9448	9447	236092	198683
DAY 3	5RG	9687	9915	9875	245642	72514
DAY 3	5RG	9426	9339	9464	235242	1
DAY 3	5RG	4594	4608	4618	115167	51275
DAY 3	HTS	12756	12878	12962	321633	149674
DAY 3	HTS	6130	6027	6115	152267	129412
DAY 3	HTS	5524	5371	5449	10287	2
DAY 3	HTS	4598	4551	4592	114508	74716
DAY 5	IGF1+dmso	9605	9471	9547	238525	280140
DAY 5	IGF1+dmso	12107	11911	12048	300550	30994
DAY 5	IGF1+dmso	12405	12497	11901	306692	2
DAY 5	IGF1+dmso	11052	10928	10995	274792	17895

DAY 5	100RG	3652	3630	3623	90875	68269
DAY 5	100RG	2993	2842	2859	72450	20332
DAY 5	100RG	2718	2670	2791	68158.3	2
DAY 5	100RG	1629	1720	1642	41591.7	11739
DAY 5	50RG	3388	3304	3336	83566.7	75488
DAY 5	50RG	3702	3512	3185	86658.3	22354
DAY 5	50RG	3539	3608	3601	89566.7	2
DAY 5	50RG	1669	1719	1671	42158.3	12906
DAY 5	10RG	7119	7046	7130	177458	217067
DAY 5	10RG	10701	10757	10702	268000	61311
DAY 5	10RG	10929	11184	10380	270775	2
DAY 5	10RG	5498	5602	7144	152033	35398
DAY 5	5RG	10503	10709	11369	271508	271946
DAY 5	5RG	11179	11129	11270	279817	53898
DAY 5	5RG	13405	13373	13290	333900	2
DAY 5	5RG	8021	8204	8082	202558	31118
DAY 5	HTS	12422	12170	12249	307008	303771
DAY 5	HTS	13505	13386	13089	333167	36322
DAY 5	HTS	13209	12810	12761	323167	2
DAY 5	HTS	9926	10170	10113	251742	20970
DAY 7	IGF1+dmso	18167	17884	18644	455792	448381
DAY 7	IGF1+dmso	16120	17202	17244	421383	20622
DAY 7	IGF1+dmso	18905	18733	18813	470425	2
DAY 7	IGF1+dmso	18074	17804	17633	445925	11906
DAY 7	100RG	5429	5501	5471	136675	123779
DAY 7	100RG	7821	7924	7979	197700	57657
DAY 7	100RG	3933	3889	3855	97308.3	2
DAY 7	100RG	2555	2560	2497	63433.3	33288
DAY 7	50RG	6808	6626	6645	167325	186856
DAY 7	50RG	9444	9750	9403	238308	50570
DAY 7	50RG	8772	8430	8761	216358	2
DAY 7	50RG	5081	5022	4949	125433	29197
DAY 7	10RG	15482	15458	15250	384917	420506
DAY 7	10RG	16995	17138	16923	425467	28244
DAY 7	10RG	18443	18083	17907	453608	2
DAY 7	10RG	16651	16702	16811	418033	16306
DAY 7	5RG	18935	18703	17959	463308	438513
DAY 7	5RG	20981	20478	20635	517450	78022
DAY 7	5RG	17555	17721	17714	441583	2
DAY 7	5RG	13047	13410	13348	331708	45046
DAY 7	HTS	15980	15854	15831	397208	410479
DAY 7	HTS	18629	18829	18501	466325	45668
DAY 7	HTS	16788	16893	16869	421250	2
DAY 7	HTS	14157	14357	14342	357133	26366

Data sheet 15 Growth of MKN 45 cells with RG 13022 and 10nM IGF-1 40,000 cells per well

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES
DAY O	HTS	1689	1738	1714	42841.7	49921
DAY O	HTS	2098	2048	2180	52716.7	4752
DAY O	HTS	2047	2125	2008	51500	2
DAY O	HTS	2125	2084	2106	52625	2743
DAY O	HTS+DMSO	2155	2194	2109	53816.7	59250
DAY O	HTS+DMSO	2356	2351	2347	58783.3	4737
DAY O	HTS+DMSO	2351	2419	2313	59025	2
DAY O	HTS+DMSO	2628	2612	2605	65375	2735
DAY O	100RG	2026	1948	2007	49841.7	55063
DAY O	100RG	2110	2117	2229	53800	4078
DAY O	100RG	2291	2377	2314	58183.3	2
DAY O	100RG	2367	2289	2355	58425	2355
DAY O	100RG+IGF1	2006	1776	1933	47625	53867
DAY O	100RG+IGF1	2006	2000	1985	49925	6726
DAY O	100RG+IGF1	2317	2159	2139	55125	2
DAY O	100RG+IGF1	2525	2543	2467	62791.7	3884
DAY O	50RG	2145	2198	2113	53800	49938
DAY O	50RG	1909	1846	1831	46550	3796
DAY O	50RG	2150	2040	2121	52591.7	2
DAY O	50RG	1935	1840	1842	46808.3	2192
DAY O	50RG+IGF1	1935	1693	1839	45558.3	45769
DAY O	50RG+IGF1	2008	1913	2052	49775	4680
DAY O	50RG+IGF1	1906	2000	1911	48475	2
DAY O	50RG+IGF1	1584	1562	1566	39266.7	2702
DAY O	10RG	3761	3777	3673	93425	56956
DAY O	10RG	2624	2692	2649	66375	28770
DAY O	10RG	1514	1420	1437	36425	2
DAY O	10RG	1272	1260	1260	31600	16610
DAY O	10RG+IGF1	2182	2109	2077	53066.7	46088
DAY O	10RG+IGF1	2460	2403	2439	60850	13813
DAY O	10RG+IGF1	1624	1649	1660	41108.3	2
DAY O	10RG+IGF1	1185	1145	1189	29325	7975
DAY O	5RG	2241	2152	2185	54816.7	39207
DAY O	5RG	2291	2319	2284	57450	24923
DAY O	5RG	1651	1669	1621	41175	2
DAY O	5RG	1122	1148	1077	3387	14389
DAY O	5RG+IGF1	2470	2430	2475	61458.3	47433
DAY O	5RG+IGF1	2172	2229	2168	54741.7	14514
DAY O	5RG+IGF1	1863	1801	1802	45550	2
DAY O	5RG+IGF1	1120	1077	1161	27983.3	8380
DAY O	IGF1	2055	1983	1967	50041.7	38258
DAY O	IGF1	2079	2062	2001	51183.3	14722
DAY O	IGF1	1236	1207	1196	30325	2
DAY O	IGF1	890	793	895	21483.3	8500
DAY O	IGF1+DMSO	1649	1589	1640	40650	27727
DAY O	IGF1+DMSO	1127	1080	1089	27466.7	9595
DAY O	IGF1+DMSO	1016	998	1010	25200	2
DAY O	IGF1+DMSO	697	736	678	17591.7	5540
DAY 3	HTS	6449	6681	6799	166075	116242
DAY 3	HTS	5413	5380	5530	136025	42452
DAY 3	HTS	3598	3628	3430	88800	2

Data sheet 15 Growth of MKN 45 cells with RG 13022 and 10nM IGF-1 40,000 cells per well

DAY 3	HTS	2903	2977	3008	74066.7	24510
DAY 3	HTS+DMSO	8224	8204	8106	204450	163617
DAY 3	HTS+DMSO	8290	8351	8578	210158	57550
DAY 3	HTS+DMSO	6170	6162	6116	153733	2
DAY 3	HTS+DMSO	3547	3557	3231	86125	33227
DAY 3	100RG	2534	2462	2363	61325	45865
DAY 3	100RG	2272	2265	2300	56975	17311
DAY 3	100RG	1716	1639	1704	42158.3	2
DAY 3	100RG	928	914	918	23000	9994
DAY 3	100RG+IGF1	1734	1659	1625	41816.7	37283
DAY 3	100RG+IGF1	1850	1860	1840	46250	9583
DAY 3	100RG+IGF1	1466	1470	1503	36991.7	2
DAY 3	100RG+IGF1	969	940	980	24075	5533
DAY 3	50RG	2414	2360	2329	59191.7	51052
DAY 3	50RG	2547	2498	2529	63116.7	14801
DAY 3	50RG	2049	2089	2094	51933.3	2
DAY 3	50RG	1204	1185	1207	29966.7	8545
DAY 3	50RG+IGF1	953	975	965	24108.3	24360
DAY 3	50RG+IGF1	1263	1233	1209	30875	4637
DAY 3	50RG+IGF1	788	796	832	20133.3	2
DAY 3	50RG+IGF1	899	870	910	22325	2677
DAY 3	10RG	1572	1480	1528	38166.7	47871
DAY 3	10RG	2780	2732	2787	69158.3	15406
DAY 3	10RG	2006	1993	1889	49066.7	2
DAY 3	10RG	1415	1374	1422	35091.7	8895
DAY 3	10RG+IGF1	4025	3986	4026	100308	82656
DAY 3	10RG+IGF1	4705	4685	4630	116833	30778
DAY 3	10RG+IGF1	2367	2425	2387	59825	2
DAY 3	10RG+IGF1	2124	2139	2176	53658.3	17770
DAY 3	5RG	1451	1408	1432	35758.3	48596
DAY 3	5RG	2286	2413	2395	59116.7	9664
DAY 3	5RG	2004	2001	1846	48758.3	2
DAY 3	5RG	2088	2024	1978	50750	5579
DAY 3	5RG+IGF1	1671	1553	1663	40725	44323
DAY 3	5RG+IGF1	1895	1871	1861	46891.7	4591
DAY 3	5RG+IGF1	1687	1543	1593	40191.7	2
DAY 3	5RG+IGF1	1975	2031	1932	49483.3	2651
DAY 3	IGF1	1628	1527	1574	39408.3	48964
DAY 3	IGF1	2253	2246	2183	55683.3	8500
DAY 3	IGF1	2038	2096	2082	51800	1
DAY 3	IGF1	2102	2072	2130		6010
DAY 3	IGF1+DMSO	2255	2246	2239	56166.7	59806
DAY 3	IGF1+DMSO	2484	2265	2376	59375	5589
DAY 3	IGF1+DMSO	2172	2280	2249	55841.7	2
DAY 3	IGF1+DMSO	2672	2781	2688	67841.7	3227
DAY 5	HTS	11721	11737	11369	290225	315850
DAY 5	HTS	12960	13158	13320	328650	47751
DAY 5	HTS	10799	10789	10545	267775	2
DAY 5	HTS	15043	15141	15026	376750	27569
DAY 5	HTS+DMSO	14177	14188	14389	356283	331358
DAY 5	HTS+DMSO	13393	13314	13460	334725	34803
DAY 5	HTS+DMSO	11144	11386	11208	281150	2
DAY 5	HTS+DMSO	14152	14280	13961	353275	20094

DAY 5	100RG	4340	4324	4358	108517	66940
DAY 5	100RG	2713	2765	2707	68208.3	30258
DAY 5	100RG	2072	2131	2101	52533.3	2
DAY 5	100RG	1614	1456	1550	38500	17469
DAY 5	100RG+IGF1	1951	1885	1939	48125	40017
DAY 5	100RG+IGF1	1552	1534	1543	38575	5480
DAY 5	100RG+IGF1	1460	1483	1444	36558.3	2
DAY 5	100RG+IGF1	1496	1452	1469	36808.3	3164
DAY 5	50RG	3433	3356	3383	84766.7	64696
DAY 5	50RG	3004	3028	3004	75300	22561
DAY 5	50RG	2548	2673	2676	65808.3	2
DAY 5	50RG	1359	1295	1295	32908.3	13025
DAY 5	50RG+IGF1	1884	1844	1926	47116.7	33667
DAY 5	50RG+IGF1	1417	1347	1355	34325	10425
DAY 5	50RG+IGF1	1259	1279	1226	31366.7	2
DAY 5	50RG+IGF1	891	879	853	21858.3	6019
DAY 5	10RG	10744	10668	10613	266875	172733
DAY 5	10RG	7980	7818	7803	196675	75934
DAY 5	10RG	5314	5534	5327	134792	2
DAY 5	10RG	3812	3629	3670	92591.7	43841
DAY 5	10RG+IGF1	4393	4181	4076	105417	111265
DAY 5	10RG+IGF1	7993	8055	8296	202867	64334
DAY 5	10RG+IGF1	3206	3347	3158	80925	2
DAY 5	10RG+IGF1	2263	2199	2240	55850	37143
DAY 5	5RG	4837	4841	4701	119825	91035
DAY 5	5RG	5718	5533	5736	141558	48687
DAY 5	5RG	2829	2720	2668	68475	2
DAY 5	5RG	1341	1398	1375	34283.3	28109
DAY 5	5RG+IGF1	3983	3937	3997	99308.3	86433
DAY 5	5RG+IGF1	3320	3337	3452	84241.7	20566
DAY 5	5RG+IGF1	4080	4191	4198	103908	2
DAY 5	5RG+IGF1	2297	2288	2408	58275	11874
DAY 5	IGF1	4666	4823	4775	118867	81598
DAY 5	IGF1	2952	2996	3038	74883.3	25185
DAY 5	IGF1	2699	2582	2556	65308.3	2
DAY 5	IGF1	2643	2718	2719	67333.3	14541
DAY 5	IGF1+DMSO	4107	4110	4019	101967	85640
DAY 5	IGF1+DMSO	3108	3212	3143	78858.3	10938
DAY 5	IGF1+DMSO	3209	3207	3213	80241.7	2
DAY 5	IGF1+DMSO	3263	3332	3184	81491.7	6315
DAY 7	HTS	18421	18560	18702	464025	537994
DAY 7	HTS	22278	22290	22119	555725	49712
DAY 7	HTS	22266	22198	22890	561283	2
DAY 7	HTS	22845	23000	22668	570942	28701
DAY 7	HTS+DMSO	22115	22351	22110	554800	546865
DAY 7	HTS+DMSO	24608	24633	24541	614850	62298
DAY 7	HTS+DMSO	22105	21973	22408	554050	2
DAY 7	HTS+DMSO	18451	18719	18481	463758	35968
DAY 7	100RG	6995	6929	6939	173858	90583
DAY 7	100RG	3431	3405	3331	84725	59509
DAY 7	100RG	2843	2796	2763	70016.7	2
DAY 7	100RG	1346	1328	1374	33733.3	34358
DAY 7	100RG+IGF1	3024	3074	3038	76133.3	51810

DAY 7	100RG+IGF1	2248	2287	2299	56950	21888
DAY 7	100RG+IGF1	2062	2038	2015	50958.3	2
DAY 7	100RG+IGF1	982	915	887	23200	12637
DAY 7	50RG	4937	4991	4918	123717	80879
DAY 7	50RG	3008	3107	3021	76133.3	33893
DAY 7	50RG	3292	3325	3296	82608.3	2
DAY 7	50RG	1711	1568	1648	41058.3	19568
DAY 7	50RG+IGF1	3130	3128	3014	77266.7	55238
DAY 7	50RG+IGF1	2460	2468	2416	61200	18408
DAY 7	50RG+IGF1	1925	1939	1950	48450	2
DAY 7	50RG+IGF1	1357	1365	1362	34033.3	10628
DAY 7	10RG	9868	9927	10078	248942	239263
DAY 7	10RG	13971	13892	13944	348392	87979
DAY 7	10RG	9110	9065	8881	225467	2
DAY 7	10RG	5294	5519	5297	134250	50795
DAY 7	10RG+IGF1	11828	11766	11676	293917	266735
DAY 7	10RG+IGF1	13750	13665	13507	341017	114021
DAY 7	10RG+IGF1	13330	13467	13220	333475	2
DAY 7	10RG+IGF1	3948	3983	3893	98533.3	65830
DAY 7	5RG	14225	14180	14050	353792	298010
DAY 7	5RG	16749	16627	16300	413967	129910
DAY 7	5RG	12563	12368	12322	310442	2
DAY 7	5RG	4725	4592	4344	113842	75004
DAY 7	5RG+IGF1	10554	10501	10477	262767	225627
DAY 7	5RG+IGF1	11769	12004	11915	297400	75735
DAY 7	5RG+IGF1	8774	8820	8792	219883	2
DAY 7	5RG+IGF1	5010	4797	4888	122458	43726
DAY 7	IGF1	9594	9322	9277	234942	199692
DAY 7	IGF1	9194	8972	9021	226558	38667
DAY 7	IGF1	7618	7389	7293	185833	2
DAY 7	IGF1	6165	6100	5907	151433	22324
DAY 7	IGF1+DMSO	8550	8551	8542	213692	200365
DAY 7	IGF1+DMSO	9663	9662	9589	240950	33461
DAY 7	IGF1+DMSO	7175	7282	7115	179767	2
DAY 7	IGF1+DMSO	6782	6753	6511	167050	19319

Data sheet 16 Growth of MKN 45 cells with RG 13022 and 10nM IGF-1 40,000 cells per well

DAY	TREATMENT	COUNT 1 DILUTION	COUNT 2 25	COUNT 3	MEAN	MEAN OF REPLICATES
DAY O	HTS	3632	3589	3614	90291.7	90540
DAY O	HTS	4689	4715	4654	117150	19706
DAY O	HTS	3409	3392	3369	84750	2
DAY O	HTS	2803	2803	2790	69966.7	11377
DAY O	100uM RG	4084	4177	3777	100317	94631
DAY O	100uM RG	5257	5258	5188	130858	30949
DAY O	100uM RG	3690	3735	3580	91708.3	2
DAY O	100uM RG	2218	2183	2276	55641.7	17868
DAY O	100RG+10nM IGF1	4680	4948	5012	122000	91619
DAY O	100RG+10nM IGF1	4043	4102	4152	102475	27903
DAY O	100RG+10nM IGF1	3429	3416	3464	85908.3	2
DAY O	100RG+10nM IGF1	2265	2275	2191	56091.7	16110
DAY O	10uM RG	4121	4011	3931	100525	87925
DAY O	10uM RG	4271	4213	4119	105025	19382
DAY O	10uM RG	3515	3363	3191	83908.3	2
DAY O	10uM RG	2525	2494	2450	62241.7	11190
DAY O	10RG+10nM IGF1	4330	4292	4358	108167	98315
DAY O	10RG+10nM IGF1	5383	5435	5476	135783	33948
DAY O	10RG+10nM IGF1	3789	3880	3744	95108.3	2
DAY O	10RG+10nM IGF1	2133	2203	2168	54200	19600
DAY O	10nM IGF1	3327	3369	3332	83566.7	72706
DAY O	10nM IGF1	3496	3370	3396	85516.7	16892
DAY O	10nM IGF1	2891	2988	2880	72991.7	2
DAY O	10nM IGF1	1949	1917	1984	48750	9753
DAY 3	HTS	12751	12702	12615	317233	288177
DAY 3	HTS	12478	12446	12603	312725	52693
DAY 3	HTS	12659	12592	12376	313558	2
DAY 3	HTS	8444	8341	8318	209192	30423
DAY 3	100uM RG	7511	7542	7494	187892	178846
DAY 3	100uM RG	8250	8446	8485	209842	52955
DAY 3	100uM RG	8684	8616	8636	216133	2
DAY 3	100uM RG	4253	3969	3960	101517	30574
DAY 3	100RG+10nM IGF1	6507	6397	6424	161067	130501
DAY 3	100RG+10nM IGF1	7256	6901	6969	176050	85182
DAY 3	100RG+10nM IGF1	7229	7406	7145	181500	2
DAY 3	100RG+10nM IGF1	3505	3388	3387	3387	49180
DAY 3	10uM RG	11706	11786	11383	290625	313008
DAY 3	10uM RG	15307	15047	15242	379967	67007
DAY 3	10uM RG	14159	14100	13972	351925	2
DAY 3	10uM RG	9237	9044	9261	229517	38686
DAY 3	10RG+10nM IGF1	10801	10765	10713	268992	284925
DAY 3	10RG+10nM IGF1	13024	13060	13002	325717	58826
DAY 3	10RG+10nM IGF1	13406	13380	13576	336350	2
DAY 3	10RG+10nM IGF1	8381	8458	8198	208642	33963
DAY 3	10nM IGF1	12281	12212	12069	304683	290863
DAY 3	10nM IGF1	14052	13750	13753	346292	58559
DAY 3	10nM IGF1	12155	12221	12144	304333	2
DAY 3	10nM IGF1	8465	8485	8027	208142	33809
DAY 5	HTS	12843	13299	13279	328508	432188
DAY 5	HTS	18240	17243	17641	442700	83082
DAY 5	HTS	21189	21238	21323	531250	2

DAY 5	HTS	17347	17014	16794	426292	47968
DAY 5	100uM RG	6354	6323	6361	158650	166354
DAY 5	100uM RG	7059	6903	6845	173392	6180
DAY 5	100uM RG	6712	6747	6742	168342	2
DAY 5	100uM RG	6648	6286	6870	165033	3568
DAY 5	100RG+10nM IGF1	5633	5661	5518	140100	167821
DAY 5	100RG+10nM IGF1	7255	7171	7078	179200	26909
DAY 5	100RG+10nM IGF1	8097	8015	7873	199875	2
DAY 5	100RG+10nM IGF1	6095	6147	6011	152108	15536
DAY 5	10mcM RG	16593	16467	16399	412158	421046
DAY 5	10mcM RG	17364	17245	17282	432425	56510
DAY 5	10mcM RG	19577	19590	19422	488242	2
DAY 5	10mcM RG	14101	14064	13998	351358	32626
DAY 5	10RG+10nM IGF1	13366	13535	13737	338650	342319
DAY 5	10RG+10nM IGF1	16388	16424	16319	409425	52936
DAY 5	10RG+10nM IGF1	14894	13496	12559	341242	2
DAY 5	10RG+10nM IGF1	11199	11166	11230	279958	30563
DAY 5	10nM IGF1	15482	15541	15213	385300	396138
DAY 5	10nM IGF1	17221	17047	17121	428242	37718
DAY 5	10nM IGF1	17248	16782	16795	423542	2
DAY 5	10nM IGF1	13606	14116	13974	347467	21777
DAY 7	HTS	15041	15720	15344	384208	427458
DAY 7	HTS	18969	18867	18878	472617	39782
DAY 7	HTS	16043	16375	16309	406058	2
DAY 7	HTS	18093	17620	17921	446950	22968
DAY 7	100uM RG	7701	7680	7646	191892	151515
DAY 7	100uM RG	6864	6822	6909	171625	49731
DAY 7	100uM RG	6623	6715	6274	163433	2
DAY 7	100uM RG	3136	3173	3184	79108.3	28712
DAY 7	100RG+10nM IGF1	6358	6418	6348	159367	148290
DAY 7	100RG+10nM IGF1	7526	7272	6768	179717	46282
DAY 7	100RG+10nM IGF1	6941	6931	7009	174008	2
DAY 7	100RG+10nM IGF1	3228	3187	3193	80066.7	26721
DAY 7	10uM RG	15559	15692	15803	392117	449713
DAY 7	10uM RG	18567	18666	18977	468417	73292
DAY 7	10uM RG	21963	21881	21630	545617	2
DAY 7	10uM RG	15799	15933	15392	392700	42315
DAY 7	10RG+10nM IGF1	17270	17527	17849	438717	436631
DAY 7	10RG+10nM IGF1	17811	18505	16282	438317	3273
DAY 7	10RG+10nM IGF1	16833	17280	17830	432858	1
DAY 7	10RG+10nM IGF1					2314
DAY 7	10nM IGF1	19910	20317	20698	507708	486873
DAY 7	10nM IGF1	20619	21181	21233	525275	35063
DAY 7	10nM IGF1	18279	18071	18098	453733	2
DAY 7	10nM IGF1	17768	18416	19109	460775	20243