Fluorescence in situ hybridisation (FISH) analysis of chromosomal aberrations in gastric tissue: The involvement of <italic>Helicobacter pylori</italic>.

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Fluorescence *In Situ* Hybridisation (FISH) analysis of chromosomal aberrations in gastric tissue: the involvement of *Helicobacter pylori.*

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Abstract

Gastric cancer is a common cause of cancer death in the UK. It most often presents in patients when the disease is advanced, and hence treatment options are limited. As such, studies on the pre-malignant stages of gastric cancer, and interest in the mechanisms of the carcinogenic process (reactive oxygen species, ROS) and the agents that may drive the carcinogenic pathway (*Helicobacter pylori* infection), are important, with a view to improving disease outcome. This series of experiments has firstly shown, using CBMN assay +/- kinetochore staining and interphase FISH, that ROS causes aneuploidy of chromosomes 4, 8, 20 and 17(p53) in a human cell line. Secondly, gastric cells have been collected using endoscopic cytology brush techniques, and prepared, such that interphase FISH could be performed. Again, aneuploidy of chromosomes 4, 8, 20 and 17(p53) were detected in normal gastric mucosa, gastritis and intestinal metaplasia. The level of aneuploidy detected increased as disease severity increased. Amplification of chromosome 4, amplification of chromosome 20 and deletion of chromosome 17(p53) were the more significant findings. The degree of chromosomal abnormalities detected increased further, in a stepwise manner, when gastric dysplasia and gastric adenocarinoma cells were studied. Hence, a role for these abnormalities may exist in the initiation of, and the progression to, gastric cancer. The presence of *H. pylori* was also determined in the gastric tissue studied using histological analysis and PCR technology. Detection rates were comparable. The more virulent strain of *H. pylori*, Cag A, was found to be associated with increased disease pathology and chromosomal abnormalities, yet numbers were small. The amplification of chromosome 4 in gastric tissue was again highlighted in association with *H. pylori* infection, hence it may reflect a role for chromosome 4 in the initiation of gastric cancer.

Chapter 1

General Introduction

1.1 Gastric cancer

1.1.1 Introduction to gastric cancer

Gastric cancer is often diagnosed at an advanced stage when symptoms such as weight loss, abdominal pain, vomiting and gastrointestinal bleeding finally alert patients to seek medical advice. Treatment regimens, such as surgery, chemotherapy and radiotherapy, are therefore limited by advanced local disease and metastatic spread. In fact, only 55-65% of gastric cancers are surgically respectable at diagnosis (Keighley 2003). Hence, the prognosis of gastric cancer is poor, helping to make gastric cancer a leading cause of cancer death worldwide (Parkin *et al.*, 1999). 5 year survival rates for gastric cancer in Wales are currently estimated at 13%, with a median survival time of 4 months (www.wcisu.nhs.wales). Other countries in Europe have similar 5 year survival rates, but rates can increase up to 28% in Spain and Austria (Keighley 2003). Japanese 5 year survival rates are significantly higher, up to 95%, but reflect high disease prevalence and are associated with a surveillance programme to detect early gastric cancers. Early gastric cancers only account for 10% of the European gastric cancers diagnosed (Keighley 2003).

1.1.2 Incidence of gastric cancer

Gastric cancer is regarded as the second commonest cancer worldwide (Parkin *et al.*, 1985) and there are 755,000 incident cases in the world each year (Sugimura and Sasako 1997). There is a significant geographical variation in the incidence of gastric cancer worldwide. In the UK, including Wales, it is the sixth most frequent

cancer in men and the seventh in women (Cancer research Campaign 1995, www.wcisu.nhs.uk). In China, it is the commonest cancer diagnosed, and it has been estimated that 38% of all gastric cancers worldwide develop in China (Parkin *et al.*, 1999). In Japan, incidence rates are high at 78 per 100,000 in males, but in Northern Africa the rate falls to 2.6 per 100,000 in females, reflecting geographical and sex differences (Parkin *et al.*, 1999). In Wales, incidence rates are estimated at 25.9 per 100,000 in males, and 10.6 per 100,000 in females, which is comparable to other European countries (www.wcisu.nhs.uk).

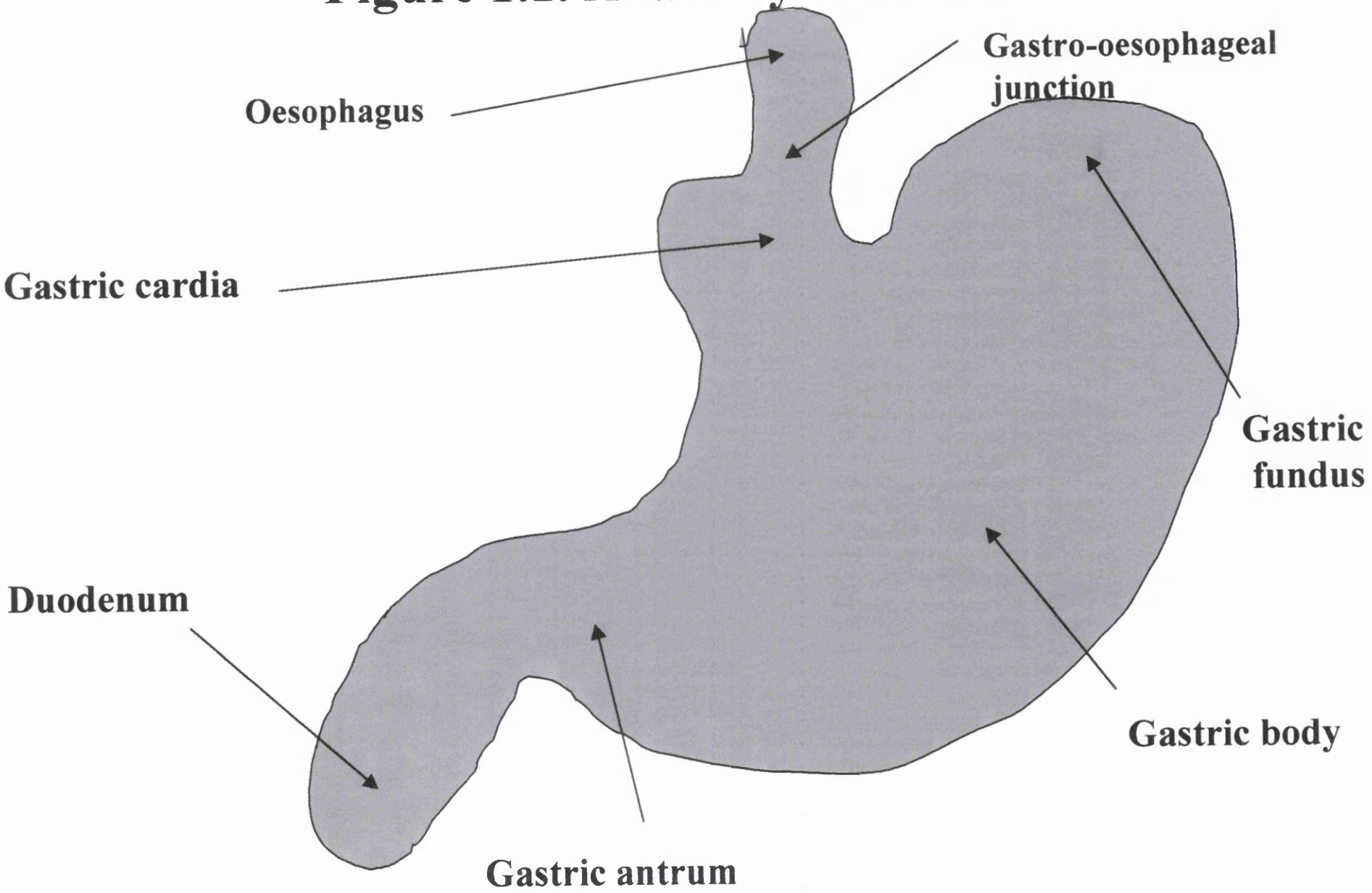
Gastric cancer is one of the main cause of deaths associated with cancers worldwide, accounting for 12.1% of cancer deaths in 1999, and responsible for 628,000 recorded deaths (Parkin *et al.*, 1999, Grady 2001). In 2001 in Wales, 300 deaths in men and 190 deaths in women were attributed to gastric cancer, with a mortality:incidence ratio of 73% in men and 70% in women (www.wcisu.nhs.uk).

However, the incidence of gastric cancer has been declining on an annual basis, and in the USA the male incidence has decreased from 38 to 5.2 per 100, 000 between 1930 and 1994, and in women from 28 to 2.3 per 100,000 (Landis *et al.*, 1998). In Wales, there has also been a significant decrease in the incidence of gastric cancer (1992-2003), 3.8% decline in men per annum, with a 3.1% decline noted in women (www.wcisu.nhs.uk).

1.1.3 Classification of gastric cancer

Gastric cancers can be classified by a number of methods. They can be subdivided according to anatomical site. Figure 1.1 illustrates the different areas of the stomach. What has been traditionally referred to as gastric cancer is in fact cancer of the distal stomach. These distal cancers are declining in incidence, with the

Figure 1.1. Anatomy of stomach



incidence of the more proximal cancers increasing (Grady 2001). Proximal gastric cancers of the cardia are often difficult to distinguish from oesophageal adenocarcinomas, as presentation is usually at an advanced stage of the disease, hence the term gastro-oesophageal cancer is used. The incidence of Barretts metaplasia has increased in developed countries and hence the incidence of Barretts associated adenocarcinoma of the oesophagus has also increased (Blot *et al.*, 1991, Clark *et al.*, 1994, Kabat *et al.*, 1993). The decline in the incidence of distal gastric cancer may reflect the improvements in lifestyle and the socio-economic climate, which have been evident over the last few decades, particularly in developed countries. Later in this chapter the contribution a number of environmental factors make to gastric cancer incidence, is discussed further.

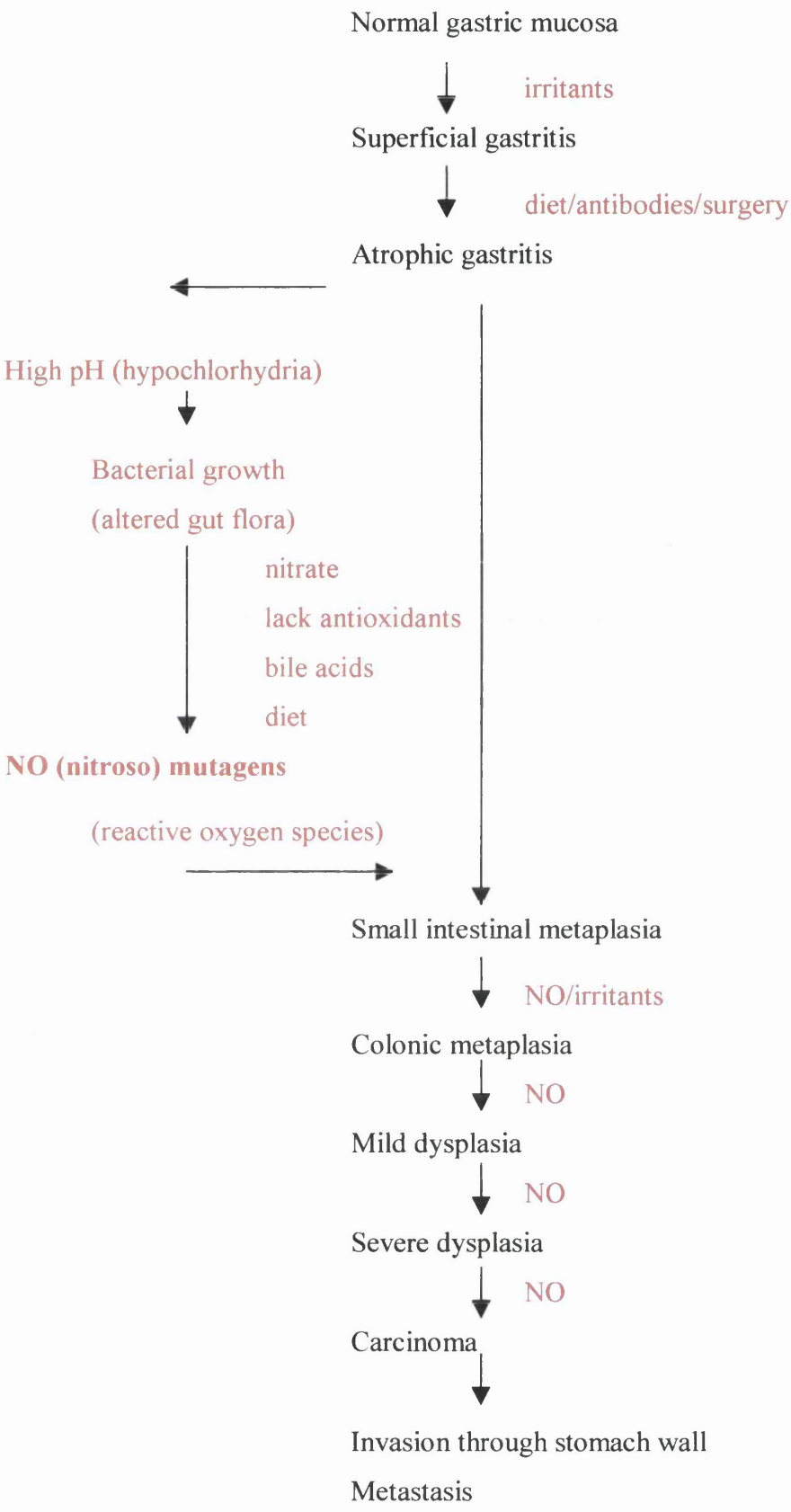
Gastric tumours can also be classified according to cell of origin and histological patterns. 90% of malignant gastric tumours are adenocarcinomas, with lymphomas, carcinoid tumours and leiomyosarcomas making up the residual tumour types (Luk *et al.*, 1998). The Lauren classification of adenocarcinomas is universally accepted, dividing cancers into intestinal and diffuse tumours (Lauren 1965, Grady 2002). Intestinal tumours are composed of well-differentiated cells arranged in glands, unlike the diffuse tumour type in which cells show little glandular formation and cells infiltrate in sheets or in single file. There are not only histological differences between these two types of gastric adenocarcinomas, but also differences in the demographic characteristics of the individuals affected, and possibly even in their prognosis. There may be variations in their aetiologies also, and hence the genetic makeup of the tumours. This is discussed in more detail in Chapter 5 when specimens of each cancer types were studied.

1.1.4 Multistep pathway of gastric carcinogenesis

The pathway leading to gastric cancer from normal gastric tissue, and the sequence of genetic changes involved at each stage remains unclear. However, Correa has proposed a multistep pathway to intestinal cancer, including pre-cancerous stages and the possible environmental insults sustained, illustrated in Figure 1.2 (Correa 1988).

Gastritis is an inflammation of the gastric tissue, and may be acute or chronic. Gastric atrophy is the loss of gastric glands due to ulceration or a prolonged inflammatory process. Cancer risk increases as gastric atrophy becomes more widespread and extends proximally into the corpus (Sipponen *et al.*, 1998). Intestinal metaplasia represents a non-neoplastic change in cell phenotype usually due to a sustained adverse environment, and is caused by alteration of the stem cell lineage or epigenetic changes. It is not always recognisable macroscopically, but may be seen as a plaque or non-homogeneous area of the gastric mucosa (Walker 2003). Intestinal metaplasia is associated with an increased risk of cancer (Morson *et al.*, 1980), however intestinal metaplasia is common and gastric cancer is not. Intestinal metaplasia may progress from small bowel metaplasia to colonic metaplasia, and can be divided into 3 types (1) complete – (small intestine) – containing goblet, Paneth, endocrine and brush bordered cells (enterocytes), (2) incomplete – (goblet cell metaplasia) containing goblet and mucous cell, no enterocytes, plus sialomucins, (3) incomplete – (colonic type) – containing goblet and mucous cells, no enterocytes, plus sulphomucins (Correa 1988). Type 3 intestinal metaplasia, has been shown to contain the most genetic changes and therefore may harbour an increased risk of cancer (Dixon *et al.*, 2001). Dysplasia is the term used to describe a histological abnormality in cells associated with progression to cancer (Morson *et al.*1980). Dysplastic cells

Figure 1.2 Correa hypothesis of gastric cancer aetiology. (Correa 1988)



have enlarged hyperchromatic nuclei with coarse chromatin and irregular nucleoli. Apoptosis and karyorrhexis can be seen together with irregular crypt architecture. These features are associated with a failure of cells to mature as they migrate away from the stem cell compartment.

1.1.5 Aetiology of gastric cancer

The aetiology of gastric cancer is multifactorial. Host genetics and environmental agents have been implicated through epidemiological and laboratory studies.

Helicobacter pylori was declared a class 1 carcinogen in 1994 due to its association with gastric cancer (IARC 1994) and, because of the large amount of experimental and epidemiological evidence, it is considered to be a major epidemiological factor in gastric carcinogenesis. This will be discussed in greater detail later in this chapter. Other bacteria have been implicated in gastric carcinogenesis, such as *Helicobacter heilmannii* and mycoplasma infection, but their correlation is weak (Stadtlander and Waterbro 1999). The importance of viral, fungal and parasitic infection has also been investigated. Epstein-Barr viral infection, already recognised as a human carcinogen because of its relationship to other cancers (Hodgkins disease, sinonasal carcinoma) (IARC 1997), is one of the infections which has been implicated in gastric cancer development (Stadlander and Waterbro 1999).

Dietary compounds have been linked to gastric cancer and its pre-malignant stages. A wealth of epidemiological and experimental studies have shown that certain foods can be protective against gastric carcinogenesis, and include fruit (especially when containing ascorbic acid) and vegetables. Other foods containing nitrosamines and salt, together with alcohol, are all agents associated with increased rates of gastric

carcinogenesis (Chan *et al.*, 1999, Correa 1988, Osaka *et al.*, 1997, UK Subgroup of EPC-EURONUT-IM Study Group 1991, Sobala *et al.*, 1992, Neuget *et al.*, 1996). Epidemiological studies from 20-30 years ago have shown an association between excessive salt intake and gastric cancer (Joosens and Geboers 1981), as well as excessive salt intake and gastritis/intestinal metaplasia (Stemmermann *et al.*, 1977, Correa *et al.*, 1975). Populations at high risk of gastric cancer, such as those in Asia, have been found to have disproportionately high levels of nitrosoamines in their diet (Wakabayashi *et al.*, 1987). You *et al.* 2000 have noted, in a follow up study to identify people at risk of developing gastric cancer, that higher levels of dietary ascorbic acid decreased the risk of progression to cancer by up to 80%. Previous work by the same author has also shown a similar negative correlation with pre-malignant gastric disease i.e. chronic gastritis, intestinal metaplasia and dysplasia (You *et al.*, 1993). Studies have shown too, that a low intake of fruit and vegetables is associated with higher levels of p53 mutation in gastric cancers (Fedriga *et al.*, 2000). You *et al.* 2000 have also documented a positive association with smoking (20 cigarettes per day) and pre-malignant gastric diseases. Smokers have further been suggested to have a moderately increased risk of gastric cancer (IARC 1985).

Both socio-economic factors and geographic differences influence the incidence rates of gastric cancer (Chan *et al.*, 1999, Correa 1988). Stadlander and Waterbro have reviewed a large number of epidemiological studies, and surmise that there is a geographical variation in the gastric cancer incidence amongst and within countries. The highest incidence rates of gastric cancer exist in Asian countries, with significant disease also occurring in European countries, Latin America and the USA (Stadlander and Waterbro 1999). Parkin *et al.* 1999 have also noted that the incidence of gastric cancer in the Far East was far higher than that in the Western populations,

with 38% of all stomach cancers occurring in China. An association has also been shown for higher levels of p53 mutation in gastric cancers and lower social class (Fedriga *et al.*, 2000).

Family history is associated with increased risk of gastric cancer (Chan *et al.*, 1999, Correa 1988, La Vecchoa *et al.*, 1992, Zaghieri *et al.*, 1990, Graham and Lilenfeld 1958), and with an increased risk of pre-cancerous abnormalities (Carneiro *et al.*, 1993). Nomura demonstrated a 2-3x increased risk of gastric cancer in relatives of patients with gastric cancer (Nomura 1982). El –Omar *et al.* have shown that relatives of patients with gastric cancer have increased prevalence of pre-cancerous gastric abnormalities, although only predominantly in patients with *Helicobacter pylori* infection (El-Omar *et al.*, 2000). Brenner *et al.* have also noted that increased levels of *H. pylori* exist in children of gastric cancer patients (Brenner *et al.*, 2000). Thus, family clustering of gastric cancer may not only be due to shared genes but might be due to environmental factors such as shared *H. pylori* infection.

We know that age is important in the progression to gastric cancer (Chan *et al.*, 1999, Correa 1988). As the population ages so the incidence of intestinal type gastric cancer increases (Sipponen *et al.*, 1998). Younger people commonly exhibit inflammation and gastritis with intestinal metaplasia, whereas the older population show gastric atrophy and colonic type intestinal metaplasia, hence increasing the risk of developing gastric cancer (Asaka *et al*, 1997, Tahara *et al.*, 1994, Lengauer *et al.*, 1999).

Sex differences also exist in intestinal type gastric cancer. The male incidence increases after the 3rd decade, but the female incidence only starts to increase after the 6th decade (Sipponen *et al.*, 1998). Possibly a protective effect is exerted by female hormones present until the menopause. No difference in gender is seen in the pre-

malignant stages of gastric cancer, or in the incidence of *Helicobacter pylori* infection. Blood group A has also been shown to be positively associated with gastric cancer (Nomura 1982), although the mechanisms behind this are not well understood.

There is significant overlap between all these aetiological agents, and determining the effect of individual risk factors on gastric carcinogenesis has proven difficult. Of clinical importance is the theory that the prevention of gastric cancer in a large number of populations should be possible by addressing the different aetiological factors discussed, although this is obviously more complicated in practice.

1.1.6 Familial gastric cancers

The majority of gastric cancers described above arise as a result of a number of sporadic genetic events arising in gastric epithelial cells in people with no inherited predisposition to gastric cancer. The accumulation of a number of genetic abnormalities in tumour suppressor genes and oncogenes underlies the multistep pathway of gastric cells to gastric adenocarcinoma (Correa 1998). Abnormalities are often the result of a failure in the normal mechanisms present in the cell to conserve DNA fidelity. Clonal selection of the abnormalities favouring tumourigenesis then occurs to further promote cancer development.

A small number of gastric cancers are however familial. Inherited predisposition syndromes include hereditary non-polyposis colon cancer syndrome (HNPCC). HNPCC is caused by a germline mutation of a mismatch repair gene, and this allows errors in DNA replication to persist. These are characterised by frameshift mutations in microsatellite repeats i.e. microsatellite instability. This genomic instability predisposes to mutational inactivation of tumour suppressor genes

containing microsatellites in their coding regions (Grady 2001). Up to 79% of the associated gastric cancers in HNPCC are of the intestinal type, and they occur in 11% of HNPCC families with MLH1 or MSH2 germline mutations (Aarnio *et al.*, 1997, Vasen *et al.*, 1996). Other syndromes that have increased risks of gastric cancer include hereditary diffuse gastric cancer (HDGC). HDGC has been attributed to a germline mutation of CDH1, the gene for E-cadherin whose role includes intercellular adhesion (Bevan and Houlston 1999, Guilford *et al.*, 1998). HDGC is also associated with breast and colon cancer (Keller *et al.*, 1999). Li-Fraumeni syndrome includes predisposition to soft tissue sarcomas, leukaemia, brain tumours, breast cancer and gastric cancer, and is caused by p53 germline mutations (Grady 2001). Peutz-Jeuger syndrome, characterised by gastrointestinal hamartomatous polyps, is associated with ovarian tumours, pancreatic cancers, melanoma and gastric cancer (Grady 2001) and has germline mutations of a serine threonine kinase (STK 11) (Hemminke *et al.*, 1998). In Japan, familial adenomatous polyposis (FAP) families, have had reported associations with gastric cancer (Bevan and Houston 1999). These families have abnormalities of the tumour suppressor gene APC.

1.1.7 p53 and gastric cancer

P53 is a well known tumour suppressor gene located on chromosome 17 (17p13.1). It acts as a transcription factor, maintaining cell stability through its control of cell cycle progression and apoptosis in response to DNA damage (Grady 2001). The ability of p53 to activate transcription in genes containing p53 binding sites appears to be required for this response (Sud *et al.*, 2001). Loss of p53 function is most commonly induced through point mutation (Bates and Vousden 1996), and there

are over 15,000 different types of p53 mutations described in cancers (www.iarc.fr). It is the most frequently mutated gene in human cancers (Levine *et al.*, 1991) and is mutated in up to 60% of all human tumours (Tamura *et al.*, 1991, Sipponen *et al.*, 1998, Levine *et al.*, 1991, Vogelstein and Kinzer 1992). It is commonly found to be abnormal in 30-58% of intestinal and diffuse gastric tumours (Grady 2001). Mutations of p53 have been found in advanced gastric cancer (Romiti *et al.*, 1998, Brito *et al.*, 1994, Joypaul *et al.*, 1993), whereas other authors have found it to be present in early gastric cancer (Uchino *et al.*, 1993, Tohodo *et al.*, 1993, Morgan *et al.*, 2003). Uchino *et al.*, found that abnormalities of p53 were present in 25% of early gastric cancers and 42% of the advanced stage (Uchino *et al.*, 1993). There is quite a large variation in the frequency rate of p53 mutation quoted in the literature, and this may occur due to the different methods of mutation detection and the different methods of tissue preparation. Sud *et al.*, noted a p53 mutation rate of 31% in the tumours they studied. This is similar to other studies – 33-38% (Tamura 1991, Uchino *et al.*, 1993), but some studies quote higher figures of 65% (Imazeki *et al.*, 1991). Loss of heterozygosity (deletion of one copy of gene) of p53 can be seen in 60% of gastric tumours and this is most often accompanied by mutation of the other copy of p53 (30-50%), therefore causing complete inactivation (Sano *et al.*, 1991, Sud *et al.*, 2001).

The presence of p53 mutations in gastric cancer has been associated with a worse prognosis than those cancers without p53 abnormalities, 5 year survival rates of 24% compared to 56% respectively have been reported (Martin *et al.*, 1992). Association between p53 abnormalities and metastatic spread has also been noted (Monig *et al.*, 1997).

Abnormalities of p53 have been found in pre-malignant gastric cancer tissue and this is discussed in greater detail in Chapter 4.

1.1.8 Aneuploidy and gastric cancer

It is widely accepted that gastric cancer results from genetic alterations, and studies have shown that genetic instability can lead to accumulation of somatic mutations and chromosomal instability (Grady 2001, Kabayashi *et al.,* 2000). There is now a hypothesis that levels of aneuploidy are related to tumour instability (Lengauer *et al.*, Nardone *et al.*, 1999). Aneuploidy is the term used to describe numerical aberrations in chromosomes, producing cells with chromosome number greater or smaller than the diploid complement.

Cytogenetic studies can be used to identify a broad range of chromosome aberrations, both structural and numerical. As the techniques to look at chromosome patterns improve, so the information available regarding the aberrations in cancer increases. Numerical and structural chromosomal abnormalities have been found in gastric cancer tissue and gastric cancer cell lines, with high levels of chromosome rearrangement demonstrated (Bertoni *et al.*, 1998). Genomic ploidy measurements can be made with flow cytometry, and Giemsa banding (G banding) can look at changes in the whole karyotype. The development of Comparative Genomic Hybridisation (CGH) and in situ hybridisation has allowed better resolution of the chromosomal aberrations present in tumour cells (Sen 2000). CGH has shown an increase in DNA content (up to 4 fold) in 86% of gastric cancer tissue studied, with the majority of chromosomes being involved (Kokkola *et al.*, 1998, Han *et al.*, 1998, Kitayama *et al.*, 2000). G banding has also shown altered karyotypes in gastric cancers (Panini *et al.*, 1995).

The developments in Fluorescence in Situ Hybridisation (FISH) and the increasing number of centromeric probes and gene specific probes now available, allow the accurate characterisation of numerical and structural genetic changes. Difficulties in performing these techniques on solid tissue are slowly being overcome, and techniques to produce a monolayer of cells have now been developed, as shown later in this thesis. FISH studies in gastric cancer have shown that certain chromosomes and regions of chromosomes are affected more commonly than others, although different studies implicate chromosomes with differing frequencies. Table 1.1 illustrates the results of a number of cytogenetic studies on gastric cancer cells. A more detailed table is presented in Chapter 4. Aneuploidy of varying degrees has been documented in all stages of gastric cancer (Beuzen *et al.*, 2000). Chromosome 3 involvement is seen commonly, not only in studies of gastric cancer but also of solid tumours elsewhere in the body (Pathak 1992). Sex chromosomes are lost in gastric cancers in up to 70% of cases, but sex chromosome loss can be seen in normal individuals as they age, and therefore may not be relevant to carcinogenesis (Yadav *et al.*, 1996, Beuzen *et al.* 2000, Nowinski *et al.*, 1990). The list of cytogenetic studies given is obviously not exhaustive but illustrates the problems arising when correlating study results. What is obvious is the variation in the results shown, although some overlap is seen between studies. A large proportion of the studies on gastric cancer genetics feature Japanese patients, as gastric cancer is the most prevalent cancer in East Asia (Chun *et al.*, 2000). As already has been explained, host genetics and socio-economic features play a part in the aetiology of cancer, hence comparing the results of these studies to those of a Western population may not be straightforward. It is also important to remember that these studies are usually of advanced tumours and that sample numbers are often small. This, together with the knowledge that gastric

Table 1.1: The results of a number of cytogenetic studies looking at chromosomal aberrations in gastric cancer.

Author	Sample size	Cancer Type	Chromosome
Panini 1995 (Greece)	11	10/11 intestinal (3 metastatic)	**3**
Kokkola 1998(Finland)	22	intestinal	gain-**20q** loss-**18q**
Koo 2000 (South Korea)	37	50% intestinal (22/37 metastatic)	gain-**8q** loss-**17p**
Yadav 1996 (USA/Japan)	4	1 metastatic	3, 7, 11, X
Fringes 2000(Germany)	20	intestinal/diffuse	gains-**1**
Beuzen 2000 (France)	60	cardia, antrum & oesophageal	7, 8, 11, 17, Y (40-65%allcancers)
Okada 2000 (Japan)	5	diffuse (80% metastatic)	loss-18q gain-7, 20
Bertoni 1996 (Italy)	2	metastatic	6, karyotype rearrangement
Han 1996 (South Korea)	18	diffuse	widespread numerical rearrangement

Table 1.1 (cont'd): The results of a number of cytogenetic studies looking at chromosomal aberrations in gastric cancer.

Author	Sample size	Cancer Type	Chromosome
Chun 2000 (South Korea)	6	advanced	loss-**17p**
Igashari 2000 (Japan)	39		1p lost in advanced
Kitiyama 2000(Japan)	24		1, 2 - early 1, 2, 4, 20 - later
Sud 2001 (UK)	9	17 intestinal 9 diffuse	loss - **22q**
Wu 2001 (Taiwan)	53	65% advanced 55% intestinal 45% diffuse	gains – **8q** loss – **16q**
Sugai 1999 (Japan)	99	65 intestinal 34 diffuse	aneuploidy in 73%

cancer cells are inherently unstable, makes the characterisation of a simple step-wise genetic pathway to gastric cancer difficult.

Opinions vary as to whether intestinal and diffuse gastric cancers have different chromosomal abnormalities, and hence different genetic pathways (Kokkola *et al.,* 1998, Pathak 1992, Nowinski *et al.*, 1990, Hoshi *et al.,* 1999 Yasui W *et al.*, 2000, Sugai *et al*, 1999). As different phenotypic pathways are said to exist for each cancer, and as patient characteristics vary, then it would be plausible to believe those who favour two distinct genetic aetiologies. It is also not clear as to whether site of tumour influences chromosomal changes (Beuzen *et al.*, 2000, Nowinski *et al.*, 1990). However, there is agreement that advanced cancers have more extensive genetic change than early cancers (Kitayama *et al.*, 2000, Koo *et al.*, 2000, Kim *et al.*, 2000, Lee *et al.,* 1999, Igarashi *et al.,* 2000, Brito *et al.*, 1994, Fonesca *et al.*, 1994). For example, a Japanese study of intestinal type cancers has found aneuploidy in 55.6% of early cancers, compared to 86.4% of advanced cancers (Sugai *et al.,* 1999). Chromosomal instability has certainly been identified as an indicator of aggressive tumour behaviour (Yadav *et al.*, 1996).

A number of researchers have also looked at pre-malignant states and found that some of the genetic changes present in the most advanced gastric cancers are present in these early stages too (Tahara 1995), indicative of important driving changes in carcinogenesis. This is discussed in more detail in Chapter 4, where the results of a cytogenetic analysis study on pre-malignant gastric tissue is described.

1.2 Aneuploidy

1.2.1 Aneuploidy and cancer

A number of genetic changes have been identified in cancers. These include point mutations in genes, gene expression alterations, chromosomal translocations causing structural rearrangement of genes, and finally, numerical changes in part or whole chromosomes. In 1936, abnormal amounts of DNA were first recognised in human cancers (Caspersson 1936), and almost 100 years ago it was thought that chromosomal imbalance was the cause of human cancers (Boveri 1914). Today, aneuploidy is commonly seen involving one or more chromosomes in human cancers (Sen 2000), and it is the most prevalent genetic abnormality seen in solid tumours (Heim and Mitelman 1995). The association of aneuploidy and cancer is therefore undisputed, but the mechanism for aneuploidy induction and indeed carcinogenesis in most tissues is still unresolved.

The identification of aneuploidy early in cancer development, has suggested that altered DNA content, and its underlying cause may play an important role in the development and progression of cancer (Barret *et al.*, 1999, DeAngelis *et al.*, 1999). DNA aneuploidy has been associated with poor prognosis in a number of cancers (Ross *et al.*, 1996, Macgennis *et al.*, 1997). There is also a hypothesis that tumour instability is related to chromosome aneuploidy (Lengauer *et al.*, 1999, Nardonne *et al.*, 1999). *In vitro* studies show that aneuploidy is required for neoplastic transformation, and its development is necessary for cell immortilisation. Aneuploidy in cells has been shown to be induced by chemical carcinogens and oncogenic viruses (Namba *et al.*, 1996, Li *et al.*, 1997).

The association of aneuploidy and GI cancer is strong. Aneuploidy in colorectal cancers, oesophageal adenocarcinomas and gastric adenocarcinoma is common. Tumours with aneuploidy have been shown to behave more aggressively, suggesting a clinical use to establish risk and prognosis of disease. For example, DNA aneuploidy has been shown to be a possible indicator of disease progression in ulcerative colitis to colonic cancer (Lindberg *et al.*, 1999). Similar observations as to the importance of aneuploidy in progression to cancer have been noted in Barretts disease of the oesophagus (Teodori *et al.*, 1998, Doak *et al.*, 2003). In gastric cancer, higher levels of aneuploidy have been associated with advanced tumours (Sugai *et al.*, 1999), and metastatic spread of gastric cancer (Sasaki *et al.*, 1999). The association of gastric cancer and aneuploidy has been discussed in detail earlier in this chapter.

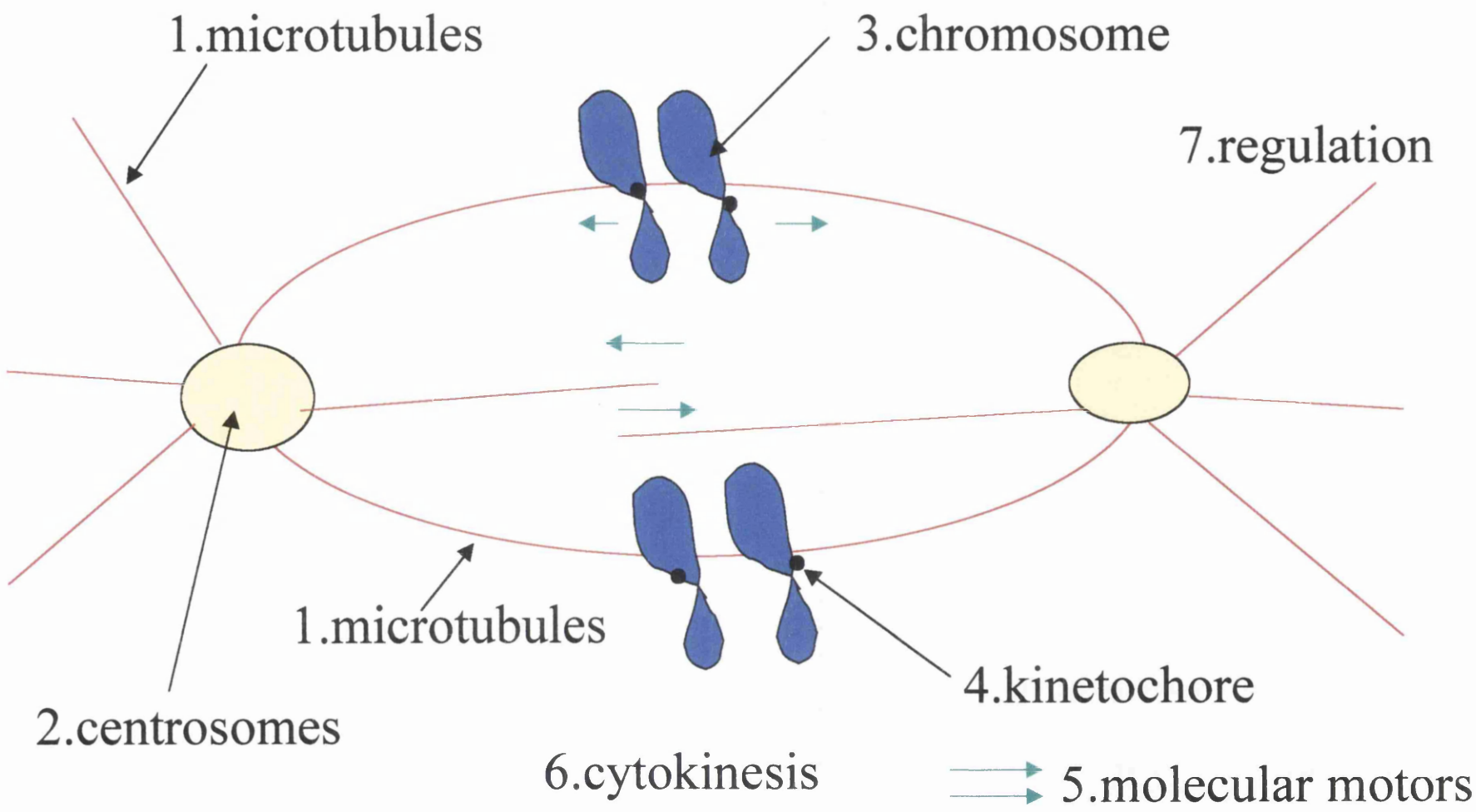
1.2.2 Mechanisms of aneuploidy development

The development of aneuploidy is possible through three broad mechanisms. Firstly, it could arise from the sporadic missegregation of chromosomes leading to stable cells with a relatively homogenous DNA content. Secondly, the development of polyploidisation leads to aneuploidy, which can be caused by multiple rounds of replication, mitotic failure (spindle failure, cytokinesis failure), or methods such as cell fusion. This again would result in tumour cells with stable chromosome number. A third mechanism, and the most likely to occur in cancers, involves the acquisition of a permanent defect in the ability to segregate chromosomes. This leads to persistent changes in chromosome numbers within tumour cells, therefore producing a heterogeneous cell population undergoing constant change (Pilan and Doxsey 1999). This is seen in a number of different human cancers (Lenguaer *et al.*, 1997) and could explain the variety of karyotypes in tumours. Persistent missegregation of

chromosomes could cause, accelerate or contribute to tumourigenesis, by facilitating the accumulation of chromosomes housing genes which have the ability to potentiate growth, and also by losing chromosomes with growth restraining genes. Hence, cells possessing a growth advantage are selected. This is also an important consideration in the treatment of cancers, as chemoresistant cancer cells would need to possess similar characteristics.

Figure 1.3 illustrates the different components in mitosis where defects can lead to chromosome missegregation and hence aneuploidy (Pihan and Doxsey 1999). Centrosomes, or spindle poles, are microtubular barrels surrounded by a protein matrix. Their function is to organise the microtubular structures of interphase cells and the mitotic spindle during cell division. They have a role in the nucleation, severing, movement and anchoring of microtubules, and also provide a support for mitotic regulatory activities. Abnormalities of centrosome function (extra copies, aberrant size and shape, acentriolar) can cause aberrant spindle formation and result in chromosome missegregation and high rates of aneuploidy, and this has been demonstrated in many tumours, including GI tumours (Pihan *et al.*, 1998, Lenguaer *et al.*, 1997). The identification of a family of serine/threonine protein kinases, Aurora kinases, with a role in the regulation of cell division has proved interesting (Carmena and Earnshaw 2003). There are 3 main types of Aurora kinases, A, B and C. Aurora A kinases are linked to centrosome separation and spindle assembly through microtubular function (Carmensa and Earnshaw 2003). One such centrosome associated kinase, STK15/BTAK/aurora2, has been found to be amplified and overexpressed in human cancers (Zhou *et el.*, 1998). This has led to the suggestion that this kinase is involved in abnormal centrosome function and hence, chromosome

Figure 1.3. Components of mitotic machinery and sites of disruption to cause aneuploidy (Pihan and Doxsey 1999)



missegregation. *In vitro* experiments have correlated this kinase activity with both aneuploidy and tumourigenesis (Giet *et al.*, 1999).

Microtubule spindle proteins provide the structural framework for mitosis, and the tracks for chromosomes during their separation. Hence, defects in these structures could upset chromosome segregation. Experiments have shown that chemicals affecting microtubular function result in aneuploidy (Oshimura and Barret 1986). Changes in the expression of tubulin, the subunit of microtubules, and mutation in the tubulin genes, lead to chromosomal missegregation (Burke *et al.*, 1989, Huffaker *et al.*, 1988).

Motor proteins provide the force for chromosome separation, condensation and segregation, and also the elongation and positioning of the spindle. Disruption of any number of these microtubular motor proteins could lead to chromosome missegregation and hence, aneuploidy (Pihan and Doxsey 1999).

Kinetochores are proteins found around the chromosome centromeres. These proteins allow spindle attachment and also anchor motor proteins to allow chromosome movement. They also possess checkpoint proteins that ensure compete chromosome separation prior to separation into daughter cells. The disruption of these proteins could result in aneuploidy. In fact, centromeres lacking kinetochore proteins have been shown in cancer cells (Vig and Sternes 1991).

Abnormalities in chromosome condensation can lead to fragmentation and missegregation during mitosis. Abnormalities of topoisomerases, whose usual function is to help avoid chromomosomal entanglement, have been demonstrated in tumour cells (Hashimoto *et al.*, 1995).

Cohesion of sister chromatids is needed during mitosis. Mutations in several yeast genes have shown that early and late separation of these chromatids results in

non-disjunction of chromosomes, and hence aneuploidy ((Miyazaki and Orr-Weaver 1994). Similar events have been shown in cancer (Gebhart 1989). The overexpression of a protein called securin, which is known to inhibit chromatid separation, has been found in human cancer cells (Zou *et al.*, 1999).

The disruption of cytokinesis, the process of cell separation into two daughter cells, also has potential to be implicated in carcinogenesis. It has been observed in cancer cells and implicated in polyploidisation (Shackney *et al.*, 1989).

The regulation of mitosis involves a number of pathways including regulatory circuits, mitotic checkpoints and apoptotic checkpoints. Entry into and exit from mitosis is controlled by a number of regulatory pathways, and abnormalities seen in some of these proteins have been shown in cancer cells with polyploidisation (Yamamoto *et al.*, 1998). Mitotic checkpoints exist throughout mitosis, and proteins thought to be important in avoiding chromosome missegregation include the Mad and Bub proteins. Defects of these checkpoint proteins have been demonstrated in cancers with chromosomal instability (Cahill *et al.*, 1998). The apoptotic checkpoint ensures that any cells do not survive if they have an aberrant chromosome number. Cancer cells have been shown to possess increased amounts of a protein called survivin, an inhibitor of apoptosis, and hence this may represent a mechanism whereby cells with aberrant chromosome copy number survive (Ambosisni *et al.*, 1997).

1.3 *Helicobacter pylori*

1.3.1 Introduction to *Helicobacter pylori*

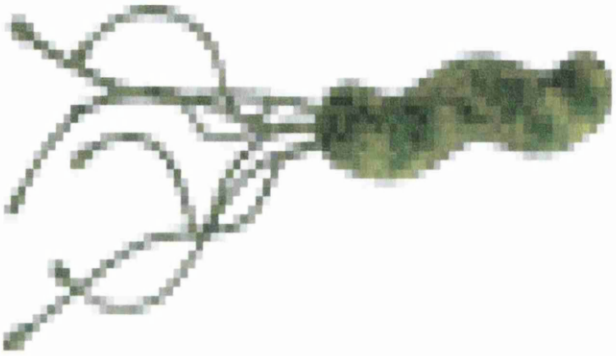
Helicobacter pylori is a member of a large family of Gram negative bacilli known for many decades to inhabit the human host. In 1983, Warren and Marshall

described this spiral bacterium, which is similar to Campylobacter, in the mucosa of patients with chronic gastritis (Warren and Marshall 1983). See figure 1.4. Since then its presence has been implicated in the aetiology of many diseases. Peptic ulcer disease, mucosa associated lymphoma (MALTomas) and gastric cancer, have been causally linked to *H. pylori* infection (Cover and Blaser 1995). In the case of other diseases such as non-ulcer dyspepsia, oesophageal disease (gastrooesophageal reflux disease/GORD, Barretts oesophagus) and atherosclerotic disease, the link is less clear. The presence of *H. pylori* has been suggested as being protective against GORD, Barretts disease and hence adenocarcinoma of the oesophagus. Asymptomatic carriage of the infection is also common (Blaser 1998), with only an estimated 20% of infected individuals developing clinical disease (Kuipers *et al.*, 1995, Uemura *et al.*, 2001). Cofactors both in the host and the environment may help to explain why not all cases of *H. pylori* infection result in disease, and why different diseases are seen (Graham 2000, Sipponen *et al.*, 1998, Blaser 1998).

1.3.2 Incidence of *H. pylori* infection

The prevalence of *H. pylori* infection worldwide has been estimated at 50% (Marshall 1994, Nepomnayshy and Burkitt 2000), but different rates occur depending upon geographic location. A review by O'Connor and Sebastian 2003, suggested 20% of people under 40 years of age in a developed country would carry *H. pylori* infection, increasing to 50% in those over 60 years, whereas in developing countries 80% of the population by age 20 would already be infected. This geographical variation would partly reflect the differences in the socio-economic conditions of the different populations. The bacterium is transmitted via the faecal oral route, and as such, high rates of infectivity in developing nations may reflect large family units and

Figure 1.4 ***Helicobacter pylori***



poor hygiene practices. Obviously the use of antibiotics will also partly be responsible for the lower prevalence rate in developed countries. The number of positive *H. pylori* serological tests increases with low socio-economic class (Logan and Walker 2001).

A decline in the incidence of *H. pylori* has indeed been noted over time. A Scandinavian study noted *H. pylori* antibody levels in their population fell between 1973 and 1994 from 56% to 31% respectively (Kosunen *et al.*, 1997). In fact, in developed countries the increased infection rates seen with age, may represent the childhood acquisition of the infection at a time when prevalence rates of *H. pylori* were higher (Logan and Walker 2001, O'Connor and Sebastian 2003).

Infection in early childhood is the usual scenario. In developed countries primary infection in adults is rare, as is re-infection after eradication in adults, although this is not always the case in developing nations (Logan and Walker 2001, O'Connor and Sebastian 2003).

1.3.3 Mechanism of action of *H. pylori*

Helicobacter pylori organisms are located in the mucus lining of the stomach. They affect the gastric tissue by inducing gastric inflammation, disrupting the gastric mucosal barrier and altering gastric acid metabolism (Dunn *et al.*, 1997).

Cellular hyperproliferation is seen in the infected gastric tissue and this is linked with increased apoptosis (Nardonne *et al.*, 1999, Moss *et al.*, 1995). Inflammation is associated with increased cytokine release, such as interleukin 6 and 8 (Graham 2000, Sipponen *et al.*, 1998, Blaser 1998, Kuniyasu *et al.*, 2000), and indeed there are increased numbers of inflammatory cells in the gastric mucosa of *H. pylori* infected individuals (Correa and Shiao, 1994). These inflammatory leucocytes release Reactive Oxygen Species (ROS), such as superoxide and H_2O_2, as a defence

mechanism against the bacterial infection. Reactive oxygen species are discussed in more detail later in this introduction. Infection is known to be a risk factor for many types of cancer as it increases the ROS levels as a result of the inflammatory immune response (Ames *et al.*, 1993). Increased levels of ROS in the gastric mucosa, and therefore the levels of oxidative DNA damage, are known to be highest in the gastritis stage of gastric cancer and are associated with the greatest inflammatory response (Morgan *et al.*, 2003, Marnett, 2000). *H. pylori* has been shown capable of inducing ROS production both directly (Bagchi *et al.*, 1996, Obst *et al.*, 2000), and indirectly through activation of polymorphonuclear leukocytes (Obst *et al.*, 2000, Farinatti *et al.*, 1998). ROS levels have been shown to correlate with *H. pylori* presence in the gastric mucosa (Baik *et al.*, 1996) and, in fact the level of ROS has been shown to be proportional to the bacterial load (Dunn *et al.*, 1997). *H. pylori* is linked causally to gastric cancer and we know that the production of ROS is one way the bacterium exerts its effect on gastric cells (Chan *et al.*, 1999, Correa, 1988, Asaka *et al.*, 1997, Wagner *et al.*, 2000). Studies have shown abnormalities at the cytogenetic level and the molecular level in pre-malignant gastric tissue infected with *Helicobacter pylori* (Nardonne et al., 1999).

1.3.4 *H. pylori* and gastric disease

H. pylori is capable of inducing a number of phenotypic changes in the gastric mucosa of infected individuals. Superficial gastritis, chronic gastritis, atrophic gastritis and intestinal metaplasia have all been demonstrated (Graham 2000, Sipponen *et al.* 1997, Kuniyasu *et al*, 2000). All these histological changes can be found in the pre-malignant stages of gastric carcinogenesis (Correa 1988). *H. pylori* infection has been causally associated with Maltomas, peptic ulcer disease and gastric

cancer. *H. pylori* has been found in 70% of gastric antrum samples of patients with chronic active gastritis/gastric ulcer, and in 90% of patients with duodenal ulceration (Pakodi *et al.*, 2000), although American studies suggest lower rates of infection (Nepomnayshy and Birkett 2000).

15% of infected individuals will develop peptic ulcer or gastric cancer as a consequence of longterm infection. The site of infection seems to be important in determining the type of disease. (See Figure 1.2.) Infection of the corpus, and then acute pangastritis, can lead to mild gastritis with no upset in gastric acid secretion and hence the patient will remain asymptomatic. However, chronic infection can lead to loss of acid secreting cells, hypochlorhydria, bacterial overgrowth and, hence an increased risk of gastric ulcer and gastric cancer (Logan and Walker, 2001, Faraji and Frank 2002). An association has been suggested with repeated infection as ageing occurs, and also with poor nutrition. A similar effect of pangastritis is seen in autoimmune gastritis (pernicious anaemia), whereby hypochlorhydria develops with bacterial overgrowth and conversion of nitrates to nitrosamines, giving an increased risk of gastric cancer in the order of 1.5-3 fold (Faraji and Frank 2002).
Predominantly antral *H. pylori* infection, leads to antral gastritis with an intact acid secreting corpus, and hence results in an increased parietal cell mass and increased acid secretion due to the disruption of the regulatory feedback pathway by gastrin produced in the antrum. This process results in a high acid load developing in the duodenum and leads to duodenal pyloric metaplasia. These islands of pyloric metaplasia are colonised by *H. pylori,* and duodenitis and ulceration occurs (Logan and Walker 2001, Faraji and Frank 2002). In summary, *H. pylori* infection of the antrum results in hyperacidity and duodenal ulcer formation, whereas corpal infection is associated with hypoacidity and gastric ulcer and gastric cancer formation.

Chronic *H. pylori* infection can occur when the host immune response generated by the bacteria is not sufficient to clear the infection. Concurrent infection with different strains is possible and produces a polyclonal infection, hence allowing DNA exchange and the development of more virulent strains (Logan and Walker 2001).

Environmental factors and host factors determine how strong the association is between *H. pylori* and gastric disease (Graham 2000). HLA status affects the severity of disease associated with *H. pylori* infection (Azuma *et al.*, 1998), as do the different polymorphisms known to exist in interleukin production (El Omar *et al.,* 2000). Hence, the risk of gastric cancer risk in individuals with *H. pylori* infection is probably also affected by host factors.

1.3.5 Association of *H. pylori* with gastric cancer

In 1994, the International Agency for Research on Cancer (IARC) declared the bacterium *Helicobacter pylori* a class one human carcinogen, capable of inducing changes leading to gastric cancer (IARC 1994). *H. pylori* is linked causally to gastric cancer and we know that the production of ROS is one way the bacterium exerts its effect on gastric cells (Chan *et al.*, 1999, Correa, 1988, Asaka *et al.*, 1997, Wagner *et al.*, 2000). O'Connor and Sebastian 2003 suggested that *H. pylori* may account for 60% of all gastric cancers worldwide. There is now a large body of evidence linking *H. pylori* to gastric cancer with epidemiological studies showing a 2.7-12 fold increased risk of gastric cancer in those people infected with *H. pylori* (Cover and Blaser 1995). The EuroGast Study Group found that incidence rates of *H. pylori* infection, as determined by the presence of antibodies, was significantly higher in patients with gastric cancer than in controls (EuroGast 1993), and estimated a 6 fold

increased cancer risk associated with *H. pylori* infection. A UK study found infection rates of 69% in gastric cancers as compared to 47% in controls (Forman *et al.*, 1991). Asaka *et al.* determined an odds ratio of 2.6 when comparing *H. pylori* antibodies in controls to gastric cancer patients (Asaka *et al.*, 1997). Rugge *et al.* determined an odds ratio of *H. pylori* infection and gastric cancer of 2.9, and they found an association with both diffuse and intestinal gastric cancer (Rugge *et al.*, 1999). A Chinese study suggested that *H. pylori* infection increased the risk of gastric dysplasia, as well as gastric cancer (You *et al.*, 2000). A meta analysis of 42 epidemiological studies found an overall increased risk of gastric cancer in *H. pylori* infected people of two fold (Eslick *et al.*, 1999).

Animal models have demonstrated a whole spectrum of gastric disease is associated with *H pylori* infection, the endpoint being gastric carcinoma (Honda *et al.*, 1998).

1.3.6 *H. pylori* virulence factors

A number of virulence factors have been described in *H. pylori* and can be divided into colonisation factors and disease associated factors (McGee and Mobley 1999). Colonisation factors, present in all strains of *H. pylori*, enable the bacteria to invade the host, and proliferate unharmed in the gastric environment. Well-recognised examples include, urease activity to neutralize acidity and reduce inflammation, and the presence of flagella to enhance motility. Disease associated factors are expressed only in certain strains of *H. pylori,* and cause increased virulence. The two major virulence factors are the vacuolating cytotoxin (VacA) and the cytotoxin associated protein (CagA) (Cellin and Donelli 2000). The importance of CagA positive *H. pylori* infection is discussed in greater detail in Chapter 6.

1.3.7 Diagnosis of *H. pylori*

There are a number of invasive and non-invasive tests to determine *H. pylori* infection in patients. Chapter 6 outlines the different methods available, commenting upon their clinical usefulness and their relative sensitivities.

1.3.8 Eradication of *H. pylori*

The treatment of *H. pylori* is best approached by adopting a 'test and treat' or more correctly a 'diagnose, treat and confirm eradication' strategy (Graham 2000). A combination treatment of an acid suppressor i.e. a proton pump inhibitor (or ranitidine bismuth) and 2 antibiotics (amoxicillin/clarithromycin, metronidazole/clarithromycin or amoxicillin/metronidazole) is a popular and effective therapy, quoting eradication rates of 90% (Unge 1998). Quadruple therapy has also proven to be an effective treatment using a proton pump inhibitor, tetracycline, metronidazole and bismuth and is often used if triple therapy fails (Graham 2000, O'Connor and Sebastian 2003). Successful eradication of *H. pylori* leads to peptic ulcer healing and a decrease in ulcer recurrence. It also has symptom benefit in those patients with non-ulcer dyspepsia. It can cause complete resolution of most low grade MALT lymphomas.

In the pre-malignant stages of gastric cancer, eradication of *H. pylori* has been shown to reverse certain genetic abnormalities, with the degree of genetic change returning to that of the non-infected tissue after bacterial eradication (Blaser 1998, Nardonne *et al.*, 1999, Walker 2002). The degree of cell proliferation visible in *H. pylori* associated chronic gastritis, has been shown to be less after successful eradication, as has the degree of gastric atrophy. There was also noted to be an improvement in the genomic instability of the cells after *H. pylori* eradication, specifically the DNA content and mutations of bcl-2, p53 and the c-myc genes

(Nardone *et al.*, 1999). Furthermore, the level of ROS has been shown to drop after eradication of *H. pylori* (Drake *et al.*, 1995). A Japanese study has gone one step further and suggested that in a series of patients with early gastric cancer the successful eradication of *H. pylori* was associated with a reduced risk of cancer recurrence (Uemura *et al.*, 1997). Treatment of people with relatives known to have gastric cancer is also now a consideration (O'Connor and Sebastian 2003).

Widespread antibiotic usage and the ability of the bacteria to mutate, has been blamed for the increasing resistance seen in developed countries (Baser 1998). Patient compliance is now improved with the introduction of the pre-packed medication regimen, 'Heliclear', which is now commercially available. There is geographic variation in the success rate of *H. pylori* eradication, and anitibiotic resistance has been shown to vary between different populations (Owen 2002). Previous studies have proved that eradication rates fall as bacterial resistance increases (Van der Wouden *et al.*, 1999). Resistance to amoxicillin or tetracycline is rare (Owen 2002), however resistance to metronidazole has been reported in 10-70% of cases (Canton *et al.*, 2001). This possibly reflects the popularity of metronidazole as a drug to combat non- *H. pylori* infections. As the full eradication of *H. pylori* is not always achieved the resistant strains of *H. pylori* are commonly selected for, and hence they increase in frequency. In a European study, resistance to both clarithromycin and metronidazole was found in 86% of *H. pylori* isolates resulting in failure to eradicate the infection (Heep *et al.*, 2000). Another study suggested rates of combined antibiotic resistance in *H. pylori* infection of 72% (Owen 2002).

Vaccinations against *H. pylori* infection are being considered as some success has been shown with animal vaccinations against *H felis*, a bacterium similar to *H. pylori* (Czinn *et al.*, 1993). Difficulties with human vaccination studies exist however.

The identification of a single oral vaccination able to provide full protection, as well as producing good rates of eradication and complete disease resolution, together with a minimal side-effect profile, has not yet been developed. Also, determining the subset of the population who would benefit from the vaccination is still very much in question (Czinn and Nedrud 1999). Developing nations may benefit most from vaccination as prevalence rates are high, but as reinfection rates exist vaccination without addressing socioeconoimc factors would be unlikely to be successful.

1.4 Oxidative DNA damage and Reactive Oxygen Species

The induction of oxidative DNA damage is an important mechanism in carcinogenesis. Indeed it has been postulated that up to 50% of all human cancers result from oxidative DNA damage (Beckman and Ames, 1997). Importantly for this thesis, oxidative damage has been proposed as a potential causative agent in gastric cancer (Correa and Shiao, 1994, Stadtlander *et al.*, 1999). Oxidative DNA damage results mainly from the leakage of Reactive Oxygen Species (ROS) within cells. ROS are produced by a number of sources both environmental and cellular. Environmental sources, include UV light and man-made pollutants, whereas cellular sources include byproducts of mitochondrial metabolism and neutrophil activation (Gracey *et al.*, 1999). During the normal metabolism of aerobic organisms, mitochondria are responsible for oxidative phosphorylation. Unfortunately, the 1-2% of oxygen that is released from mitochondria as ROS (Yakes and Van Houten, 1997) is the price to pay for the 12 additional adenosine triphosphate (ATP) molecules produced per Acetyl Coenzyme A combusted during aerobic respiration (Marnett, 2000). ROS are low molecular weight compounds and are released from the mitochondria as superoxide

radicals, hydrogen peroxide and hydroxyl radicals (Friedberg *et al.*, 1995, Gracey *et al.*, 1999).

These ROS are considered to be complete carcinogens in that they can both damage DNA (mutagenic) and stimulate cell division (mitogenic) (Perwez Hussain *et al.*, 1994), this combination being most effective at inducing cancer development after longterm exposure. ROS can interact directly with DNA, or exert their effect on DNA via cellular components such as proteins and lipids (Marnett, 2000). ROS damage DNA by modifying the base structure of DNA and by introducing strand breaks. These strand breaks can also be introduced during DNA repair processes (involving DNA glycosylases of the base excision repair pathway) (Wang *et al.*, 1998). DNA strand breaks, when occurring on opposite DNA strands in the same proximity, can lead to double stranded chromosomal breaks resulting in fragmentation of chromosomes. It has been estimated that approximately 1000 oxidative damage events occur in the DNA of each cell per day (Wang *et al.*, 1998), with more DNA damage occurring in the mitochondrial DNA resulting in higher mutational rates in mitochondrial DNA compared to genomic DNA (Richter *et al.*, 1988). ROS induced DNA damage, which escapes the DNA repair systems designed to deal with them, can cause cells to become genetically unstable and develop into tumours.

1.5 Aims/Hypothesis

Gastric cancer was chosen to study, as it is a common cancer resulting in significant mortality and morbidity. As has been discussed already in this introduction, Correa has proposed a multistep pathway of gastric carcinogenesis (Correa 1988), and gastric tissue with malignant potential, gastritis (active/chronic/atrophic) and intestinal metaplasia, has been identified. In this thesis

tissues with malignant potential have been studied, and have been collectively termed pre-malignant tissue. Numerous studies outlined in this chapter have shown chromosomal abnormalities in gastric cancer, and experiments in this thesis have attempted to determine if specific chromosomal abnormalities (aneuploidy of chromosome 20, 8, 4, 17(p53)) are important in gastric carcinogenesis, and in particular play a role in the initiation of gastric cancer.

Evidence has been reviewed in this introduction to ascertain the cause of gastric cancer, and *Helicobacter pylori* has proved to be an important aetiological agent, therefore its relationship to chromosomal abnormalities was also investigated.

Oxidative damage has been described as a mechanism for carcinogenesis, and is also present in gastric tissue infected with *Helicobacter pylori.* An attempt has been made in this thesis to determine its role in the stages of gastric carcinogenesis.

Hence the aims of the work described in this thesis were three fold.

(1) To determine the type of chromosomal damage induced by oxidative damage in a human cell line.

(2) To investigate the level of aneuploidy existing in pre-malignant and malignant gastric tissue.

(3) To correlate the degree of aneuploidy in gastric tissue with *Helicobacter pylori* infection, and also make a comparison with the chromosomal abnormalities shown to be induced by oxidative damage.

The hypothesis to be tested was that *Helicobacter pylori* infection induces oxidative damage in gastric tissue, leading to aneuploidy, and that this is important in the initiation and progression to gastric cancer.

The first series of experiments, outlined in Chapter 3, looked at the effect of ROS exposure in a human cell line. Different cytogenetic methods were used to identify chromosomal damage, CBMN assay, CBMN assay + kinetochore staining and interphase FISH, using CEN probes (4,8,20) and LSI probes (p53).

Chapter 4 investigated gastric cells at different stages of disease – normal gastric mucosa, gastritis and intestinal metaplasia. Interphase FISH was used to assess chromosomal damage (CEN 4,8,20 and LSI p53). A correlation was made with patient characteristics, histological diagnosis and *H. pylori* infection.

The third interphase FISH study, Chapter 5, was performed on surgical resections to assess the chromosomal abnormalities in malignant gastric disease, and make a comparison with the pre-malignant tissue already studied.

Finally in Chapter 6, a PCR study was undertaken to determine the level of *H. pylori* infection in the pre-malignant tissue already assessed in the Chapter 4. A comparison of the detection rates of *H. pylori* infection, using PCR and histological analysis, was made. The determination of the more virulent strain of *H. pylori*, Cag A, was also made using PCR, and an attempt to correlate disease severity and the level of chromosomal abnormalities was also made.

Chapter 2.

Materials and Methods

This chapter outlines the process involved in patient recruitment and data collection in those patients studied in the endoscopic study described in Chapter 4, and in the assessment of surgical resections in Chapter 5. The methods of cell collection from endoscopic samples and resection samples is described, as is the method of interphase FISH used to determine cytogenetic abnormalities. The statistical analysis used to assess the results for the interphase FISH is explained, as well as the process whereby the histological diagnosis of gastric biopsies was made.

2.1 Patient enrolment into the study

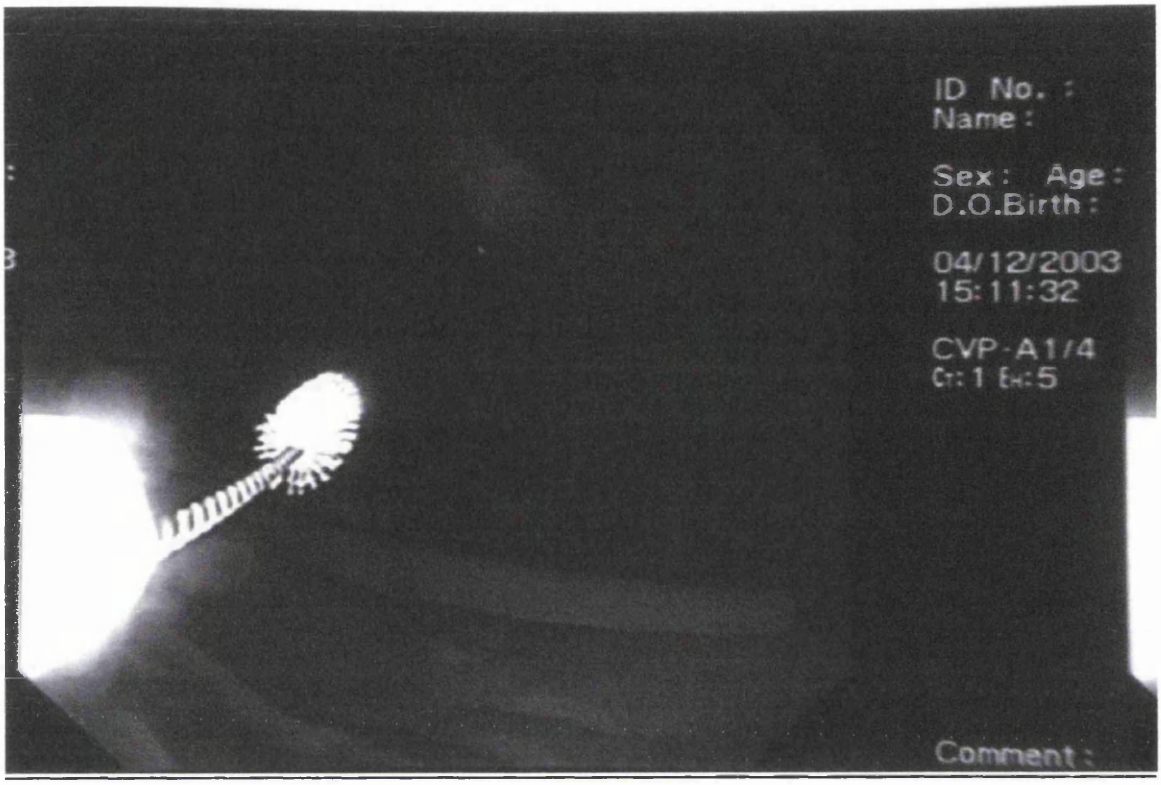
The patients who were invited to participate in this study were all attending Neath Port Talbot Hospital. The hospital is situated in a mixed rural and urban area of West Wales, and serves a socially deprived population of approximately 140,000. Prior to the start of this study Ethical Approval was obtained from the Local Research Ethics Committee, November 2001. The study was performed from January 2002 to September 2002. Patients enrolled in this study were identified during routine endoscopy lists at Neath Port Talbot Hospital. Only those people attending my endoscopy list were invited to participate, as I was the only endoscopist able to collect and transfer the samples to the university for analysis. Time only allowed a maximum of two people to be enrolled in the study at each list. Exclusions to the study were made when it was felt consent could not be adequately given (<18 years, unable to give informed consent). In an effort to achieve informed consent patient information leaflets (appendix 1.1) were sent out to all patients together with their appointment

details, and a discussion of the leaflet was made before consent was taken (appendix 1.2). Patients were not asked to consent if factors were present that were thought to modify the gastric environment (currently taking proton pump inhibitors and non steroidal anti-inflammatory drugs, recent *H. pylori* eradication and a history of previous upper GI surgery). Information was collected on sex, age, ethnicity, family history, diet, smoking, alcohol and drug intake, prior to the endoscopy and documented on Patient Case Record Forms, see appendix 1.3. The information from the patient was the only source of information collected, and no other records were reviewed.

2.2 Endoscopic cytology brushings.

At the end of an upper GI endoscopy examination, cytology brushes (Diagmed Ltd) were used to collect cells from the gastric and oesophageal mucosa. Figure 2.1 shows a cytology brush at the tip of an endoscope. The endoscopic procedure was only prolonged by 2-3 minutes to collect the brush and biopsy samples needed. Gastric brushes were taken at the same site of the gastric biopsy. If an area of the stomach was macroscopically normal the brush/biopsy was taken form the gastric antrum, otherwise the abnormal area was sampled. Oesophageal brushes were taken from macroscopically normal distal oesophagus. This methodology has been described for oesophageal samples (Doak *et al.*, 2003). Brushes/biopsies were not taken from patients on anticoagulation therapy, those with obvious bleeding lesions at endoscopy and those who showed any adverse signs during the procedure, therefore these patients were not entered into the study. No patient reported any immediate complications following any endoscopic procedure performed in the study.

Figure 2.1 Cytology brush protruding through tip of endoscope



Initially gastric cell collection on the cytology brushes was found to be poor. This improved after care was taken to adjust the angle of the brush to maintain better contact with the gastric mucosa, as the stomach is less tubular than the oesophagus. Also yield was noted to be poor if bile, residual food or blood was present in the stomach, therefore 20ml sterile water washes were used prior to brushing. Brushes were also taken prior to biopsy, as biopsy resulted in small amounts of local bleeding.

Brushes were stored in 10ml of 90% methanol, on ice, in the Endoscopy Unit for transportation to the University for laboratory analysis. Vigorous shaking of the brushes to dislodge the cells from the bristles immediately after storage in the vials was found to improve cell yield. These brushes were stored successfully for up to a week. Initially in the study, brushes were transported from the Endoscopy Unit in ETN buffer (0.1M EDTA, 0.01M Tris-HCl, 0.02M NaCl, pH 7) on ice, as described for oesophageal brushes (Doak *et al.*, 2003), but in spite of harvesting these cells within 4 hours, cell yield was unreliable and the media was therefore changed to 90% methanol.

2.3 Cytology brushes from surgical resections of gastric cancer

Patients in the Neath Port Talbot locality requiring gastrectomy for gastric cancer are referred to Morriston Hospital for surgery. Surgical resection specimens from 10 patients undergoing gastrectomy for gastric cancer at Morriston Hospital Swansea, (Consultant Surgeon Mr T Brown), were identified by Dr AP Griffiths, Consultant Pathologist. Multiple brushings (Diagmed Ltd) from the surgical resection specimens were taken, together with biopsies at the same sites, hence allowing histological diagnosis. The cytology brushes were stored in 90% methanol within 30 minutes of resection. Samples were collected between July 2002 and April 2003.

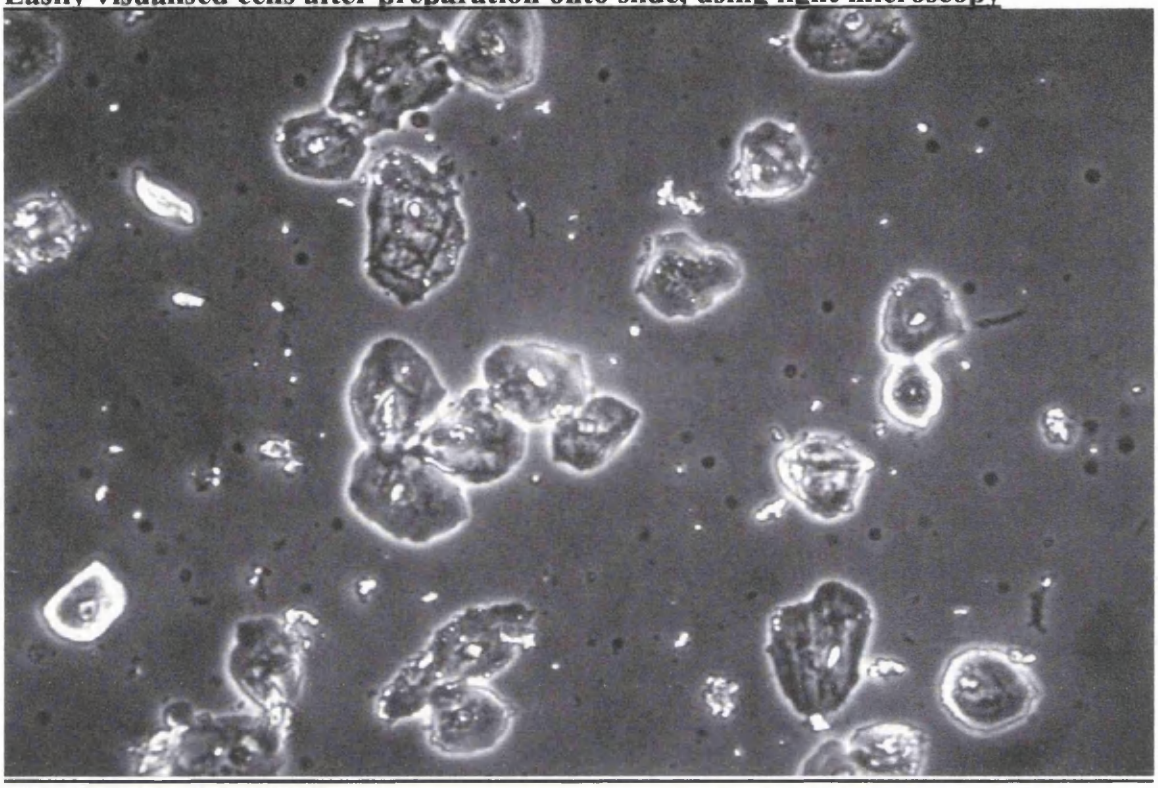
2.4 Interphase cell preparations.

The cell suspension was washed x3 by centrifugation/resuspension in ETN buffer (Doak *et al.*, 2003). If the cell pellet produced was noted to be small the third wash was omitted. The resultant cell pellet was resuspended in 0.5ml ETN buffer and a cytodot was produced on a glass slide using Cytospin 4 (ThermoShandon, Cheshire, UK). Larger pellets were resuspended in 1ml ETN buffer. The cytodot was examined with a light microscope, and cell suspensions were diluted accordingly to ensure that an adequate number of cells evenly spaced apart were present for interphase FISH to be successful. Oesophageal cells were easily visualised, but the gastric cells frequently had no clear cytoplasm/cell membrane, appearing partially digested. This improved when the brushes were stored in 90% methanol giving a better yield of cells. The poor yield in ETN was most likely due to persisting activity of endogenous enzymes present in the gastric secretions. Cell preparations on slides were fixed in 90% methanol at –20° C for 10 minutes and left to air dry. An average of 2-5 slides were produced per sample. Figure 2.2 shows examples of cells easily visualised on light microscopy, and cells obscured by debris.

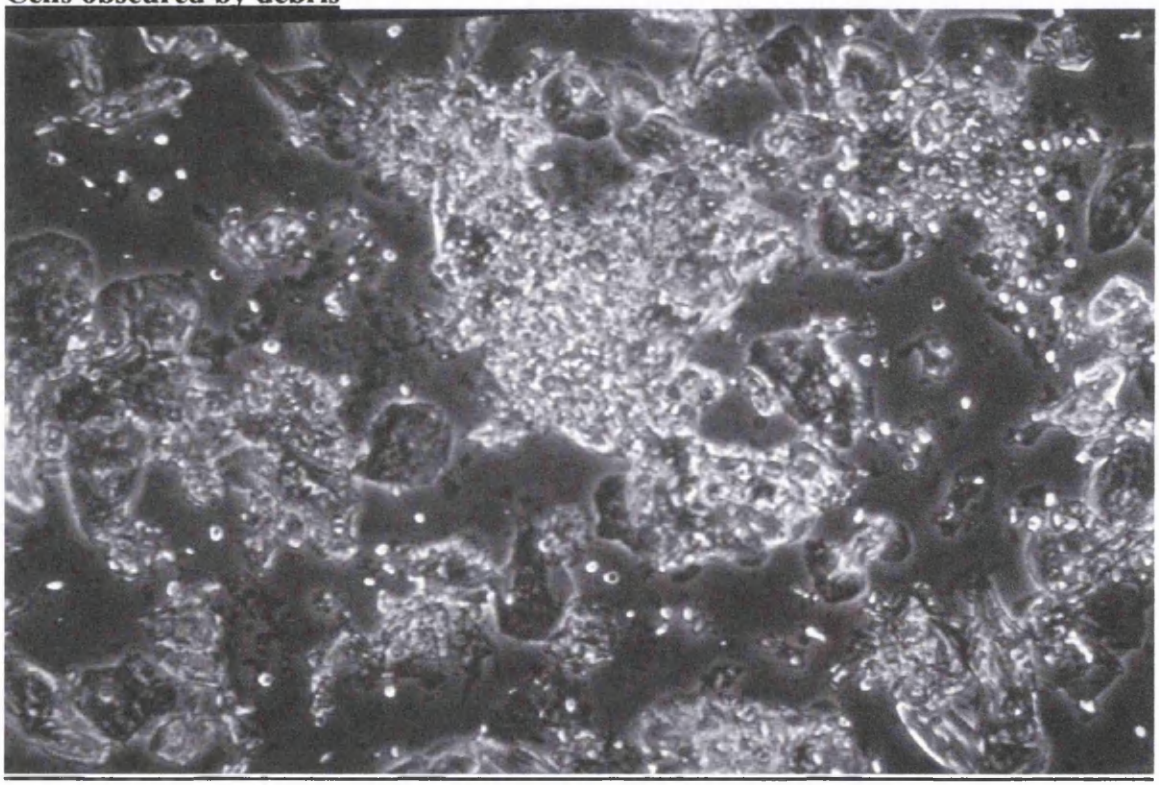
Interphase cell preparations were incubated at 37° C for 10 minutes and then treated with 300 µl /ml HCl pepsin, pH 2.7-3 (Sigma, Dorset, UK) at 37°C, to remove cytoplasmic proteins, hence improving probe penetration. Oesophageal cells were treated for 7 minutes as described by Doak *et al.*, but over this period of time overdigestion of gastric cells resulted in loss of nuclei. Again, this suggested that partial gastric cell digestion after the collection phase at endoscopy had already occurred, therefore the pepsin digestion time was reduced to 5 minutes and the yield improved. Slides were washed in PBS (5 minutes) and PBS/$MgCl_2$ (5 minutes) at

Figure 2.2.

Easily visualised cells after preparation onto slide, using light microscopy



Cells obscured by debris



room temperature to arrest the enzymatic action of pepsin. They were then dehydrated in increasing concentrations of ethanol prior to FISH.

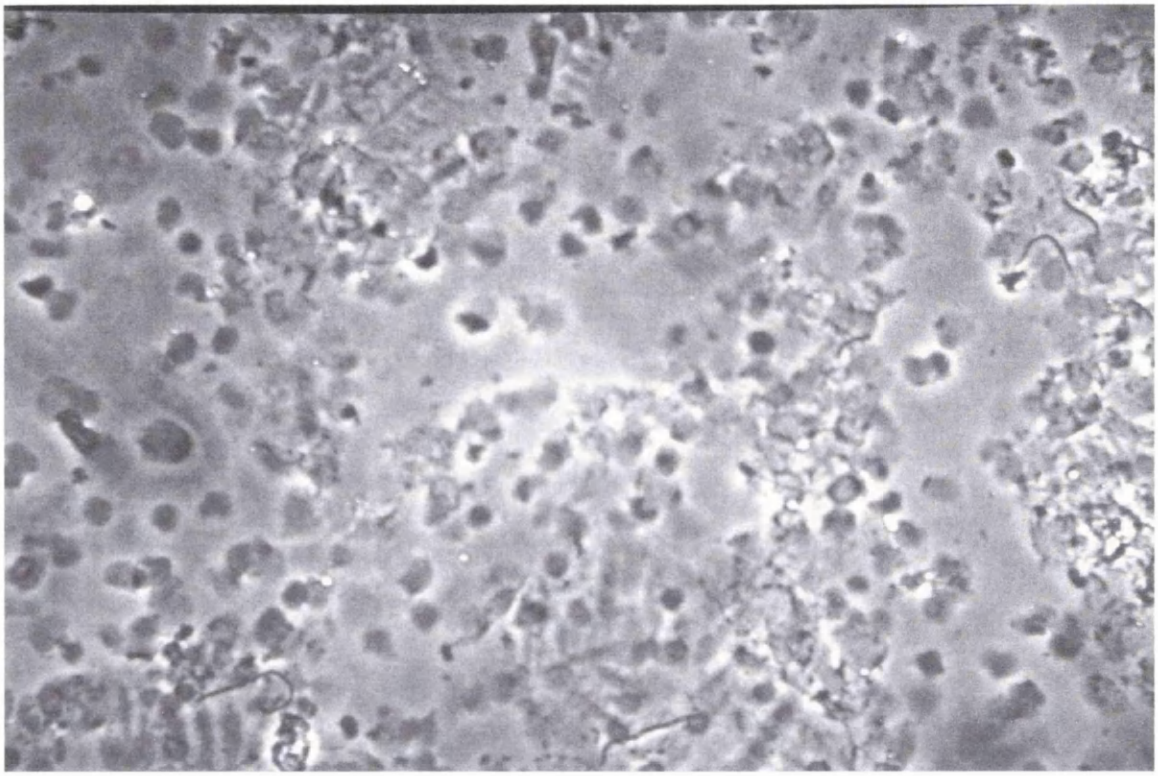
Figure 2.3 shows examples of cells after pepsin digestion.

2.5 Interphase FISH.

Centromeric enumeration probes (CEN) for chromosomes 4, 8 and 20, and a locus specific identifier probe (LSI) for p53 (Vysis, Surrey, UK) were used. Two probes were used simultaneously, 20 and 4, p53 and 8. FISH was performed according to slightly modified manufacturers instructions. 5µl of probe mixture was added to each cytodot (3.5µl hybridisation buffer, 0.5µl of each probe and 0.5µl of water). The sample and probe were co-denatured on a 75°C hotplate for 2 minutes and incubated in the dark at 37°C for 30 minutes (CEN) or 16 hours (LSI). Slides were washed for 2 minutes at 73°C with 0.4xSSC/0.3% Nonidet P-40, and for 30 seconds at room temperature in 2xSSC/0.1% Nonidet P-40, and allowed to air dry in the dark. To counter stain the nuclei 10µl Dapi Π (Vysis, Surrey, UK) was added to each slide. A 30 minutes hybridisation step using a combination of CEN 20 and CEN 4 probes worked well, as did a 16 hour hybridisation step with LSI p53 and CEN 8 (using LSI hybridisation buffer). When CEN probe 4 was left to hybridise longer than 30 minutes the probe was seen to adhere to other sites in the nucleus, as it is able to bind weakly to chromosome 9 (Vysis, Surrey,UK).

FISH probes could be washed off the slide as described by Doak *et al.*, 2003, therefore slides could be reused. However, this was only the case if the Dapi Π stain was removed immediately.

Figure 2.3. Cells after pepsin digestion.



2.6 Signal visualisation and scoring.

An Olympus BX50 microsope and Powergene 4.3 software (Applied Imaging, Newcastle Upon Tyne, UK) was used to score each slide. Slides with < 100 cells were excluded and an average of 223 cells per slide was scored (range 100-452). Nuclei were only included if they had at least one signal from each probe to avoid inclusion of results where hybridisation was inadequate. Nuclei that were smeared or overlapping were also excluded.

CEN probes highlight the centromere of the chromosome and were used to determine whole chromosome changes i.e. aneuploidy. A loss of a CEN signal was said to be a deletion of that chromosome, and more than 2 signals was determined an amplification. The LSI probe for p53 was a marker of that gene locus, and again a loss of a signal represented deletion, whereas a gain represented amplification.

Slides were coded before scoring, with no knowledge of the histological details of the tissue sample.

2.7 Statistical analysis of chromosomal abnormalities

Fisher's exact analysis was used to compare the chromosomal changes between the differing histological diagnoses. Statistical significance was achieved if the calculated p values were less than 0.05, documented as $p<0.05$ (actual values have not been given).

2.8 Reproducibilty of interphase FISH

The reproducibility of this technique has been assessed in Chapter 4, section 4.3.4.

2.9 Histological diagnosis of tissue biopsies

The histological diagnoses of both the endoscopic biopsies taken at Neath Port Talbot Hospital, and the surgical resection biopsies taken at Morriston Hospital, were determined at the Pathology Department Morriston Hospital, Dr AP Griffiths, Consultant Pathologist.

Chapter 3.

Identification of chromosomal abnormalities induced by a model Reactive Oxygen Species (ROS) in a cultured human cell line, using Fluorescence In Situ Hybridisation (FISH).

This chapter describes experiments to assess the chromosomal abnormalities seen in a human cell line exposed to a model ROS. Different cytogenetic methods were used, CBMN assay, CBMN assay + kinetochore staining and interphase FISH. Chromosomal damage was demonstrated by the production of micronuclei in the cell line exposed to oxidative damage. Kinetochore staining suggested that aneuploidy was the main type of chromosomal abnormality caused. Interphase FISH showed that aneuploidy of chromosome 20, 4, 8 and 17(p53) was seen in the cells exposed to ROS. Amplification of chromosome 4, deletion of p53 and aneuploidy of chromosome 20 were the most significant chromosomal abnormalities caused by oxidative damage.

3.1 Introduction.

3.1.1 Oxidative DNA damage

Oxidative damage is an important mechanism in carcinogenesis. It has been implicated in up to 50% of all human cancers (Beckman and Ames, 1997) and importantly, has been considered a potential causative agent in gastric cancer (Correa and Shiao, 1994, Stadtlander *et al.*, 1999). Oxidative DNA damage results mainly from the leakage of Reactive Oxygen Species (ROS) within cells and an example of ROS is hydrogen peroxide (Friedberg *et al.*, 1995, Gracey *et al.*, 1999).

Many agents are suspected of playing a role in the aetiology of gastric cancer and some are capable of producing Reactive Oxygen Species (ROS). A number of studies have demonstrated that *H. pylori* is able to exert oxidative damage on infected tissue, the evidence for which has been reviewed in the general introduction.

3.1.2 Hydrogen peroxide

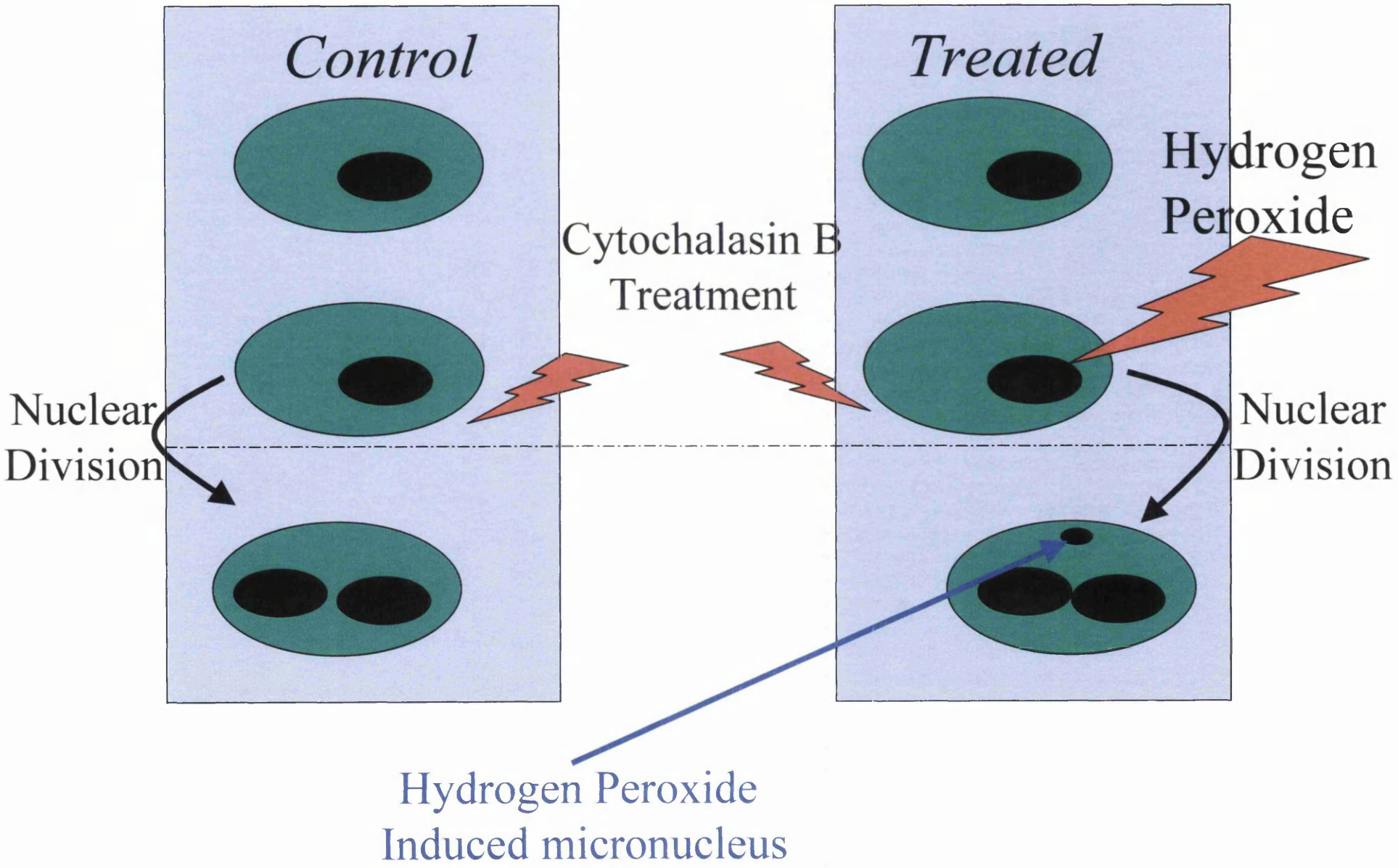
In order to mimic the ROS inducing potential of *H. pylori in vitro*, the model ROS, hydrogen peroxide (H_2O_2), which is one of the forms of ROS actually produced *in vivo* and known to be capable of causing oxidative cell damage was used (Fenech *et al.*, 1999, Jaruga and Dizdoroglu, 1996). It is a readily available ROS, accessible in liquid form, and in previous studies, it has been noted that human cells exposed to H_2O_2 show a dose dependent increase in chromosomal aberrations (Fenech *et al.*, 1999, Uemgaki and Fenech, 2000, Rueff *et al.*,1993). Similar chromosomal damage is seen when cells are exposed to activated neutrophils (Uemgaki and Fenech, 2000), hence H_2O_2 is likely to be involved in the inflammatory mediated release of ROS. In fact, H_2O_2 is likely to represent the diffusible form of ROS that is responsible for genomic DNA damage *in vivo,* as the other types of ROS formed *in vivo* are too reactive to be translocated through the cell to the nucleus (Marnett, 2000). H_2O_2 is considered to be an ideal model of ROS induced DNA damage due to its production of the hydroxyl radical, which is considered to be the ultimate ROS in terms of DNA damage induction (Henle and Linn, 1997, Dreher and Junod, 1996). H_2O_2 produces the hydroxyl radical by Fenton chemistry, involving transition metal cofactors. Iron ($Fe2^+$) reduces H_2O_2, and the extremely reactive hydroxyl radical is formed (Gracey *et al.*, 1999).

The hydroxyl radical is capable of reacting with biomolecules including DNA (Gracey *et al.*, 1999). H_2O_2 damages DNA at 11 different sites (Jaruga and Dizdoroglu, 1996). These DNA adducts have a half-life of 8-60 minutes in lymphoblastoid cells (Beckman and Ames, 1997), presumably as a consequence of their removal by efficient DNA repair pathways. H_2O_2 has been shown to be capable of inducing DNA strand breaks as measured by the Comet assay (Duthie *et al.*, 1997) and chromosomal breakage in the Cytokinesis Block Micronucleus Assay (CBMN) (Fenech *et al.*, 1997), hence showing a potential role for ROS in inducing chromosomal abnormalities. H_2O_2 is also a gene mutagen, as determined by its induction of mutations of the p53 tumour suppressor gene (Jenkins *et al.*, 2001). The doses of H_2O_2 used in these above studies to damage DNA have ranged from 100μM to 700μM.

3.1.3 The Micronucleus and FISH methodologies

The Cytokinesis-Block Micronucleus Assay (CBMN) assay is a non-specific method of estimating the degree of chromosomal damage, necrosis and apoptosis. Micronuclei are formed from chromosomal fragments, or whole chromosomes, that separate from the normal chromosomal segregation step during mitosis forming small DNA containing micronuclei in the cytoplasm (Fenech *et al.*, 1999, Albertini *et al.*, 2000). Micronuclei can be formed as chromosome fragments because of direct DNA breakage, replication of damaged DNA or inhibition of DNA synthesis. Micronuclei can also be formed from whole chromosomes because of a failure of the normal chromosome segregation mechanism at mitosis, as discussed in the chapter 1. The micronucleus assay has been shown to be an effective way of measuring general chromosome damage *in vitro* and *in vivo* (Fenech *et al.*, 1999). Figure 3.1 illustrates

Figure 3.1. The Micronucleus assay



the basic principle behind the methodology. After treatment with the test agent, cells are allowed to divide in the presence of cytochalasin B. Cytochalasin B inhibits cytokinesis (cell separation after mitosis) as it inhibits the actin polymerisation needed to form the microfilament ring that constricts the cytoplasm between the daughter nuclei (Fenech 2000). Hence, the cytochalasin B step produces binucleate cells. It is important to score the micronuclei only in binucleate cells, as a comparison of micronuclei incidence between populations of dividing cells only becomes reliable if scoring is restricted to cells that complete one nuclear division after the DNA insult (Fenech and Morley, 1985). The CBMN assay was used here as an indicator of chromosomal damage in order to establish the optimum amount of hydrogen peroxide needed to produce chromosomal damage for subsequent FISH analysis.

The addition of anti-kinetochore antibodies to the CBMN assay allows us to distinguish whole chromosome presence in the micronuclei from chromosomal fragments (Fritzer *et al.*, 1980, Vig and Swearnigin 1986). The detection of these antibodies to the kinetochore complex was first reported in 1980, in patients suffering from the autoimmune condition CREST, (calcinosis, Raynaud's, oesophageal fibrosis, sclerosis, telangiectasia) (Fritzer *at al.*, 1980). Kinetochore positive micronuclei indicate centromere presence, and therefore whole chromosome presence, in the micronuclei. Chemicals that produce mostly kinetochore positive micronuclei (>70%) are said to be aneugens, whereas those forming mostly negative micronuclei (>70%) are clastogenic compounds (Antoccia *et al.*, 1991). Parry *et al.*, in 2002, illustrated the importance of this amended CBMN assay to determine chemicals causing aneugenic chromosomal damage (Parry *et al.*, 2002). However, it should be noted that only the chromosomes with active centromeres which bind to anti-kinetochore antibodies are

detected, and that false negatives can occur if chemicals present target the kinetochore protein and stop the antibody binding (Dellarco *et al.*, 1985).

The cell line used in these studies was the AHH-1 cell line (Genetest Corporation). AHH- 1 is a human lymphoblastoid cell line, derived from a B lymphocytic cell line RPMI 1788, which expresses inducible aryl hydrocarbon hydroxylase (AHH). Experiments have shown that this cell line is sensitive to mutagens that require oxidative metabolism to exert their effect and, as such, it is a useful human cell line for studies on mixed-function oxygenases (Crespi and Thilly, 1984, Freedman *et al.*, 1979). It is a readily available human cell line, able to be grown *in vitro* with little cell death or contamination, and with a doubling time that allows the effect of chemical exposure to be assessed relatively easily (Crespi *et al.*, 1991, Doherty 1996). Analysis of the cell line has shown it to possess a stable karyotype, with 48 chromosomes due to an extra copy of chromosomes 3 and 9 (Doherty 1996).

This study allowed the optimisation of FISH methodology using the chosen centromeric probes for chromosomes 4, 8 and 20, and the locus specific probe for the p53 gene (17p13). Centromeric FISH probes can demonstrate whole chromosome changes in the cell and therefore detect chemicals producing an aneugenic effect (Parry *et al.,* 2002). The chromosomes were chosen due to their implication in gastric cancer progression in previous publications, as illustrated in Table 1.1. Later in this thesis, the specific chromosomal alterations detected here are compared to chromosomal abnormalities found in pre-malignant and malignant gastric tissue (Chapters 4 and 5), with or without *H. pylori* infection, in order to compare the chromosome changes induced by a known ROS *in vitro* (H_2O_2) and a suspected ROS *in vivo* (*H. pylori*).

3.1.4 Aims

This series of experiments was designed to illustrate the effects a model ROS (H_2O_2) has on the chromosome integrity of a human cell line as measured with Fluoresence In Situ Hybridisation (FISH) and the Cytokinesis-Block Micronucleus Assay (CBMN) assay. The aims of the work described in this chapter were three fold; initial investigations using the CBMN assay were confirmation studies to show that H_2O_2 causes chromosomal damage, as well as range finding studies for the clastogenicity / aneugenicity of H_2O_2. Secondly, the data from this study was used to choose suitable doses for the study of specific chromosomal aberrations induced by H_2O_2 with the FISH methodology. Finally, this study aimed to optimise the use of FISH on the selected chromosomes prior to studying these chromosomes in gastric epithelial cells isolated from patients with pre-malignant and malignant gastric cancer.

3.2 Materials and Methods.

3.2.1 General cell culture.

All the glassware and pipettes used were sterilised in an autoclave (Prior Clave Ltd) at 121°C and 15psi for 30 minutes. The disposable, sterile, plastic tissue culture vessels, pipettes, centrifuge tubes and universal containers, were supplied by Invitrogen and Sterilin. All cell culture manipulation was carried out in aseptic conditions using a Cytomat Pharmaceutical Safety cabinet, class 2 (Medical Air Technology Ltd). LEEC MK11 proportional temperature controller incubators at 37°C and 5% CO_2 were used for all incubations.

AHH-1 cells (Genetest Corporation) were stored in DMSO in liquid nitrogen prior to use. After the cells were defrosted, using a waterbath at 37 °C, they were

incubated at 37 °C with a 5% CO_2 atmosphere, in a medium composed of RPMI (+ glutamine) with 10% horse serum (GIBCO, Invitrogen Corporation, UK). The best cell growth was achieved if cells were cultured in plastic flat bottomed flasks, and diluted with pre-warmed medium every 48 hours to obtain a cell concentration of 1.5 - 2 x 10^5 cells per ml (Crespi *et al.*, 1991,Genetest Corporation).

Freezing of the cell stock, and thawing, was carried out as detailed in Doherty 1996.

3.2.2 AHH-1 cell culture and dosing with Hydrogen Peroxide.

AHH-1 cells were suspended in serum free medium just prior to dosing with H_2O_2. This was to eliminate any interactions between the hydrogen peroxide and the protein containing serum. In toxic fume hoods, the AHH-1 cells were dosed with differing concentrations of hydrogen peroxide (SigmaAldrich, Poole, UK) i.e. 0, 50 and 100 μM, and incubated at 37 °C for thirty minutes. This dose range was chosen to reflect the lower concentrations used in previous studies of DNA damage induction (Uemgaki *et al.*, 2000, Rueff *et al.*, 1993, Jenkins *et al.*, 2001, Fenech *et al.*, 1999, Perwez Hussain *et al.*, 1994, Duthie *et al.*, 1997). Previous work in the University of Wales Swansea has shown that higher concentrations than 100μM H_2O_2 are cytotoxic to cells and therefore chromosomal events can be confused with general toxicity (Jenkins *et al.*, 2001). Hence, in this study a sub-toxic dose range was chosen in an attempt to mimic chronic inflammation, as the actual doses of ROS *in vivo* are unknown. After exposure to H_2O_2 the cells were centrifuged using the Thermo Shandon Cytospin 3 (at 1200 rpm for 8 minutes) and re-suspended in full serum containing medium. The cells were then subjected to cytochalasin B treatment for 24 hours to produce binucleate cells for the micronucleus study.

3.2.3 Cytokinesis block micronucleus (CBMN) assay.

Cytochalasin B was added to the cells, giving a final concentration of 3μg/ml. They were then incubated at 37 °C with 5% CO2 atmosphere for 24 hours. Cells were harvested using a Cytospin to produce a cytodot on a microscope slide. A haemocytometer was employed in order to produce slides with a cell solution concentration of 1 x 10^5 cells per ml. The cells were fixed onto the slides by immersion in a solution of 90% methanol for 10 minutes at -20 °C. The slides were then stained with Giemsa before scoring for micronucleated cells.

Kinetochore labelling was performed on the the binucleate cells produced, when AHH1 cells were dosed with 0 and 100μM H_2O_2. The frozen cells were rehydrated with PBS at room temperature for 2 minutes, and then placed in primulin (0.5μg /ml) in deionised water for 30 seconds. A solution of anti-kinetochore antibody (antinuclear antibody centromere pattern from human serum in 0.1% sodium azide, MedicaEncinitas) diluted 1:1 with PBS was prepared and 50μl added to each cytodot. A plastic coverslip was used and the slides incubated in a humidified chamber for 45 minutes at 37 ^{0}C. The slides were then immersed x3 in a solution of bovine serum albumin in PBS (1g per 100ml) for 3 minutes at room temperature, and then x3 in PBS at room temperature for 3 minutes. 50μl of FITC (1μl of fluorescent anti human IgG, Sigma, + 99μl of PBS in a foil covered tube at 2-8^{o}C) was added to the cytodot, covered with a plastic coverslip and incubated at 37^0 C for 45 minutes. Again the slides were washed in albumin/PBS for 3 minutes x2, and then PBS for 3 minutes x1. They were then air dried in the dark and stored at 4C. To view 20μl of Dapi1 was added to each slide.

3.2.4 Scoring micronucleated cells.

Micronuclei cells were coded and the number of micronuclei present in the binucleated cells scored using an Olympus BH2 microsocope at 100x magnification. Binucleated cells with and without micronuclei were scored for each treatment group. Micronuclei were collectively scored whether present in the cells as single or multiple events.

Micronuclei (MN) were scored if they fulfilled the following criteria (Fenech 2000):

- MN morphologically identical to nuclei
- MN diameter between 1/16 and 1/3 of mean diameter nuclei
- MN non-refractile therefore distinguishable from artefact
- MN not linked or connected to main nuclei
- MN may touch but not overlap main nuclei, clear boundaries
- MN same staining intensity as main nuclei

Kinetochore staining was scored only in binucleate cells with micronuclei, by using the Olympus BX50 microsope and the Poweregene 4.3. Kinetochore probe was visualised as a green signal in the main nuclei +/- the micronuclei.

Figure 3.2 shows an example of a binucleate cell bearing a micronucleus and kinetochore staining.

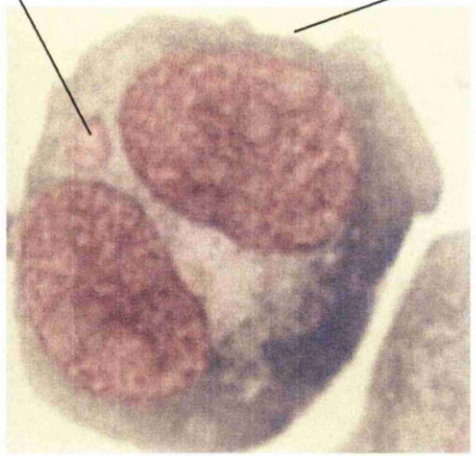
3.2.5 FISH

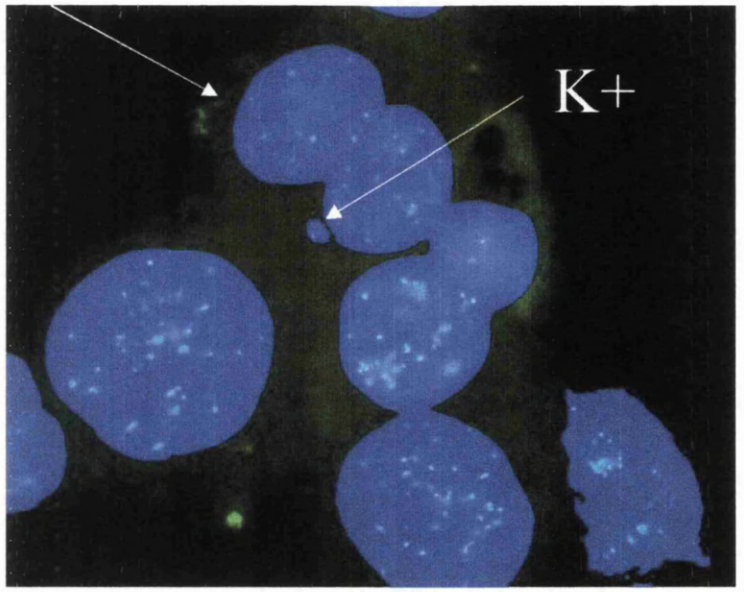
After the analysis of the micronuclei induction by H_2O_2, suitable doses were used to investigate specific chromosomal alterations induced by H_2O_2. AHH-1 cells were dosed with hydrogen peroxide at 0, 50 and 100 μM, harvested onto slides and fixed with 90% methanol as above. FISH was then performed using centromeric

Figure 3.2. A Micronucleus induced by exposure to Hydrogen Peroxide.

micronucleus

binucleate cells





K+ = Kinetochore positive micronucleus

probes for chromosomes 4, 8 and 20, and a locus specific probe for p53, see Chapter 2. At least 400 cells were scored for each probe and slide.

The reproducibility of FISH as a technique will be discussed later in chapter 4. In this series of experiments however, a duplicate experiment was performed with AHH1 cells exposed to 50μM H_2O_2, and with AHH1 cells exposed to no H_2O_2. FISH with CEN probe 20 was then performed.

3.2.6 Statistical analysis of the micronucleus test and FISH results.

For both the micronucleus test and for FISH analysis, p values of statistical significance (95% confidence intervals) were obtained using the Fisher's exact analysis.

3.3 Results

3.3.1 Cytokinesis block micronucleus assay

Table 3.1 contains the average values of duplicate CBMN experiments, and Figure 3.3 illustrates the percentage of micronuclei formed when cells were dosed with differing concentrations of hydrogen peroxide (0, 50 and 100 μM).

The percentage of micronuclei is representative of the chromosomal damage produced by hydrogen peroxide. As the dose increased so chromosomal damage increased. Statistical significance for the percentage of micronuclei formed was reached when comparing control cells to those dosed with 100 μM H_2O_2.

Kinetochore labelling results are illustrated on Table 3.1 for AHH1 cells dosed with 0 and 100μM H_2O_2. Chromosomal damage seen when cells were treated with

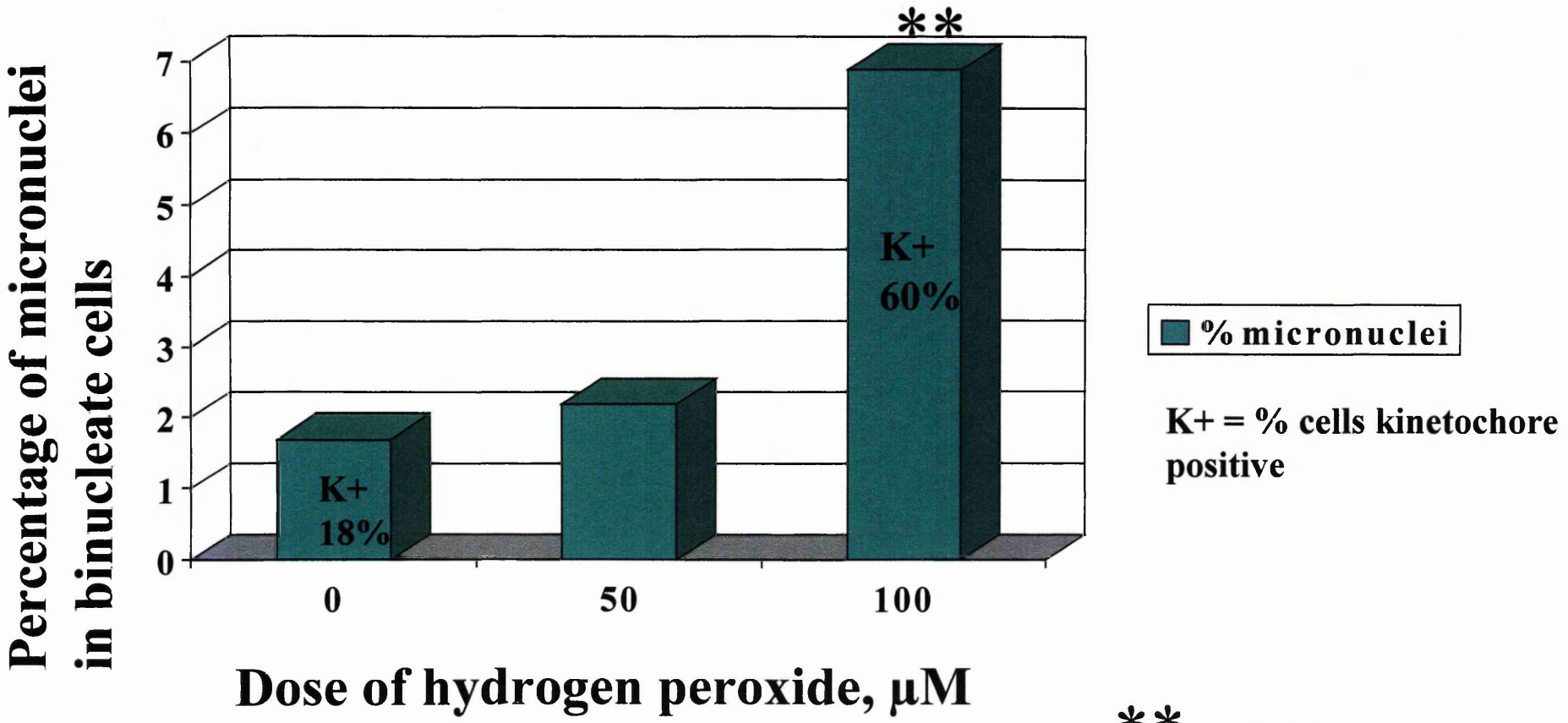
Table 3.1. Data from Micronucleus study of hydrogen peroxide effect on AHH1 cell line (results averaged from duplicate experiments)

Dose μM	mon	bi	tri	tet	total	%bi	Total MN	%bi/ MN
0	336	1011	2	1	1350	74.9	17 K^+18%	1.7
50	1281	582	7	0	1870	31.1	13	2.2
100	673	479	20	2	1174	40.8	33 K^+60%	6.9

Mon=mononucleate, bi=binucleate, tri=trinucleate, tet=tertranucleate, MN=micronucleate

K^+ = kinetochore positive micronuclei

Figure 3.3. CBMN assay to determine the percentage of micronuclei produced in binucleate cells in a AHH1 cell line treated with differing doses of hydrogen peroxide



** $p<0.005$ at doses 0 vs 100µM

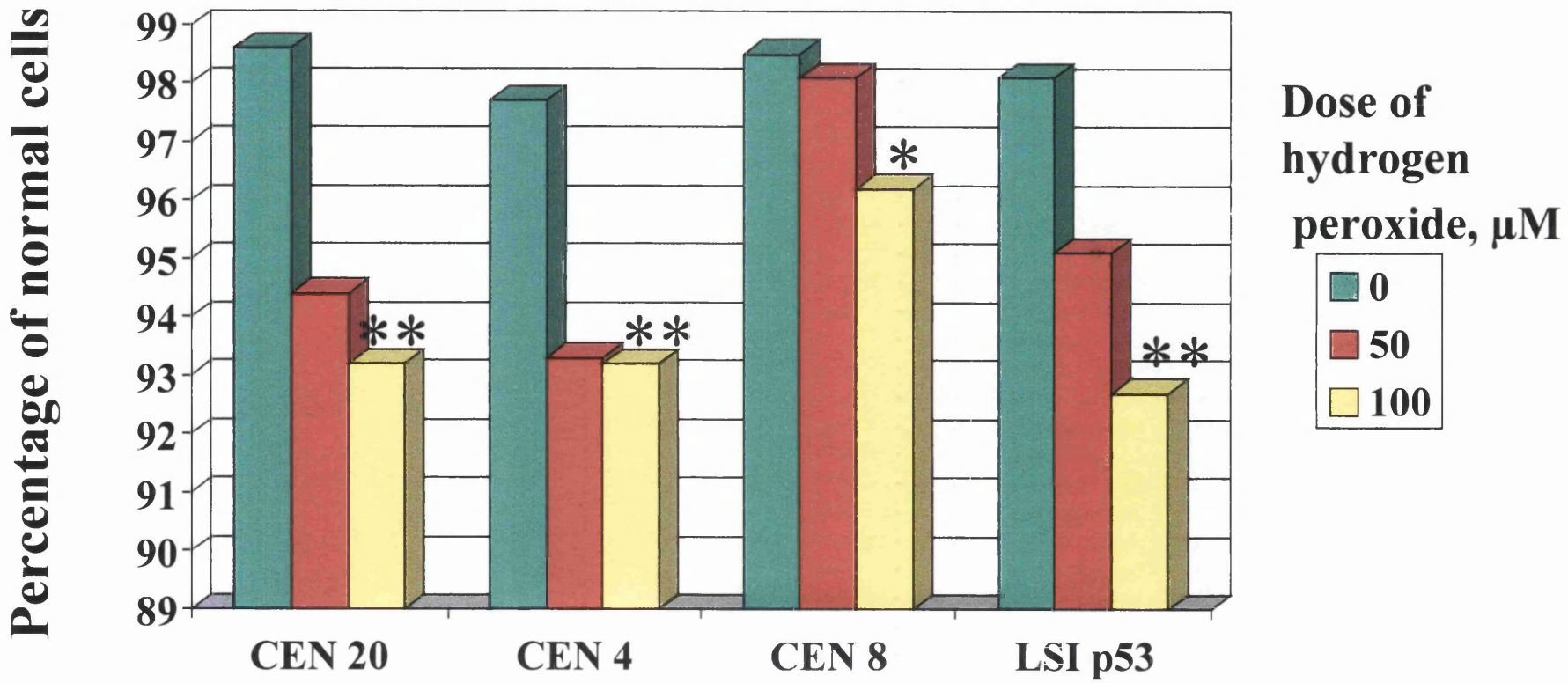
hydrogen peroxide was mostly in the form of whole chromosome loss, therefore aneugenic.

These initial experiments have confirmed that hydrogen peroxide, at these concentrations, is capable of causing chromosomal abnormalities in human cells. The finding is in keeping with a range of studies using different techniques, as discussed in the introduction. Hence, the same concentrations of 0, 50 and 100μM hydrogen peroxide were chosen to perform FISH analysis looking at targeted chromosomes.

3.3.2 FISH analysis of AHH-1 cells dosed with hydrogen peroxide.

All somatic cells should possess 2 copies of each chromosome (apart from sex chromosomes X and Y). Hence all cells should show 2 copies of each chromosome probe. If the cell has < 2 copies or > 2 copies then the normal chromosome complement has been altered. Changes in centromeric probes for chromosome 4, 8 and 20 suggest whole chromosome change, aneuploidy. Aneuploidy can have severe consequences on the individual cell, for example an extra copy of a protooncogene can result, or a loss of a copy of a tumour suppressor gene. Figure 3.4 illustrates the percentage of cells with two signals of each probe, (centromeric probes 20, 8 and 4, and locus specific probe p53) at differing concentrations of hydrogen peroxide. The percentage of cells with a normal complement of chromosomes (i.e. two chromosomes) remained above 90% for all probes and all doses, hence reflecting the stability of this particular cell line. The p values shown were calculated using Fisher's exact for control samples versus 100μM hydrogen peroxide. All values were significant suggesting that hydrogen peroxide is capable of causing these specific chromosomal aberrations, as illustrated with the centromeric and locus specific probes

Figure 3.4. Percentage of AHH1 cells with 2 signals of each FISH probe, at differing concentrations of hydrogen peroxide (µM)



0 vs 100 µM

** p< 0.005, * p<0.05

used. The general trend was for the degree of chromosomal abnormality to increase as the dose of hydrogen peroxide increased.

Table 3.2 shows the raw data and Figures 3.5, 3.6, 3.7 and 3.8 show the percentage of cells with abnormal number (gain and loss) of each specific probe. Again using the Fisher's exact test, p values were calculated. In the case of chromosome 20 (Figure 3.5), the trend was for chromosomal abnormalities of 20 to increase as dose of hydrogen peroxide increased. Significance was seen for both loss and gain of chromosome 20 when comparing control cells to cells exposed to 100μM hydrogen peroxide. In the case of chromosome 4 (Figure 3.6), the trend was for chromosomal abnormalities to increase as dose of hydrogen peroxide increased. Significance was seen for gain of chromosome 4 only. Figure 3.7 shows the abnormalities of chromosome 8 as a function of the dose of hydrogen peroxide. Again, the trend was for chromosomal damage to increase as dose of hydrogen peroxide increased, but significance was not reached for either loss or gain of chromosome 8.

As with the centromeric probes, the level of abnormalities seen with the p53 probe increased as the hydrogen peroxide dose increased. From Figure 3.8, it is possible to see that significance was obtained with p53 loss.

The effect of 50μM hydrogen peroxide, and no hydrogen peroxide, on the AHH1 cell line was reassessed in a second experiment, using CEN probe 20. There was very little difference in the percentage of chromosome 20 abnormalities between the first and second experiments. Cells with no exposure to hydrogen peroxide showed chromosomal abnormalities in 1.7% and 1.4% of cells respectively, and in those cells exposed to hydrogen peroxide chromosomal abnormalities were present in 5.6% and 5.5% of cells respectively.

Table 3.2. FISH data showing abnormalities in AHH1 cell line induced by hydrogen peroxide

a. AHH-1 cells with no exposure (repeat)

Probes	Number of cell with 2 signals	Number of cells with 1 signal	Number of cells with 3 signals	Number of cells with 4 signals
20	417(403)	3(6)	3(1)	0
4	417	7	3	0
8	395	2	3	1
p53	405	5	2	1

Table 3.2. FISH data showing abnormalities induced by hydrogen peroxide

b. AHH-1 cells dosed with 50μM H_2O_2(repeat)

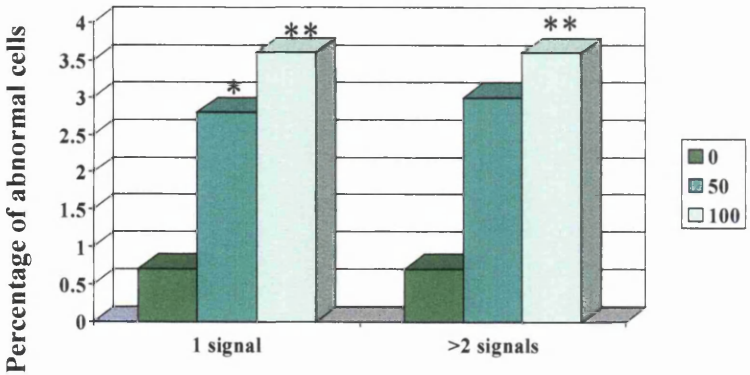
Probes	Number of cells with 2 signals	Number of cells with 1 signal	Number of cells with 3 signals	Number of cells with 4 signals
20	395(389)	11(15)	11(8)	1
4	391	7	20	1
8	405	4	2	2
p53	405	16	5	0

Table 3.2. FISH data showing abnormalities induced by hydrogen peroxide

c. AHH-1 cells dosed with 100μM H_2O_2

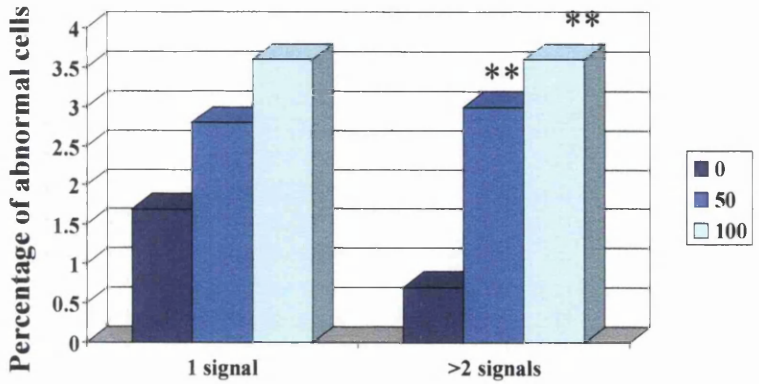
Probes	Number of cells with 2 signals	Number of cells with 1 signal	Number of cells with 3 signals	Number of cells with 4 signals
20	411	15	12	3
4	412	11	16	3
8	407	6	8	2
p53	407	26	6	0

abnormal numbers of CEN 20



Loss of 20: 0 vs 50μM, p<0.05, 0 vs 100μM, p<0.005
Gain of 20: 0 vs 100μM, p<0.005

abnormal number of CEN 4



Gain of 4: 0 vs 50μM, p<0.005, 0 vs 100μM, p<0.005

Figure 3.7. Percentage of cells with abnormal number of CEN 8

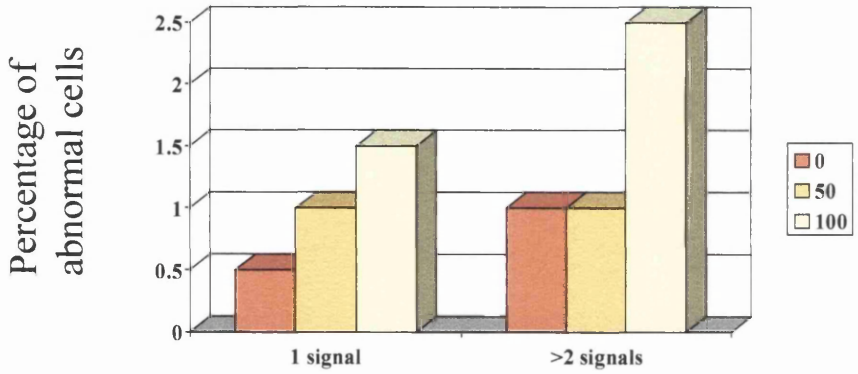
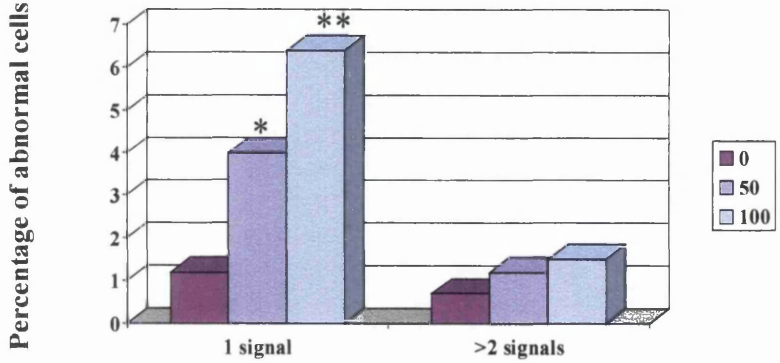


Figure 3.8.Percentage of cells with abnormal number of LSI p53



Loss of p53: 0 vs 50μM, p<0.05, 0 vs 100μM, p<0.005

3.3.3 Examples of chromosome abnormalities induced by hydrogen peroxide

Figure 3.9 shows examples of some of the specific chromosomal events induced by exposure to hydrogen peroxide and detected by FISH.

3.5 Discussion

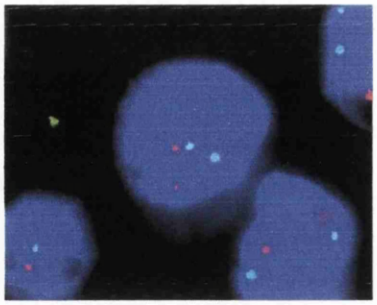
The aim of this chapter was to illustrate the chromosomal abnormalities induced in human cells by ROS *in vitro,* for comparison to the *in vivo* situation described in Chapter 4. Hydrogen peroxide is a model ROS *in vitro*, and is likely to mimic the DNA damage induced *in vivo* in inflammatory tissues, as it is actually produced during these processes and may represent the diffusible form of ROS *in vivo* (Marnett, 2000). A dose range was chosen in an attempt to mimic the *in vivo* state. Hydrogen peroxide has been shown to cause DNA damage, and differing cytogenetic techniques have shown chromosomal abnormalities when human cells are exposed to hydrogen peroxide (Duthie *et al.*, 1997, Fenech *et al.*, 1999, Jenkins *et al.*, 2001, Uemegi and Fenech, 2000).

This chapter illustrates, using two separate techniques, that chromosomal abnormalities were seen in a cultured human cell line, AHH-1, when dosed with hydrogen peroxide, and that this chemical could cause aneuploidy.

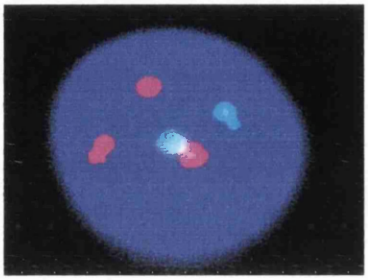
CBMN assay (+/- kinetochore staining) and interphase FISH are both observer dependent. However, the fact that two different cytogenetic techniques were employed and the results were the same, strengthens the statement that an oxidative insult results in aneuploidy. The findings of aneuploidy in the chromosome 20, 8 4 and 17(53) validates the choice of probes to assess gastric disease (+/- *H. pylori* infection).

Figure 3.9. Examples of FISH in cells treated with model ROS

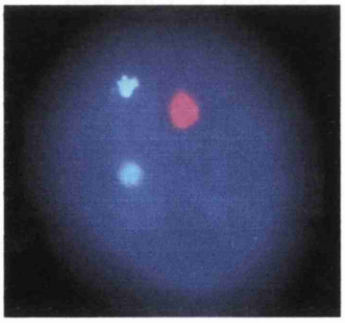
Normal complement 20 and 8 (2 signals)



Amplification of chromosome 20 (red signal) – 3 copies



Deletion of chromosome 20 (red signal) – 1 copy



Firstly, the CBMN assay illustrated chromosomal damage in AHH-1 cells exposed to hydrogen peroxide. This was illustrated by the production of micronuclei. 60% of the micronuclei were kinetochore positive, suggesting whole chromosome loss into the micronuclei. The effect was dose responsive, and the higher dose of 100μM hydrogen peroxide showed a statistically significant increase in the number of micronuclei produced. This dose was in line with the previous literature cited in the introduction to this chapter. Thus, human cells exposed to ROS *in vitro* undergo chromosomal damage. The type of chromosome damage induced by H_2O_2 shown here is mainly in the form of whole chromosome abnormality, consistent with an aneugenic effect.

Secondly, FISH was performed on AHH-1 cells dosed with the same concentration of hydrogen peroxide as in the micronucleus assay. FISH can measure aneuploidy, where non-disjunction of chromosomes occurs, and also simultaneously allows us to target particular chromosomes to monitor the effect of ROS on specific chromosome number. The overall aim of this project was to look at the chromosomes implicated in gastric cancer progression. Hence the chromosomes chosen have all been found to be abnormal in gastric cancer, or in the development of another upper GI cancer, adenocarcinoma of the oesophagus i.e. chromosome 20, 8, 4 and 17 (p53), (see Chapter 1). When AHH-1 cells were exposed to hydrogen peroxide, abnormalities in each chromosome occurred. For each chromosome investigated, the number of cells with aberrant copy number increased as the dose of hydrogen peroxide increased. When the total chromosomal damage (loss and gain) of cells exposed to the higher concentration of hydrogen peroxide was compared to those with no exposure, statistical significance was achieved for each probe used, ($p<0.05$).

Thus, human cells exposed to ROS *in vitro* undergo chromosomal damage, and specifically aneuploidy of chromosomes 20, 8 and 4 and 17(p53).

When results for individual probes were analysed more fully, statistical significance, ($p<0.05$), was achieved for loss and gain of chromosome 20, gain of chromosome 4 and loss of the p53 locus. It is possible to compare these changes to those in the literature. Doak *et al.* 2003 showed an amplification of chromosome 4 in the progression of disease from Barrett's metaplasia to adenocarcinoma. Hence, this mirrors the *in vitro* situation described here, where gain, and not loss, of chromosome 4 is induced by ROS. Given that Barrett's disease and *H. pylori* infection (and hence gastric cancer) are both inflammatory conditions, this result suggests that amplification of chromosome 4 is a non-random event, and may represent a significant event in disease progression. p53 is a tumour suppressor gene and as such its loss leads to genetic instability, including chromosomal instability and aneuploidy (Bouffler *at al.*, 1995, Fukasawa *et al.*, 1996). p53 loss has been implicated in 60% of human cancers, including gastric cancer (Levine *et al.*, 1991, Sano *et al.*, 1991, Sipponen *et al.*, 1998, Vogelstein and Kinzer, 1992). Interestingly in the present study, p53 loss is preferentially induced by H_2O_2, as opposed to p53 gain. From Figure 3.5 it is also seen that untreated cells have more p53 loss than gain, suggesting possible instability. In the case of chromosome 20, CGH and FISH studies have shown levels of chromosome 20 to be altered in gastric adenocarcinoma (Kokkala *et al.*, 1998, Han *et al.*, 1996, Koo *et al.*, 2000, Kityama *et al.*, 2000, Okada *et al.*, 2000). The data given here shows both loss and gain of chromosome 20 induced by H_2O_2, suggesting no specific role for chromosome 20, but possibly reflecting genetic instability.

The *in vitro* experiments described here allow the determination of the types of chromosomal aberrations typical of ROS exposure *in vitro*. Chapters 4 and 5 have looked at the chromosomal damage seen in gastric tissue at all stages of disease i.e. normal gastric mucosa, gastritis, intestinal metaplasia, gastric dysplasia and gastric adenocarcinoma, using the same FISH probes for chromosome 20, 8 and 4 and p53. Thus, allowing the comparison of the type of chromosomal aberrations in the model ROS *in vitro* study, with those seen in the *in vivo* study of gastric tissue.

Chapter 4

The detection of chromosomal abnormalities in gastric tissue, using Interphase Fluorescence In Situ Hybridisation

In this series of *in vivo* experiments patients were enrolled at endoscopy and patient details collected. Gastric cells were obtained from the patients and a histological diagnosis determined as well as the degree of chromosomal abnormality, using interphase FISH. The level of aneuploidy in chromosomes 20, 8, 4 and 17(p53) was found to increase as the gastric pathology worsened (normal gastric mucosa > gastritis > intestinal metaplasia). Amplification of chromosome 4, deletion of p53 and amplification of chromosome 20 were the most significant events found in the premalignant stages of gastric carcinogenesis.

4.1 Introduction

4.1.1 Gastric cancer development

Gastric cancer is regarded to be the second commonest cancer worldwide (Parkin *et al.*, 1985) and in the UK accounts for a significant proportion of national morbidity and mortality as it is the sixth most frequent cancer in men and the seventh in women (Cancer Research Campaign 1995). Presentation of gastric cancer is usually advanced and therefore prognosis is poor. Improving the outcome of cancers can often be achieved by identifying people at a high risk of developing cancer (Hussain and Harris 1996) and by early cancer detection (Matturri *et al.*, 1998). The latter has been addressed in Japan, where the incidence of gastric cancer is one of the highest worldwide, and screening programmes have been developed to diagnose lesions early.

Identifying those people who will actually progress to gastric cancer has proved more difficult, as unfortunately the actual genetic events responsible for the initiation and progression to gastric cancer are still unknown (Koo *et al.*, 2000, Sud *et al.,* 2001).

The pathways leading to gastric cancer from normal gastric mucosal tissue and the sequence of genetic changes involved, remains unclear. However, Correa has formulated a multi-step pathway to gastric cancer, including pre-cancerous stages and the possible environmental insults sustained which is illustrated in the general introduction, Figure 1.2 (Correa 1998). This pathway is described in more detail in Chapter 1. In this chapter premalignant gastric tissue will be analysed to look for aneuploidy.

4.1.2 Cytogenetics of gastric cancer

It is widely accepted that gastric cancer results from genetic alterations, and studies have shown that genetic instability can lead to accumulation of somatic mutations and chromosomal instability (Grady 2001, Kabayashi *et al.,* 2000). A stepwise pathway of genetic change in gastric carcinogenesis has not yet been identified, although some abnormalities in the karyotype have been found in gastric cancer cells and in some of the pre-cancerous stages outlined by Correa (Correa 1988).

Aneuploidy of varying degrees has been documented in all stages of gastric cancer (Beuzen *et al.*, 2000). Table 4.1 describes a number of cytogenetic studies and highlights the particular chromosomes that are found to be abnormal in gastric cancer. Chromosome 20 and 8 are frequently implicated (Kokkola *et al.*, 1998, Koo *et al.*, 2000, Wu *et al.*, 2001). A number of researchers have also looked at the pre-malignant states of gastritis and intestinal metaplasia, and found that some of the

Table 4.1: The results of a number of cytogenetic studies looking at chromosomal aberrations in gastric cancer.

Author	Sample size	Cancer Type	Chromosome
Panini 1995 (Greece)	11	10/11 intestinal (3 metastatic)	**3**, 6, 13
Kokkola 1998(Finland)	22	intestinal	gain-**20q** (55%) 17q(41%), 8q(41%) loss-**18q**(41%), 4q(32%)
Koo 2000 (South Korea)	37	50% intestinal (22/37 metastatic)	gain-**8q**, 2q, 7pq, 13q, 7q, gain - 18q, 20pq loss-**17**p
Yadav 1996 (USA/Japan)	4	1 metastatic	3, 7, 11, X
Fringes 2000(Germany)	20	intestinal/diffuse	gains-**1**(63%), 17(59%)
Beuzen 2000 (France)	60	cardia, antrum & oesophageal	7, **8**, 11, 17, Y (40-65%allcancers)
Okada 2000 (Japan)	5	diffuse (80% metastatic)	loss-18q gain-7, 20
Bertoni 1996 (Italy)	2	metastatic	6, karyotype rearrangement
Han 1996 (South Korea)	18	diffuse	widespread numerical rearrangement

Table 4.1 (cont'd): The results of a number of cytogenetic studies looking at chromosomal aberrations in gastric cancer.

Author	Sample size	Cancer Type	Chromosome
Chun 2000 (South Korea)	6	advanced	loss-**17p**(100%) gain-7
Igashari 2000 (Japan)	39		1p lost in advanced
Kitiyama 2000(Japan)	24		1, 2 - early 1, 2, 4, 20 - later
Sud 2001 (UK)	9	17 intestinal 9 diffuse	loss - **22q**, 14q, 4q, 17p
Wu 2001 (Taiwan)	53	65% advanced 55% intestinal 45% diffuse	gains – **8q**, 6q, 11q, 13q, 7p7p, 17q, 20q loss – **16q**, 19p, 5q, 3p, 4q, 1p
Sugai 1999 (Japan)	99	65 intestinal 34 diffuse	aneuploidy in 73%

(bold =commonest)

genetic changes present in the most advanced gastric cancers are present in these early stages (Tahara 1995), indicative of important driving changes in carcinogenesis. DNA copy number has also been looked at in adenomas of the stomach and both DNA increases (amplifications) and losses (deletions) were found. The more severely dysplastic adenomas had higher chromosome copy number (in almost 50%), than those with moderate dysplasia (Panani *et al.*, 1995). Studies have also looked at patients with chronic gastritis and found aneuploidy (Nardone *et al.*, 1999). Kobayashi *et al.* looked at intestinal metaplasia and early adenocarcinoma samples from the same patients and found that the same molecular changes of microsatellite instability were present in both, but to a higher degree in cancer. They also showed that the loss of heterozygosity was more prevalent in cancer, and suggested a place for chromosomal instability in the initiation of carcinogenesis (Kobayashi *et al.*, 2000).

p53 is the most frequently mutated gene in human cancers and is mutated in up to 60% of all human tumours (Sippponen *et al.*, 1998, Levine *et al.*, 1991, Vogelstein and Kinzer 1992) and a significant proportion of intestinal and diffuse gastric tumours (Grady 2001). A number of studies support the findings of p53 abnormalities in pre-malignant conditions (Dixon *et al.*, 2001, Ochiaio *et al.*, 1996, Shiao *et al.*, 1994). Rugge *et al* found abnormalities of p53 in 61% carcinomas, 64% severe dysplasia , 27% moderate dysplasia and 19% mild dysplasia (Rugge *et al.*, 1992). Brito *et al.,* also noted a stepwise elevation of p53 abnormalities from dysplasia to advanced gastric cancer (Brito *et al.*, 1994). p53 abnormalities exist at all stages of carcinogenesis, i.e. even before dysplasia has developed (Correa and Shaio 1992). p53 mutations have been detected in early stages of pre-malignant gastric tissue, both in gastritis (Stemmermann *et al.*, 1994, Kodama *et al.*, 1998, Morgan *et al.*, 2003) and in intestinal metaplasia (Shaio *et al.*, 1994, Ochai *et al.*, 1996, Zheng *et*

al., 1998). Tohodo *et al.* noted p53 abnormalities to be present in 33% of gastric adenomas and atrophic gastritis (Tohodo *et al.*, 1993). Interestingly, p53 loss often precedes and may cause chromosomal instability and hence produce aneuploidy (Bouffler *et al.*, 1995, Fukasawa *et al.*, 1996).

4.1.3 *Helicobacter pylori* and gastric cancer .

In 1994, the International Agency for Research on Cancer (IARC) classified the bacterium *Helicobacter pylori* a Class 1 human carcinogen, capable of inducing changes leading to gastric cancer (IARC 1994). Epidemiological studies have shown a 2-12 fold increased risk of gastric cancer in those infected with *H. pylori* (Blaser 1998, Forman 1998, Eurogast 1993, You *et al.*, 2000, Rugge *et al.*, 1999). As such, infection must be a significant risk factor for gastric cancer, although it should be stressed that the majority of people who acquire the infection do not develop cancer.

In the pathway outlined by Correa and illustrated in the general introduction *H. pylori* has been shown capable of inducing the tissue changes described in the pre-malignant stages (Graham 2000, Sipponen *et al.* 1997, Kuniyasu *et al*, 2000). Experimental work has also shown the release of Reactive Oxygen Species (ROS) in the presence of *H. pylori,* and this is known to lead to DNA damage (also discussed in the general introduction).

4.1.2 Aims

Chapter 3 has described the optimisation of the interphase FISH methods needed to determine chromosomal abnormalities, and also illustrated the specific abnormalities seen when cells were exposed to ROS *in vitro*. In this chapter the methods described in the *in vitro* study were modified to study the *in vivo* changes in

gastric tissue. The chromosomes studied have been chosen as they are all suspected of being important in gastric carcinogenesis. Chromosomes 20 and 4, and the p53 chromosome locus 17p13 have been found to be abnormal in gastric cancer. Chromosome 4 has been implicated in the progression of another upper GI cancer, adenocarcinoma of the oesophagus (Croft *et al.*, 2002, Doak *et al.*, 2003). The types of chromosomal abnormalities seen in a range of pre-malignant gastric tissue were investigated.

The hypothesis is that the progressive stages of gastric carcinogenesis are the consequence of genomic instability leading to aneuploidy.

Correlation with patient characteristics and *Helicobacter pylori* infection was also possible during this study.

4.2 Materials and methods

4.2.1 Patients enrolled into study.

Patients were enrolled at the Endoscopy Unit in Neath Port Talbot Hospital as outlined in Chapter 2. Samples were also received from Dr AP Griffiths, Consultant Pathologist at Morriston Hospital Swansea, from patients having undergone upper GI surgery for gastric malignancy.

4.2.2 Histological diagnoses.

Gastric biopsies (one or two) were taken at the site of the brush sample and sent for histological analysis (Pathology Department Morriston Hospital) including the determination of *Helicobacter pylori* by the designated Consultant Pathologist, Dr AP Griffiths.

Gastric biopsies were also taken during the upper GI endoscopy, stored at -70°C, and used later to determine *Helicobacter pylori* and subtype status by PCR (see Chapter 6).

4.2.3 Gastric cell collection, preparation of slides, interphase FISH, scoring

The methods of gastric cell collection using cytology brushes, the preparation of slides for FISH and the details of the FISH methodology used have been described in Chapter 2.

4.2.4 Reproducibility of FISH technique.

Duplicate gastric slides from the same patients were exposed to the same FISH probes and scored to ensure the reproducibility of the technique. Any significant statistical differences in the results were calculated using the Fisher exact test, $p<0.05$.

4.2.5 Statistical analysis.

Fisher's exact analysis was used to compare the chromosomal changes between the differing histological stages.

Standard error bars were also calculated for each histological group

4.3 Results

4.3.1 Patients enrolled in the study.

Twenty seven patients were enrolled at the Endoscopy department at Neath Port Talbot Hospital. Cytology brushes from four of these patients did not yield adequate cell numbers to perform FISH. As the study proceeded improvements in the

method of collection of cells with the cytology brushes and transport medium modification resulted in fewer samples being lost. Three additional pre-malignant patient samples were obtained from brushes taken at the Pathology Department in Morriston Hospital from surgical resections. Hence the total number of patients samples considered in this part of the study was thirty.

Table 4.2 illustrates the information collected on the case record forms of patients enrolled in the study, and shows the histological diagnoses for each patient together with *Helicobacter pylori* status. The information available was provided by the patients and not corroborated by other sources, therefore underreporting of risk factors may be present. Seventy-seven percent were female, all were white and the age range was 23 to 79 years. Alcohol excess was noted when above national recommended limits (21 units a week), and three people admitted to such an intake. Eight people admitted to a smoking history whether current or within the last 5 years. Seven people described a family history of GI cancer; medical notes were not sought to confirm this. Dietary history was determined by using patients' response to two simple questions (1) how many portions of fruit and vegetables do you eat per day? (2) Do you add salt to your food? Patients diet was regarded as poor if they had < 3 portions of fruit or vegetables daily, or added salt to their food, and 7 patients fitted these criteria.

The reason for referral was also noted for each patient involved in the study, and more than one symptom was often cited. 30% complained of dyspepsia, 26% of reflux, 26% of epigastric pain, 9% of GI bleeding (melana/haematemesis), 4% of anaemia, 4% of dysphagia, and 22% were re-referred for endoscopic follow up (ulcer/polyps/dysplasia).

Table 4.2.Patient characteristics

Patient	Age	Sex	Family history	Alcohol excess	Smoking	Diet history - Fruit/veg/ meat/salt	Race Histological diagnosis
1	55	f	Father – oesophageal cancer	/	/		White NO SAMPLE
2	49	f	/	/	Ex		White gastritis
3	23	f	/	/	/		White NO SAMPLE
4	79	f	/	/	20-day	/	White normal
5	71	m	/	/	/	Low fat	White HP gastritis
6	70	f	/	/	/	Vegetarian	White normal
7	52	m	/	/	/	no veg/fruit	White gastritis
8							NO HISTOLOGY
9	50	m	Parent-gastric cancer	80u-week	20-day	No fruit/veg, added salt	White HP, intestinal metaplasia
10	71	f	Sib-gastric cancer	/	/	Added salt	White gastritis
11	70	m	/	/	/	Added salt	White atrophic gastritis
12	45	f	Sib-ulcer	/	10-day	Added salt	White normal
13	51	f	Father – metastases ?gastric cancer	/	/	Low fat and wheat	White normal
14	68	f	? Father bowel cancer	/	/	Gluten free	White HP gastritis
15	39	f	/	/	30-day	Added salt, rarely veg, no fruit	White normal
16	66	f	Grandmother-gastric cancer	/	20-day	/	White HP gastritis
17	52	f	/	/	/	No red meat	White normal

Patient characteristics (cont'd)

Patient	Age	Sex	Family history	Alcohol excess	Smoking	Diet history- Fruit/veg/meat/salt	Race	Histological diagnosis
18	57	f	/	/	/	/	White	HP gastritis
19	34	m	/	40-week	/	Rarely fruit/veg	White	normal
20	50	f	/	/	20-day	/	White	normal
21	54	f	/	/	15-day	/	White	NO SAMPLE
22	75	f	/	/	/	vegetarian	White	HP gastritis
23	67	m	/	/	/	/	White	gastritis
24	87	f	/	/	/	No red meat	White	intestinal metaplasia
25	47	f	/	/	/	/	White	NO SAMPLE
26	78	f	/	/	/	/	White	intestinal metaplasia
27	61	m	/	32u-week	/	/	White	intestinal metaplasia
			Surgical resections (>1 diagnosis possible)					
T1	45	m					White	intestinal metaplasia, normal
T2	46	f					White	intestinal metaplasia
T3	72	m					White	intestinal metaplasia

4.3.2 Histological diagnosis.

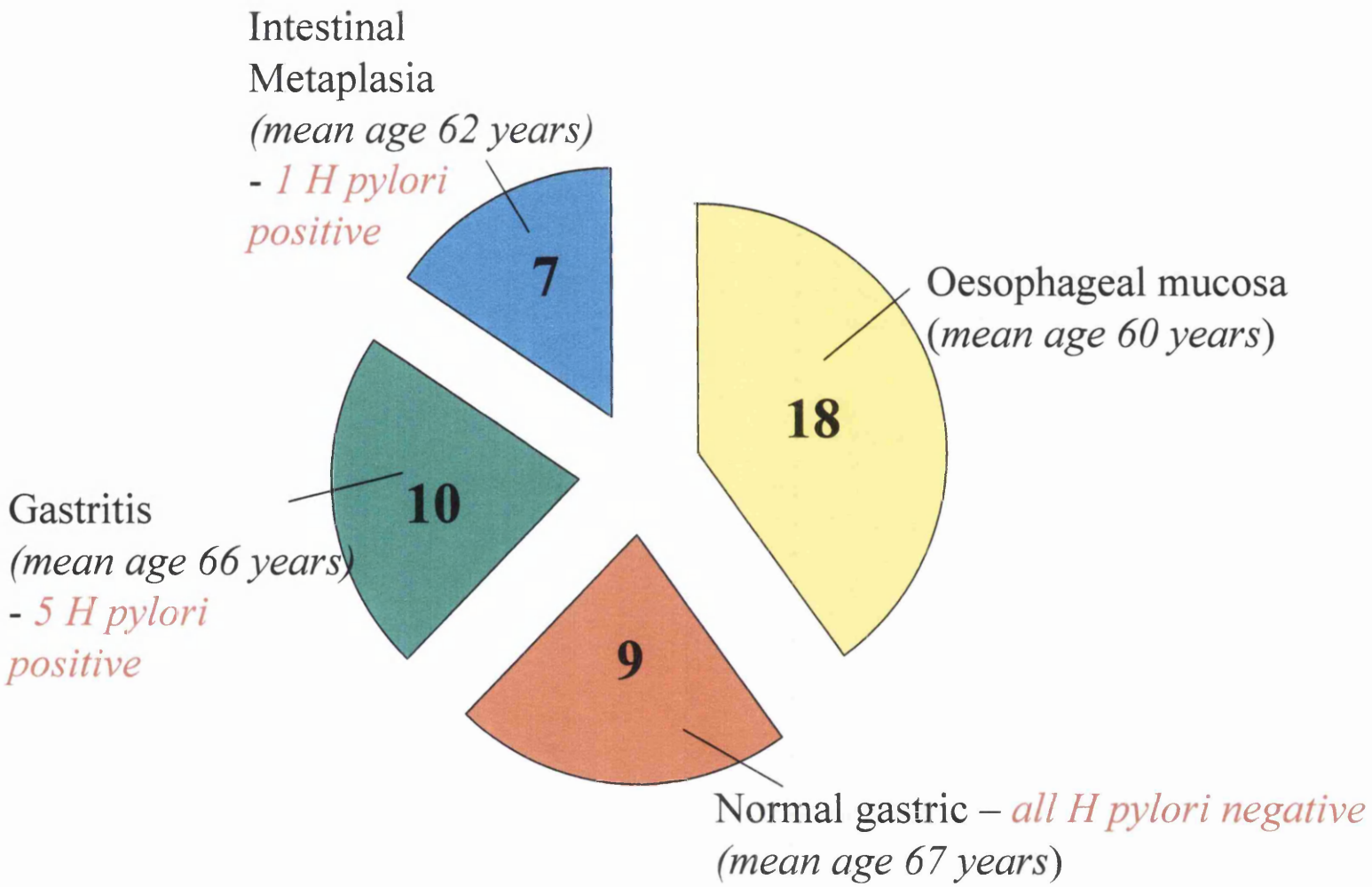
Figure 4.1 illustrates the distribution of histological diagnoses amongst the patients. Gastric samples from patients were divided into three broad groups - normal gastric mucosa, gastritis and intestinal metaplasia. Patients were not subdivided into active, chronic or atrophic gastritis as numbers were too small. Where more than one diagnosis was present the patient was assigned to the more advanced pathology in the Correa pathway. All of these histological diagnoses can be found in the pathway outlined by Correa depicting a stepwise change from normal gastric mucosa to adenocarcinoma (Correa 1988), and they represent the pre-malignant changes in the mucosa. Although, it should be remembered that the changes in the mucosa can be arrested at any point and do not always result in cancer.

One patient could not be diagnosed histologically because of inadequate biopsy size, therefore FISH results on this patient were excluded.

As has been explained in Chapter 2.2 patients with no macroscopic abnormality at endoscopy had brushes/biopsies taken from the gastric antrum. It was interesting to note that of the patients analysed in this part of the study, one patient who had macroscopically normal gastric mucosa had evidence of antral gastritis on biopsy. All of the patients with abnormal endoscopies had abnormalities limited to the gastric antrum, and therefore brushes/biopsies were taken from this area. Five people were noted to have mild antral gastritis endoscopically but histological analysis was normal.

Eighteen patients of twenty seven had their oesophageal brushes analysed in this study, and this group acted as an internal control.

Figure 4.1. Histological diagnoses of patients' tissue samples collected

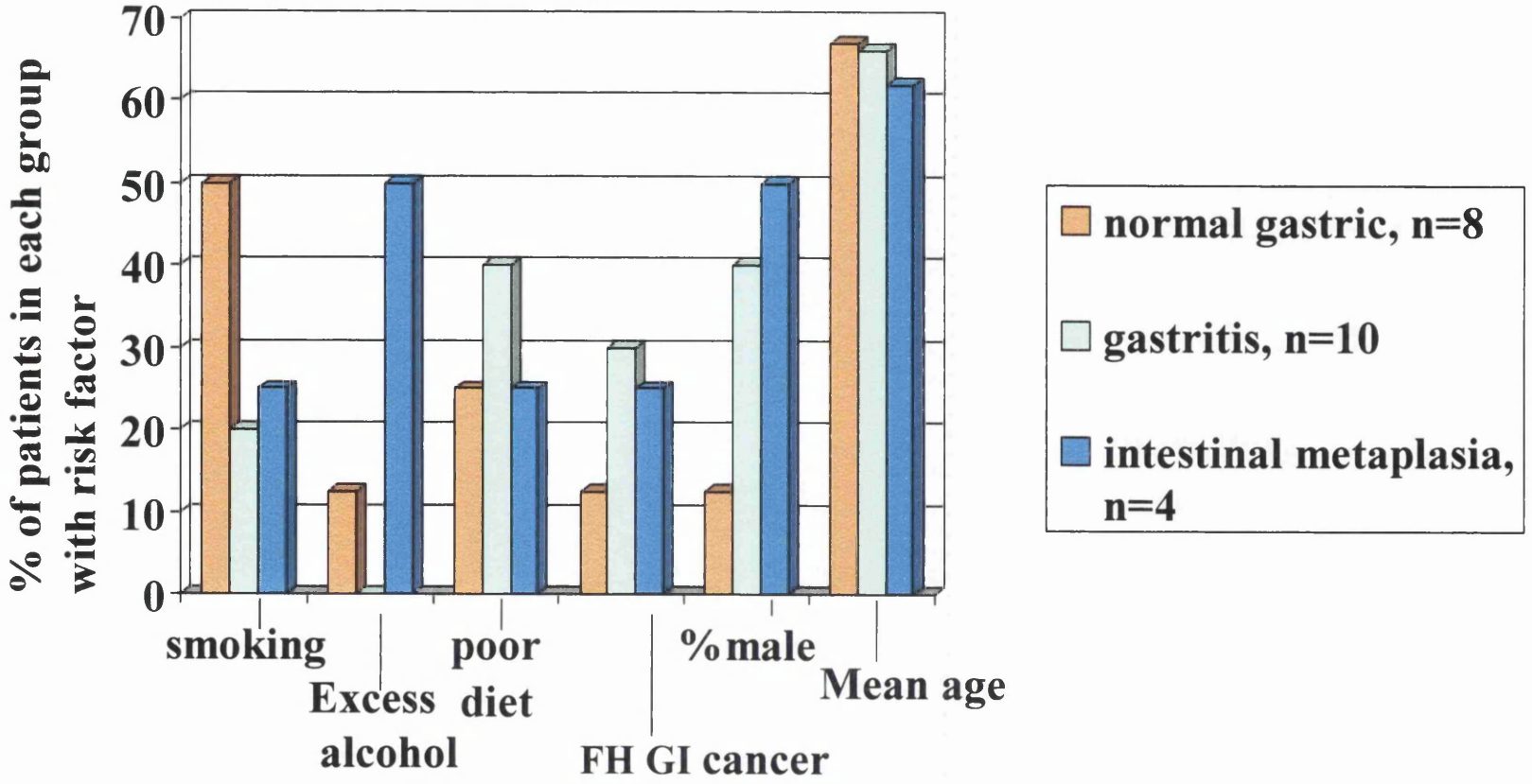


4.3.3 Risk factors associated with gastric cancer.

Family history, male sex and increasing age have all been implicated as risk factors for gastric cancer. Smoking, alcohol, and dietary habits have also been linked to the aetiology of gastric cancer. Figure 4.2 shows the frequency of these risk factors according to histological diagnosis. As can be seen the numbers were small in each group therefore no statistical significance was calculated, but it appeared that apart from male sex, there was no obvious trend to suggest that any of the risk factors were associated with the pre-malignant progression to gastric cancer. The fact that male gender showed a stepwise increase with increasing grade of histology was interesting when it was considered that in this study over three quarters of the patients were female, and gastric cancer is considered predominantly a disease of men.

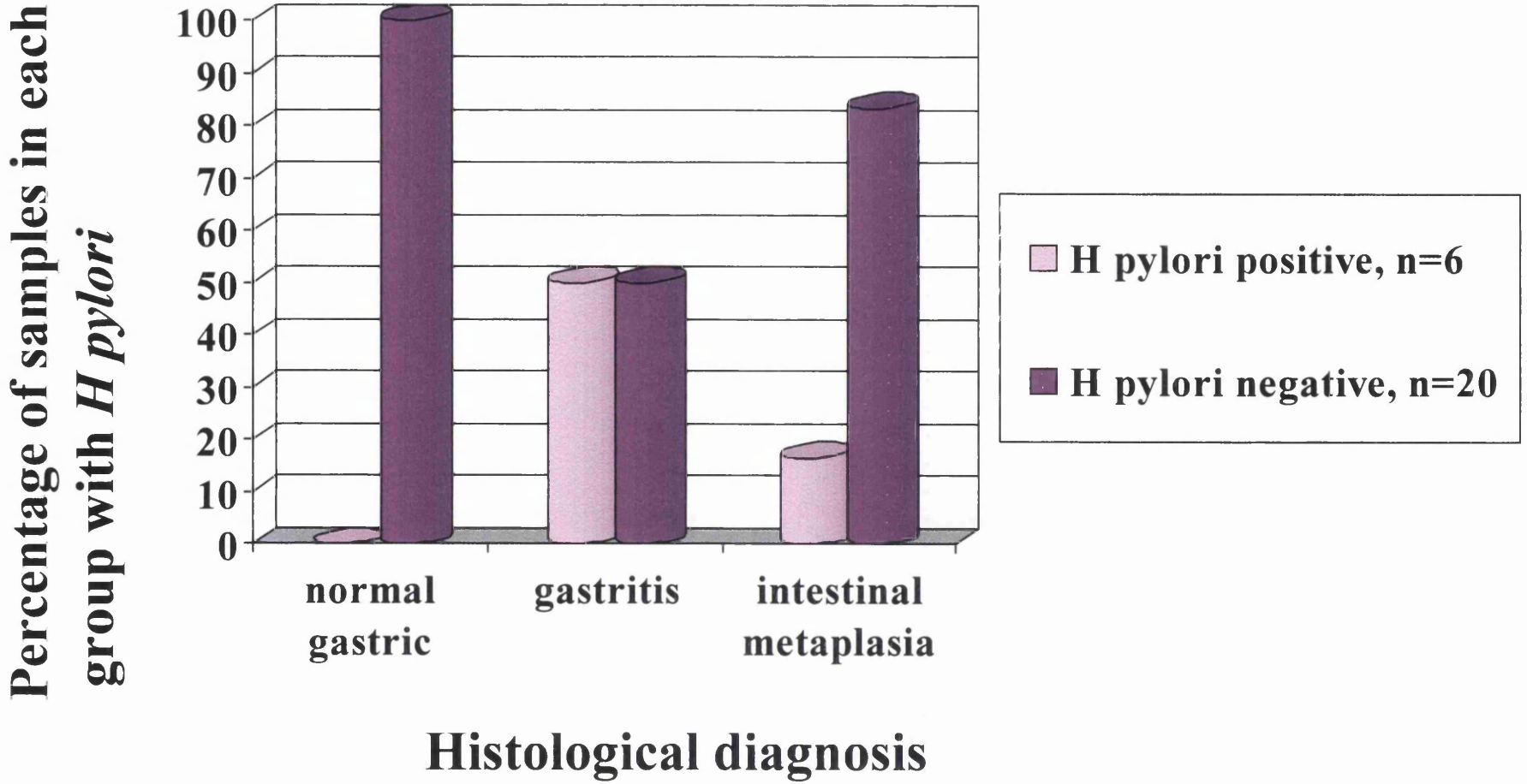
Helicobacter pylori infection has been strongly implicated as a risk factor for gastric cancer. The determination of the bacteria in the gastric mucosa of these patients was made by a Consultant Pathologist using standard haematoxylin and eosin histological preparations produced from the biopsy samples, and included searching for organisms with oil immersion lens when gastritis was present. Chapter 6 compares these findings with a more sensitive method of *H. pylori* infection, PCR analysis, using a second gastric biopsy taken alongside that used for histological purposes (Ismail 2004). However, based upon the histological diagnosis of infection given here, no normal gastric mucosa was infected, but 50% of patients in the gastritis group had *H. pylori.* This percentage falls in the intestinal metaplasia group, as one might expect to occur when the gastric environment changes and the pH alters, making the environment less optimal for *H. pylori* to adhere to the tissue and survive (Graham 2000, 5, You *et al.*, 2000). This is illustrated in Figure 4.3.

Figure 4.2 Percentage of patients enrolled at Endoscopy with proposed risk factors for GI cancer



Risk factors present in each group, numbers in each group small,
no distinctive trend to increase as disease severity increases, except from male sex

Figure 4.3. Presence of *Helicobacter pylori* diagnosed histologically in gastric samples



When the data was analysed to look for a link between *H. pylori* infection and the other risk factors for gastric cancer, none was seen.

4.3.4 Reproducibility of FISH technique.

Two slides from the gastric brushing of patient 26 were used to perform FISH using probes LSI p53 and CEN 8. The Fisher's exact test was employed using the results for both probes, in order to assess the reproducibility of the FISH technique. No statistical difference was found. The result was the same for individual probe loss or gain, as well as when any abnormality in chromosome complement was present. A further two slides from the gastric brushing of patient 15 were used with probe CEN 20, and again no statistical difference was found for altered chromosome complement, amplification or deletion. Furthermore, a brushing from a surgical resection, T7 (see Chapter 5), produced two slides and FISH was performed with CEN 4, and again the Fisher's exact test was used to show no statistical difference. Again the result indicated the same levels of abnormality and amplification /deletion. Table 4.3 shows the raw data.

This highlights the robust nature of the technique and validates the reproducibility of the scoring performed.

4.3.5 Chromosomal abnormalities between oesophageal and normal gastric tissue.

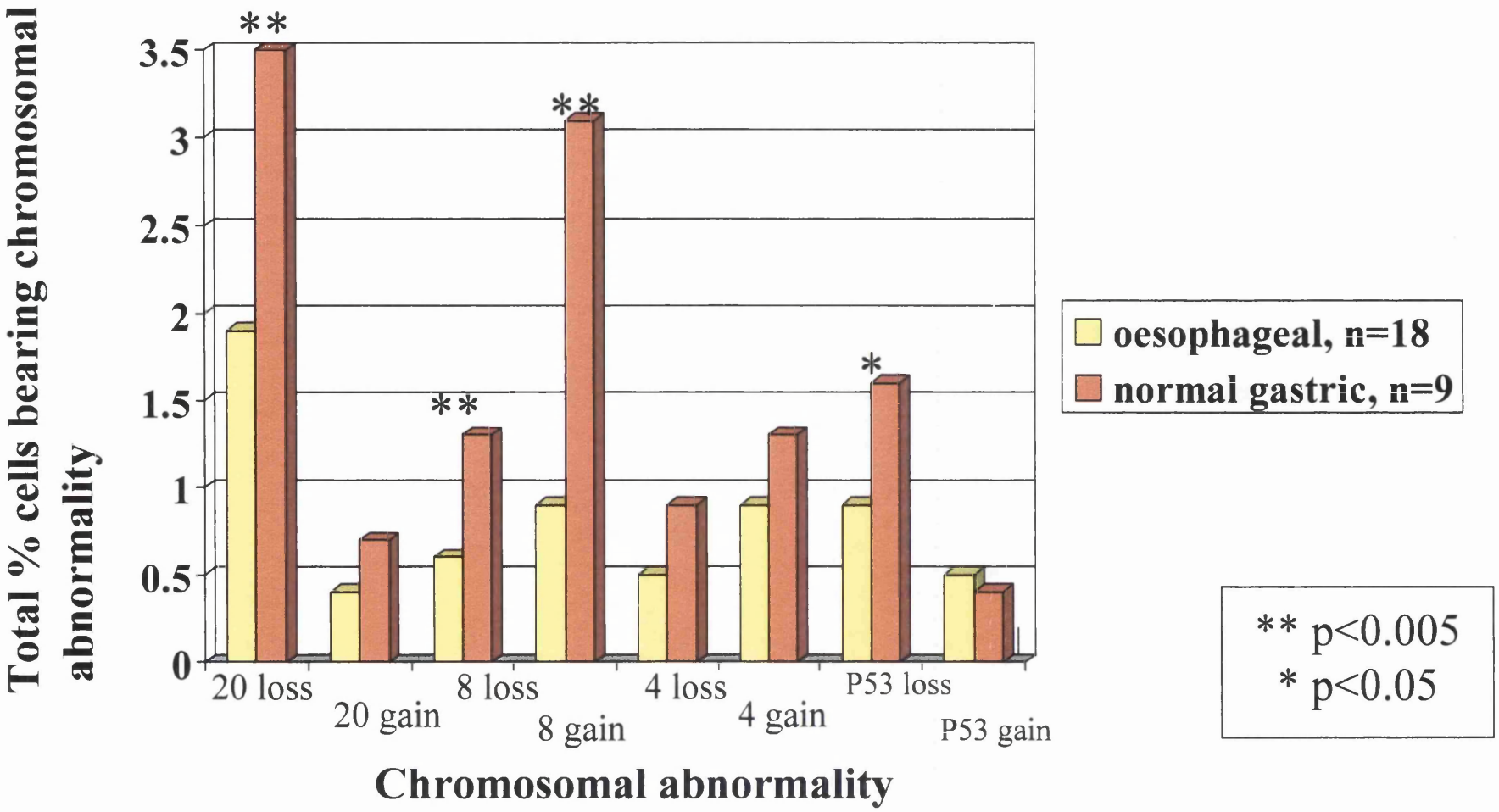
Cytology brushes were taken from the gastric mucosa, and also from macroscopically normal oesophagus in each patient to act as internal controls, ensuring any gastric abnormality seen was not widespread in the patient i.e. a germ cell abnormality. Eighteen of the twenty seven oesophageal brushings taken produced adequate cell numbers for FISH.

Table 4.3. Results of duplicate FISH experiments using the same gastric sample.

Patient & probe	Patient 26 & LSI p53 P value	Patient 26 & CEN 8 P value	Patient 15 & CEN 20 P value	Patient T7 & CEN 4 P value			
Nos cells with 2 signals	216, 202 P=0.4	214, 218 P=0.5	354, 293 P=0.5	108, 101 P=0.3			
Nos cells with <2	16, 16 p=0.5	5, 5 P=0.6	6, 10 P=0.13	2, 4 P=0.3			
Nos cells with >2 signal	5, 6 P=0.5	17, 19 P=0.6	7, 2 P=0.15	28, 18 P=0.2			

Figure 4.4 graphically represents the chromosomal abnormalities seen in the oesophageal cells and in the normal gastric cells. CEN probes highlight the centromere of the chromosome and were used to determine whole chromosome changes i.e. aneuploidy, whereas the LSI probe was a marker of the p53 locus. As is demonstrated in Figure 4.4, oesophageal cells had fewer chromosomal abnormalities detected using these probes than normal gastric cells. Oesophageal cells had generally less than 1% of cells bearing either amplifications or deletions of chromosome 20, 8, 4 and the p53 gene, therefore oesophageal cells in this study were seen to be genetically stable. When comparing chromosomal changes in oesophageal cells to gastric cells, and it should be stressed that these gastric cells were histologically normal, more chromosomal instability was seen in the gastric cells. For each type of probe abnormality, whether deletion or amplification was sought, abnormalities were higher in the gastric cells. Statistical significance was reached when loss of p53 was compared between the two groups. As p53 is a tumour suppressor gene, a loss would be expected to provide a selective growth advantage to cells, favouring tumourigenesis. Statistical significance was also reached for a deletion of chromosome 20, and a deletion and amplification of chromosome 8. This may suggest genetic instability has been induced by the stomach contents (acid, pepsin bile) in constant contact with gastric cells, unlike the transient contact that is seen for oesophageal cells.

Figure 4.4. Comparing chromosomal abnormalities in normal gastric and oesophageal tissue



4.3.6 Chromosomal abnormalities in gastric tissue at different stages of disease severity.

Cells from gastric tissue with different histological diagnoses were examined for abnormalities of chromosomes 20, 4 and 8 using FISH. The p53 LSI probe also allowed examination of the p53 locus in the different tissues.

Figure 4.5 shows the percentage of cells with abnormalities of each probe as a function of the histological diagnosis. For CEN probes 20, 8 and 4, the percentage of abnormalities (loss or gain) were higher in abnormal gastric tissue i.e. gastritis and intestinal metaplasia, as compared to normal gastric tissue, and this was observed despite the fact that normal gastric tissue has already been found to be genetically unstable. In fact, there was a clear trend for the instability to increase with histological progression, from normal gastric tissue through to gastritis and then to intestinal metaplasia. Therefore, an increase in chromosomal instability was shown through the early pre-malignant stages of gastric cancer. A similar trend was seen for the LSI p53 probe, suggesting increasing instability at the site of the p53 tumour suppressor gene through the early stages of the gastric carcinogenesis pathway.

Figure 4.6 illustrates the gains of each individual probe and thus the amplification of chromosomes 20, 8 and 4, and the p53 locus. Error bars demonstrate the standard error, showing the variation in the data. For each chromosome investigated, the percentage of amplification increased with each successive step in the Correa progression, with highest amplifications being seen in the intestinal metaplasia group. Amplifications were more frequent in the centromeric chromosome probes than in the gene specific p53 probe. The difference in the levels of chromosome amplification between normal gastric mucosa and abnormal gastric tissue (gastritis and intestinal metaplasia) was most striking in chromosome 4.

Figure 4.5. Overall chromosomal abnormalities of gastric samples in patients with differing histological diagnoses

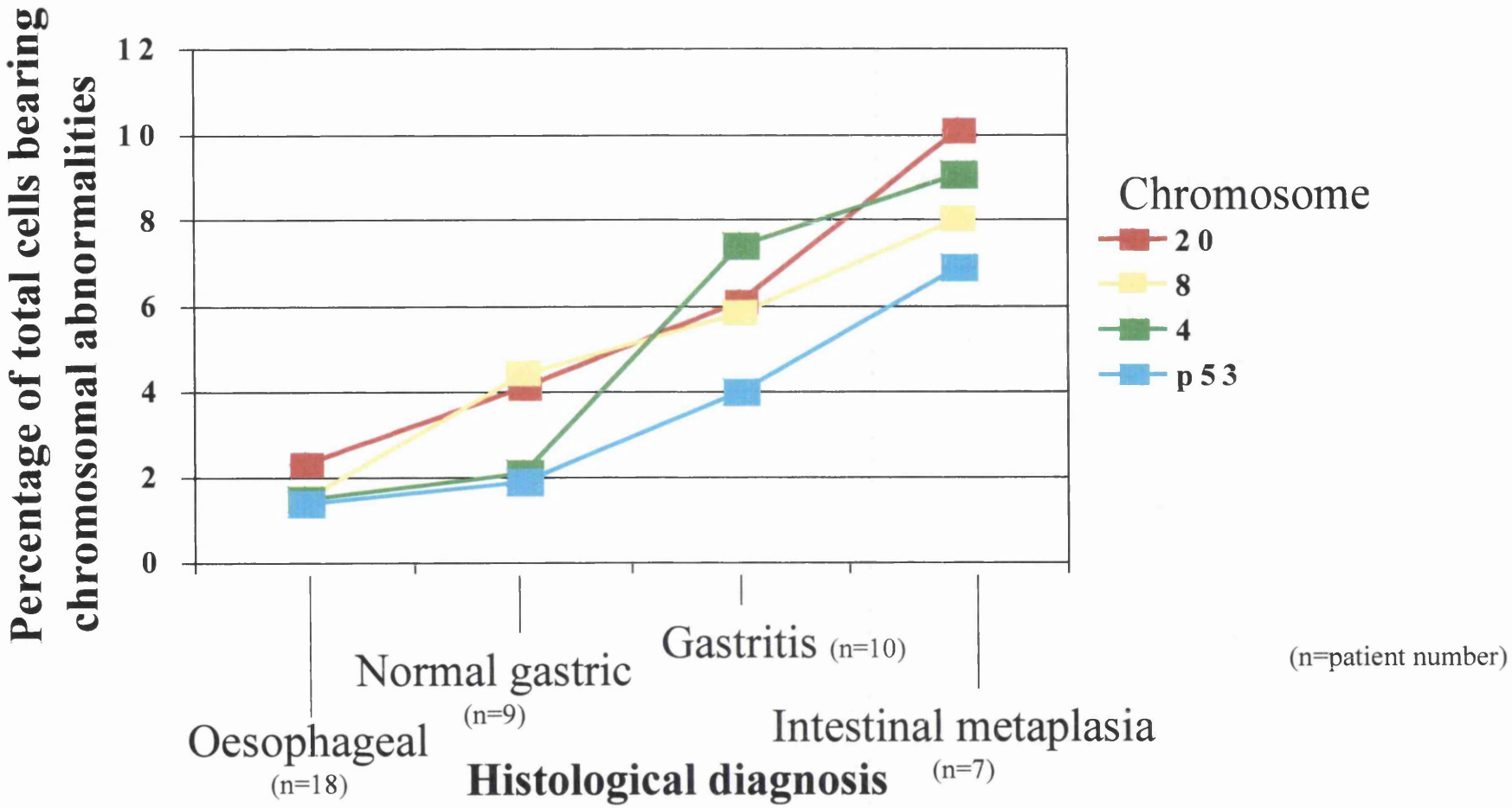
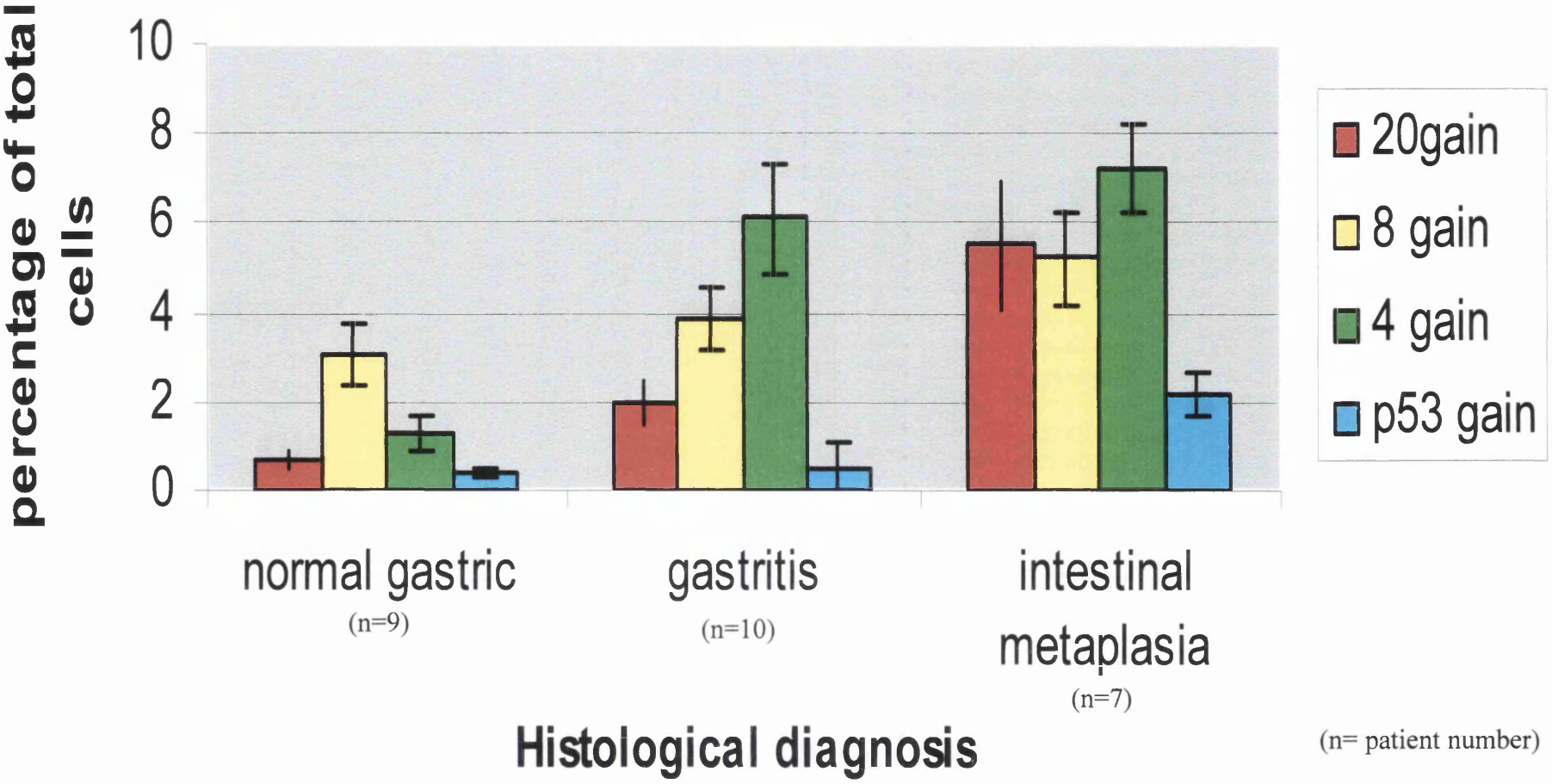


Figure 4.6. Percentage of cells in each histological group with chromosomal gains

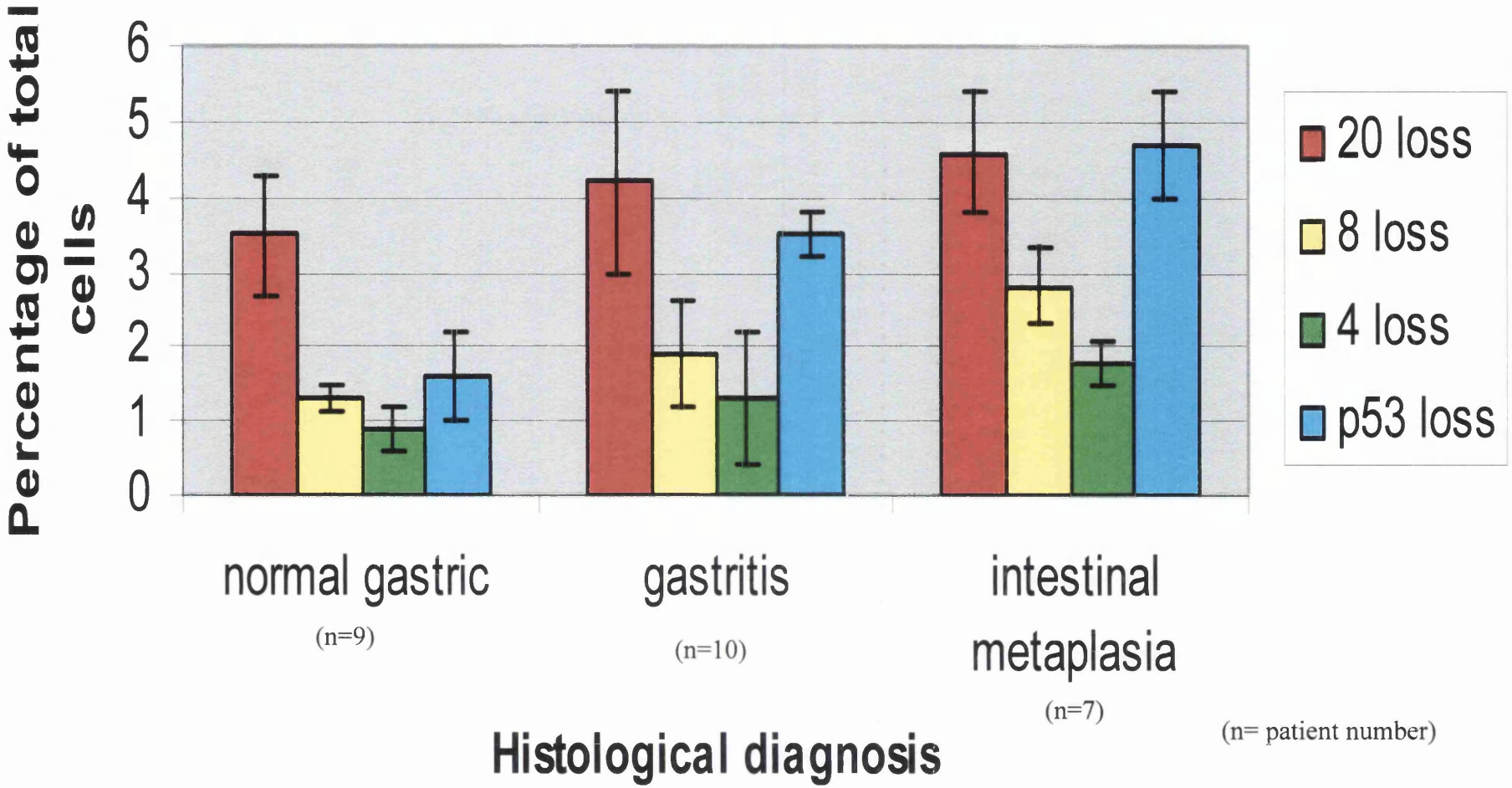


Chromosome 20 amplification showed a clear stepwise increase in the progression to metaplasia. This trend was also present for chromosome 8, yet less obvious with error bars overlapping between each group. p53 was also amplified with increasing frequency along the pathway but percentages of abnormalities were small, <2%.

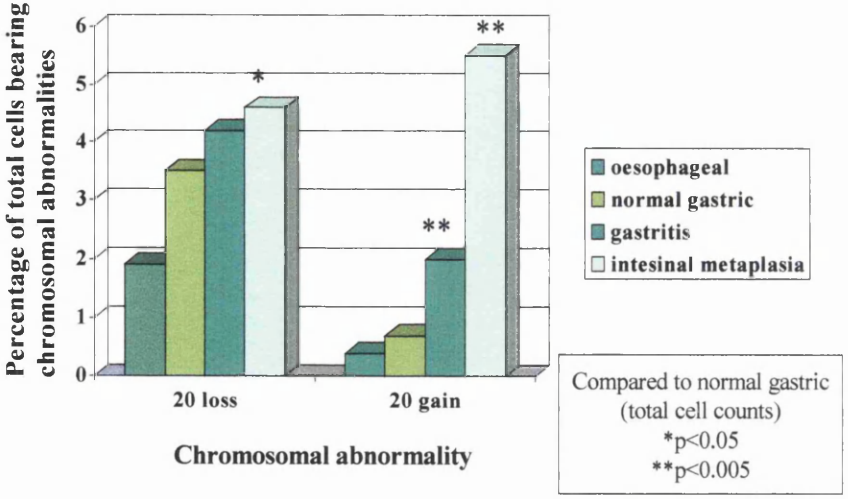
Figure 4.7 represents the loss of chromosomal probes, therefore the chromosomal deletions. As seen above with the amplifications, deletions of each probe were more frequently found in abnormal gastric tissue as compared to normal gastric tissue. A clear and significant increase in probe deletions with worsening histological progression was only seen with the p53 locus specific probe. This was not unexpected, as loss of this tumour suppressor gene would herald genetic instability and be selected for in tumour evolution. Chromosome 20, 8 and 4 deletions showed a trend to increase with histological progression but to a lesser degree, with overlapping error bars.

Figures 4.8, 4.9, 4.10 and 4.11 again illustrate the individual deletions and amplifications of each chromosomal and locus specific probe, as a function of the histology, with p values being shown calculated with Fisher's exact test. Chromosome 20 amplifications were statistically significant in the gastritis and intestinal metaplasia group, when compared to the normal gastric mucosa ($p<0.005$). Chromosome 20 deletions were only significant when comparing the most severe pathological group, intestinal metaplasia to normal gastric mucosa ($p<0.05$). Chromosome 8 amplification and deletion was significant in the intestinal metaplasia group ($p<0.005$), but deletion was also significant in the gastritis group when compared to normal gastric mucosa ($p<0.05$). Amplification of chromosome 4 was a highly significant event seen in both gastritis and intestinal metaplasia in relation to normal gastric cells ($p<0.005$). Deletion of chromosome 4 was also significant in the intestinal metaplasia group

Figure 4.7. Percentage of cells in each group with chromosomal losses



chromosome 20 in pre-malignant gastric tissue



chromosome 8 in pre-malignant gastric tissue

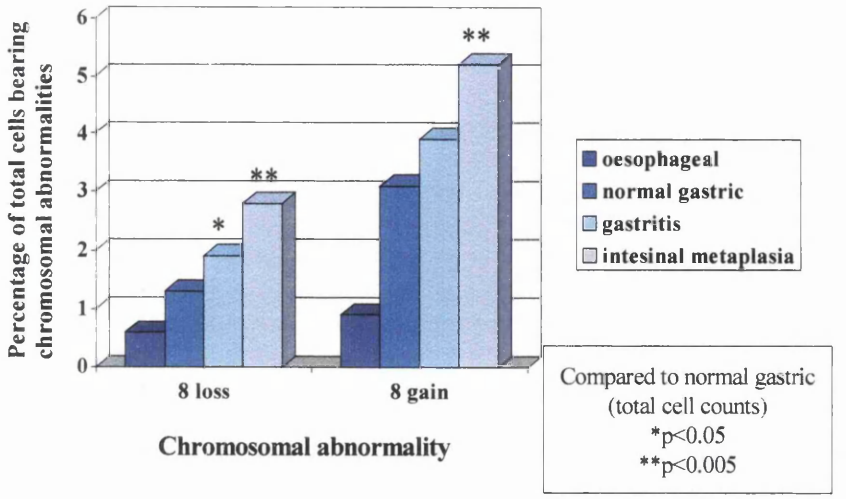


Figure 4.10. Abnormalities of chromosome 4 in pre-malignant gastric tissue

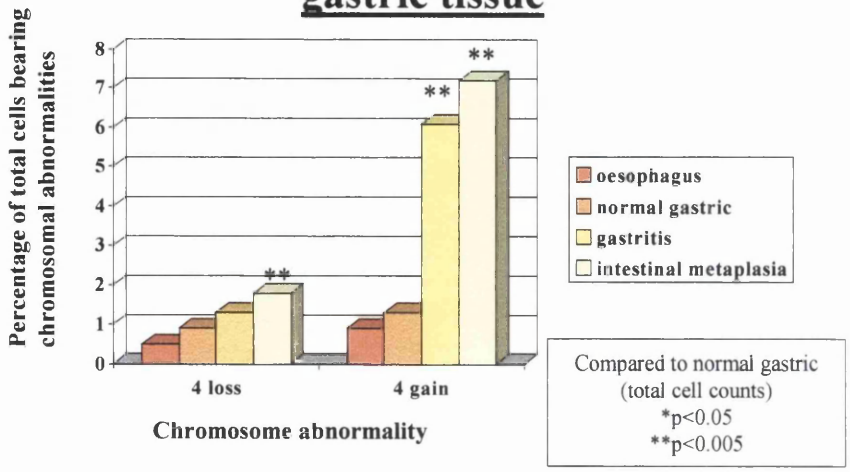
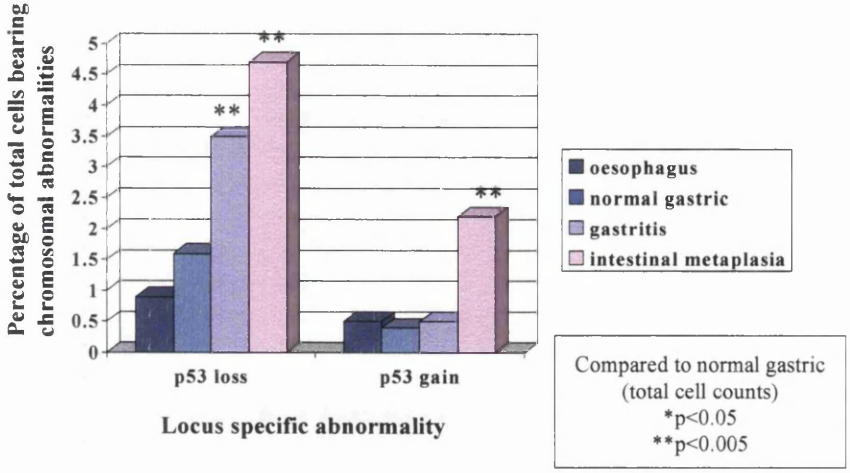


Figure 4.11. Abnormalities of p53 in pre-malignant gastric tissue



($p<0.005$). The loss of p53 was highly significant in both gastritis and intestinal metaplasia ($p<0.005$). In the intestinal metaplasia group the amplification of the p53 probe was also significant ($p<0.005$).

In an attempt to determine the rank order of importance of the chromosomal abnormalities detected in the pre-malignant tissues studied here, Table 4.4 demonstrates the fold differences in chromosomal amplification and deletion in gastritis and intestinal metaplasia compared to normal gastric tissue. As can be seen amplification of chromosome 20, amplification of chromosome 4 and deletion of p53 rank highly in both pre-malignant groups, suggesting their importance in the early stages of gastric carcinogenesis.

4.3.7 Correlation of *Helicobacter pylori* infection and chromosomal abnormalities.

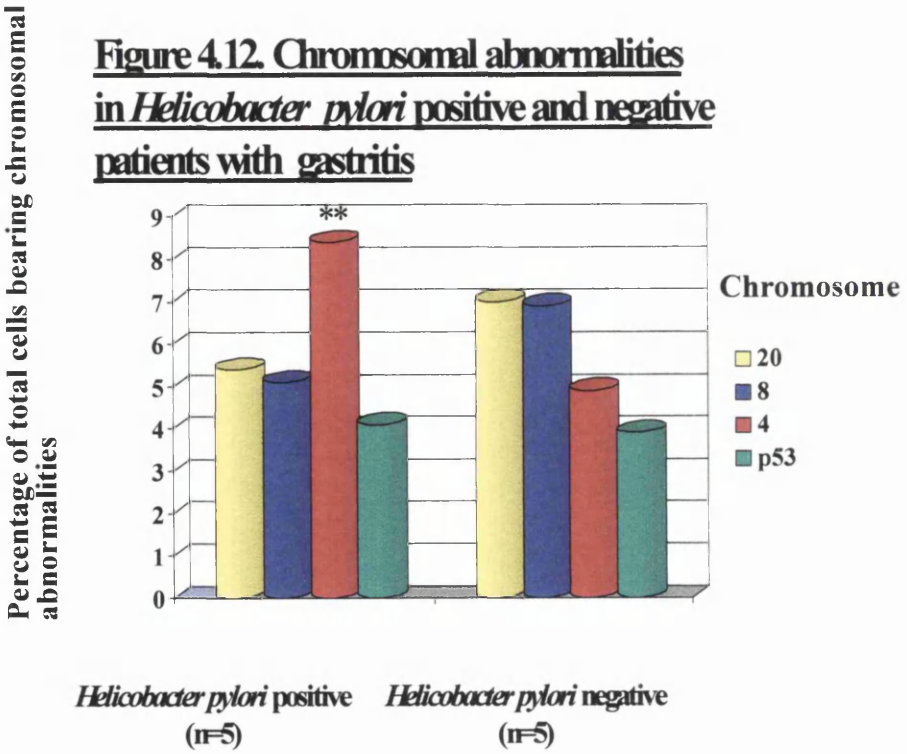
H. pylori infection in these gastric tissues has been determined histologically in this chapter. Figure 4.3 has already illustrated the different frequencies of *H. pylori* infection seen in normal gastric tissue and in different disease states, gastritis and intestinal metaplasia. I have also already discussed the fact that chromosomal abnormalities differ depending upon histological diagnosis. In an attempt to determine whether *H. pylori* presence in the tissue had an influence on the chromosomal abnormalities seen, Figure 4.12 shows the different chromosomal abnormalities seen in patients with the same diagnosis, gastritis, but with and without *H. pylori* infection. Only gastritis patients were analysed, as this is the stage most closely linked to *H. pylori*. Surprisingly perhaps, only chromosome 4 showed more chromosomal instability in *H. pylori* infected tissue compared to non-infected tissue, and this was statistically significant ($p<0.005$). In fact, chromosome 4 amplification accounted for 81% of the abnormalities seen in chromosome 4 of the *H. pylori* positive patients.

Table 4.4. Fold increase in chromosomal abnormalities with histological progression in gastric tissue

Chromosome	Increase in prevalence in gastritis cf. normal gastric
4 gain	**X 4.7**
20 gain	**X 2.9**
P53 loss	**X 2.2**
8 loss	X 1.5
4 loss	X 1.4
8 gain	X 1.3
P53 gain	X 1.3
20 loss	X 1.2

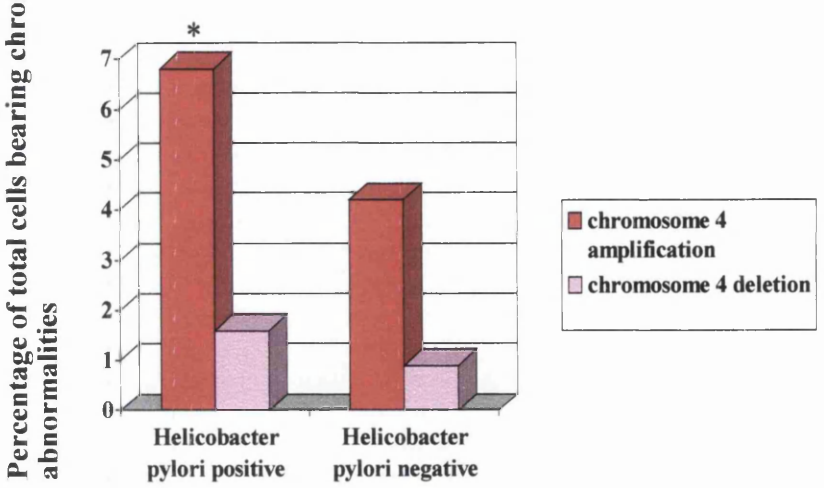
Chromosome	Increase in prevalence in intestinal metaplasia cf. normal gastric
20 gain	**X 7.9**
4 gain	**X 5.5**
P53 gain	X 5.5
P53 loss	**X 2.9**
8 loss	X 2.2
4 loss	X 2.0
8 gain	X 1.7
20 loss	X 1.3

Figure 4.12. Chromosomal abnormalities in *Helicobacter pylori* positive and negative patients with gastritis



** p<0.005

Figure 4.13. Chromosome 4 abnormalities in *H pylori* positive and negative patients with gastritis.



* p<0.05

When the levels of amplification of chromosome 4 were compared between *H. pylori* positive and negative patients, a statistical significance was still seen ($p<0.05$). Figure 4.13.

Obviously numbers were small in the groups compared (5 in each group), and infection was only diagnosed with one technique, histology. Therefore a comparison of the *H. pylori* positive and negative patients in Chapter 6, with the additional benefit of *H. pylori* diagnosis using the more sensitive PCR techniques, may provide further interesting information.

4.4 Discussion

The experiments described in this chapter have attempted to study the chromosomal abnormalities present in gastric tissue *in vivo*, using interphase FISH, and as such investigate the hypothesis that as gastric carcinogenesis progresses, so genomic instability leading to aneuploidy increases. Interphase FISH was successfully performed on gastric cell preparations to determine chromosomal abnormalities. The level of aneuploidy found in chromosomes 4, 8, 20 and 17(p53) of gastric cells increased as disease severity increased. Amplifications of chromosomes 4 and 20, together with deletions of chromosome 17(p53), were the most significant findings.

As explained in Chapter 2, the patients were enrolled from my routine open access endoscopy lists at Neath Port Talbot Hospital, and as no selection policy for appointing them to my list was in place they represent the population referred with upper GI symptoms by general practitioners in that area. A maximum of two patients per list were enrolled due to time constraints on a busy open access endoscopy list. It may have been possible to screen patients for suitability to the study by interview

prior to their arrival at endoscopy, and thereby improve efficiency of enrolment. However, this would have required an additional hospital visit by the patient and therefore it was not done. Information was collected from each patient on known gastric cancer risk factors to determine if any one factor stood out. The information collected was provided by patients' alone, and as such underreporting of risk factors may be present, in particular alcohol and smoking history. The accuracy of information collected on risk factors such as family history has to be questioned as no other source (GP/medical case notes) was used to confirm the information given. Interviewing the patients prior to their endoscopy date and allowing more time to answer the questions may have improved accuracy. Also it was evident that when the data was analysed the numbers in each group were too small to show significant differences, and a larger data collection was needed. Again, with improved efficiency of enrolment and the optimisation of transport methods to the laboratory, this would be possible.

Solid tissue has often proven difficult to manipulate into a form such that FISH techniques can be employed. A monolayer of cells needs to be generated onto a glass slide such that FISH probes can be added, and then detected in the nuclei of these cells using microscopy. Therefore, initially in this study the development of a successful methodology to deliver cells suitable for FISH analysis was paramount. The modification of a technique described by Doak *et al.* 2003 has shown that endoscopic cytology brushing is a reliable and safe method of cell collection from the gastric mucosa. Furthermore, it allows the preparation of cytodots with adequate cell numbers for analysis using interphase FISH. Interphase FISH is an observer dependent technique and in order to check the validity of the results from this study, a reproducibility exercise was performed, 4.3.4. The histological diagnosis of the tissue

studied was determined from biopsies taken at the same site as the brushings to allow direct comparison (Doak *et al.*, 2003). In order not to prolong the endoscopic procedure, whereby increased risk to patients could result, only one site was analysed. Hence sampling error in this study, particularly in the determination of *H. pylori* infection, may be a problem. However, in Chapter 6 *H. pylori* identification was determined from a second biopsy and using a second method of detection.

Using these cell preparations, the purpose of the study was to determine the level of chromosomal abnormality present in pre-malignant gastric tissue samples from patients. The chromosomes targeted have all been implicated as being important in gastric or oesophageal adenocarcinoma, as outlined in the introduction. A variety of histological stages were obtained for investigation. All fitted into the early stages of the multi-step model for gastric carcinogenesis, as described by Correa i.e normal gastric mucosa, gastritis and intestinal metaplasia (Correa 1988).

Initially, a comparison was made of normal oesophageal tissue and normal gastric tissue from the same patients. The oesophageal squamous tissue, which was macroscopically normal, had little evidence of aneuploidy in chromosomes 20, 4 and 8 and also little instability of the p53 locus, broadly speaking <1%. A small percentage of probe abnormalities could be expected due to the experimental processes, in particular losses of probe can occur due to poor probe penetration and labelling inside the cell nuclei, and also because of shielding of signals. This low incidence of chromosomal abnormality in the oesophageal tissue suggests a very stable tissue, and is comparable with the results shown by Doak *et al.*2003.

Histologically normal gastric tissue however showed significantly more chromosomal instability. This may be explained by considering the different environments in which the two tissues exist. Gastric cells live in an acidic medium

and are bathed in gastric secretions often containing a combination of acid, bile, food, mucus, enzymes and bacteria (*H. pylori*), whereas the contact of these agents with the oesophageal tissue is transient only during reflux episodes. In this study oesophageal brushings were from the mid oesophagus where contact with gastric secretions would be minimal. Hence, the suggestion is that acid or bile, key agents in gastric secretions, may play a role in inducing aneuploidy in gastric tissue. Bile and acid have been implicated as having a role in the mechanism of upper GI cancer (Triadafilopououlos 2001). Bile acids in particular have been shown to cause DNA damage (Scates *et al.*, 1995, Scates *et al.*, 1996) and chromosomal aberrations (D'Souza *et al.*, 2003).

Against the unstable background found in gastric tissue, specific chromosomal abnormalities and possible correlations with histological diagnosis were sought. Chapter 1 has already discussed p53 abnormalities as being implicated in not only cancer, but specifically in gastric cancer. In this study, deletions of p53 were detected using a FISH probe for the p53 locus in pre-malignant gastric tissue. Deletions of p53 were found more commonly than amplifications. p53 function involves control of cell cycle progression and apoptosis in response to DNA damage, therefore loss of the function would lead to cell instability (Grady 2001). Deletions of p53 were found in all types of gastric tissue, with a significant stepwise trend to increase with histological progression along the pathway to cancer. Thus higher numbers of p53 abnormalities were seen in the intestinal metaplasia group. Previous studies have shown p53 abnormalities in the various stages of pre-malignant disease, commonly dysplasia and intestinal metaplasia (Tamura *et al.,* 1991, Uchino *et al.*, 1993). Becker *et al.* demonstrated levels of p53 mutation/LOH to be 30% in cancers, 30% in adenomas and 10% in intestinal metaplasias (Becker *et al.*, 2000). Other studies support the findings of p53 abnormalities in pre-malignant conditions (Dixon *et al.*,

2001, Nardone *et al.*, 1999, Brito *et al.*, 1994, Ochiaia *et al.*, 1996, Shiao *et al.*, 1994). This study however, has also shown p53 losses to be significantly increased in gastritis when compared to normal tissue, suggesting important abnormalities of this tumour suppressor gene are present very early in the progression to carcinoma. In fact, p53 losses were present to a lesser degree in normal gastric tissue.

Alterations in chromosome 8 copy number were also found in pre-malignant gastric tissue as early as gastritis. The trend involving chromosomal 8 abnormalities increased with histological progression. Chromosome 8 copy number is very often abnormal, usually amplified, in all types of gastric cancer (see Table 4.1). Some authors suggest that its amplification is more frequent in advanced cancers and therefore may be a late event (Wu *et al.*, 2001). This is in contrast to the data shown here in pre-malignant tissue, where chromosome 8 abnormalities appeared early. Both deletions and amplifications were seen in this study in all tissue types investigated. Chromosome 8 houses an important oncogene, c-myc, which acts as a proliferative transcription factor. Its amplification and rearrangement has been observed in many tumour types (Grady 2001). C-myc over-expression has been reported in gastric cancers with varying frequencies, 16-67% (Grady 2001). There are some reports in the literature of abnormalities of c-myc being present in some pre-malignant stages of gastric cancer. Panani *et al.* have shown gains of chromosome 8 in gastric adenomas (Panani *et al.,* 1995). One study reports frequencies of c-myc over-expression in 36% of cancers, and 15% of chronic gastritis. All these abnormalities were also associated with abnormal p53 expression (Nardone *et al.*, 1999). Further work will need to be performed on the pre-malignant histological types in this study to see if the amplification of chromosome 8 is associated with an upregulation of c-myc, and therefore implicating a role in early gastric carcinogenesis. Also it was not possible to

determine from the data shown here how frequently chromosome 8 amplification co-existed with p53 deletion, as suggested by Nardone *et al.* (Nardone *et al.*, 1999). This may prove a worthwhile follow up.

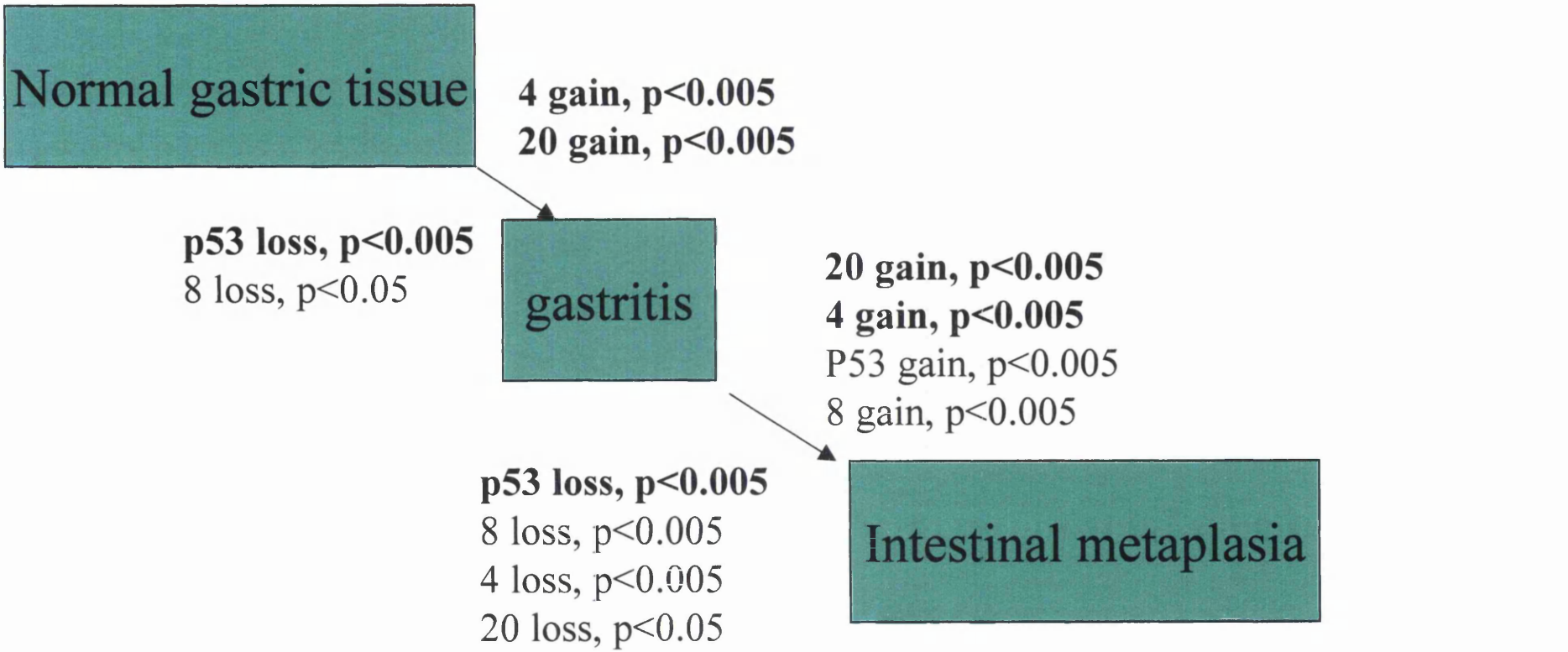
Abnormalities of chromosome 20 were found to be present in pre-malignant tissue, with both amplifications and deletions existing, although amplification showed more statistically significant differences between the tissue types as pathology progressed. Chromosome 20 is implicated in a number of CGH and FISH studies of all types of gastric cancer (Table 4.1 in the introduction), and again amplification is more usual. Chromosome 20 does not have any of the classic cancer related genes, but the Cancer Genetics Web (www.cancer-genetics.org) does give information on certain genes located on chromosome 20 being over-expressed in gastric cancers - TNFRSF6B – 20q13.3, PYGB – 20p11.2-11.1. Panani has shown with CGH that amplification of chromosome 20 can occur in adenomas as well as carcinoma (Panani *et al.*, 1995), a stage further along the carcinogenesis pathway than gastritis or intestinal metaplasia. In this study, abnormalities of chromosome 20, both losses and gains, were usually found to be more frequent than other chromosomal abnormalities, also having a higher prevalence in normal gastric tissue. This could therefore be representative of general instability of the tissue, and be a marker of aneuploidy, i.e. chromosome non-disjunction, due to increased cell proliferation. It is interesting to note that Aurora kinase A has been located on chromosome 20q13.2. Aurora kinases have been discussed in the general introduction as having a possible role in causing cells to develop aneuploidy, and Aurora kinase A has a function associated with centrosomes during chromosome separation (Carmena and Earnshaw 2003). In fact, this protein kinase has been amplified and overexpressed in cancers and cell lines (Zhou *et al.*, 1998, Giet *et al.*, 1999). It may be that abnormality of this protein has a

role in the further development of aneuploidy, and hence cell instability, in gastric carcinogenesis.

Chromosome 4 aneuploidy was highlighted in this study. As with the other chromosomal abnormalities, the frequency of chromosome 4 aneuploidy increased as histology progressed, and there were significant changes of chromosome 4 in even the earliest step along the pathway to gastric cancer. Amplifications of chromosome 4 were the most striking abnormalities to exist in gastritis and intestinal metaplasia, when compared to normal gastric mucosa. This may suggest an important role for chromosome 4 amplification in the initiation of gastric carcinogenesis. Chromosome 4 amplification has been recently shown to be a significant event in the progression of disease from Barretts oesophagus to adenocarcinoma, especially in the early histological changes of this pathway (Croft *et al.*, 2002, Doak *et al.*, 2003). The level of chromosome 4 amplification in the intestinal metaplasia samples studied here, 7%, was less than, but nonetheless comparable with, that seen in the metaplasia patients with Barretts disease i.e. 12% (Doak *et al.*, 2003). Further work by the same group has linked the amplification of chromosome 4 to the protein Nuclear Factor κB (NFκB) (Jenkins *et al.*, 2003). NFκB resides on chromosome 4 and acts as an anti-apoptotic factor, as well as influencing the pro-inflammatory action of interleukin-8 (IL-8) (Rayet and Gelinas 1999). The increased copy number of NFκB allows continued cell survival with avoidance of the usual cell regulators. Could a similar situation be present in gastric pre-malignant tissue? NFκB has already been linked to many cancers including gastric cancer (Rayet and Gelinas 1999).

Figure 4.14 shows a modified Correa model of gastric carcinogenesis (Correa 1988). An attempt has been made to fit the chromosomal abnormalities identified in

Figure 4.14. Modified model of Correa's pathway of gastric carcinogenesis with the chromosomal abnormalities identified



Bold = most prominent abnormalities

this study into the pathway, implicating their possible role in early gastric carcinogenesis. As is shown, amplification of chromosome 4 and 20, together with deletion of p53, are significant events in the sequential change from normal gastric mucosa through gastritis to intestinal metaplasia.

Chapter 3 has shown the types of chromosomal abnormalities induced by Reactive Oxygen Species (ROS). To recapitulate, out of the chromosomes studied, amplification of chromosome 4 and deletion of p53 were the most prominent abnormalities induced by ROS, together with instability of chromosome 20. These ROS induced changes in chromosomal copy number are in concordance with the types of aneuploidy that I have demonstrated in pre-malignant gastric tissue, suggesting maybe that ROS could be a causative agent of the aneuploidy shown in pre-malignant gastric tissue. *Helicobacter pylori* infection has been well documented as causing oxidative DNA damage through activation of ROS released by the immune system, some of the evidence to support this statement has been summarised in chapter 1. The final experiments in this chapter have attempted to determine if a correlation exists between chromosomal abnormalities and *H. pylori* infection. As the histological stage of gastric tissue itself affects the level of aneuploidy present, and also as *H. pylori* infection can be lost in gastric tissue with changed phenotype (Graham 2000, Grady 2001), the comparison of aneuploidy in tissue with and without *H. pylori* infection was restricted to gastritis tissue samples only. Also, Morgan *et al.* 2002 noted that in tissue with gastritis, higher levels of ROS, and therefore oxidative DNA damage, were present. As such, any chromosomal damage present due to ROS would be enhanced in this group. Again it should be noted that diagnosis of *H. pylori* was on histological grounds only, although this is discussed further in Chapter 6 when more sensitive PCR techniques are used to detect *H. pylori* in the same tissue samples.

However in the results illustrated here, the frequency of abnormalities in chromosome 4 was found to be significantly higher in the *H. pylori* positive patients, and specifically amplification of chromosome 4. Therefore, it could follow that amplification of chromosome 4 is associated with ROS, but a causative role is still only suggestive. Also it should be reiterated that numbers in this study group are now small, only 10.

Gastric cells exposed to *H. pylori* have been shown to have an upregulated expression of IL-8, and it is known that IL-8 is a transcription factor of NFκB (Jenkins *et al.*, 2003, Maeda *et al.*, 2001). It is therefore possible to hypothesise that *H. pylori* infection acts as a trigger to activate NFκB, and that cells with amplification of chromosome 4 and NFκB possess a distinct survival advantage, hence favouring tumourigenesis. This would support a role for *H. pylori* in gastric carcinogenesis.

Other reports in the literature discuss increased specific genetic abnormalities associated with pre-malignant gastric disease and *H. pylori* infection. Nardone *et al.* found that in atrophic gastritis, aneuploidy and c-myc and p53 expression were only found if *H. pylori* infection was also present (Nardone *et al.*, 1999).

Chapter 5 will look at the level of aneuploidy in gastric tumours from a patient cohort within the same geographic location as those patients studied in this chapter, as we know external factors influence gastric cancer incidence. This can allow a comparison of the chromosomal abnormalities present in tumours with those chromosomal abnormalities identified in this chapter in pre-malignant tissues. In other words, to establish whether the levels of aneuploidy continue to increase in a stepwise manner as pathology progresses as would be expected in the Correa model of gastric carcinogenesis, and also to identify any specific chromosomal abnormalities that are

strongly associated with progression to cancer, and therefore are suggestive of a driving force in cancer.

Chapter 5

Detection of chromosomal abnormalities in gastric cancer, using Interphase Fluorescence in Situ Hybridisation

In this chapter tissue samples of gastric cancer were obtained from surgical resections of 10 patients undergoing gastrectomy for gastric cancer. Cytology brushings were used to collect cells, and interphase FISH was performed with CEN probes 20, 4 and 8 and LSI 17(p53). All the chromosomes studied showed aneulpoidy, and in greater frequencies than that already found to exist in pre-malignant tissue (Chapter 4). Intra-tumoural heterogeneity was also found in some of the tumours studied, and the effects of chemotherapy discussed.

5.1 Introduction.

Gastric cancer is a common cancer worldwide and a significant cause of death in the UK (Parkin *et al.*, 1995, Cancer Research Campaign 1995). The general introduction outlines what is known of the evidence available on the incidence and aetiology of gastric cancer.

Ninety percent of all gastric cancers are adenocarcinomas (Luk 1998). Gastric carcinoma can be divided into 2 types histologically, using the Lauren classification, intestinal and diffuse (Lauren 1965, Grady 2002). The intestinal type is composed of gland-like tubular structures that often form a mass or an ulcer (Faraji and Frank 2002). Correa hypothesised that intestinal type gastric cancer was preceded by atrophic gastritis and intestinal metaplasia (Correa 1988). These pre-malignant stages are described in detail in Chapter 1 and Chapter 4. Diffuse type gastric cancer is a poorly differentiated cancer that infiltrates and thickens the gastric wall, and tends to

arise without a precursor lesion (Faraji and Frank 2002). These two types occur with differing frequencies worldwide, and also differ in their patient profile. The intestinal type of gastric cancer is more prevalent in the 7th decade in men, and diffuse cancer is seen predominantly in a younger population with no sex difference. Also importantly, the diffuse type of gastric cancer has a poorer survival rate than the intestinal type of cancer (Faraji and Frank 2002, Sipponen *et al.,* 1998, Grady 2002). Genetic studies on both types of cancers have been reported but whether intestinal and diffuse gastric cancers have different chromosomal abnormalities and therefore different genetic pathways remains controversial (Kokkola *et al.*, 1998, Pathak 1992, Nowinski *et al.*, 1990, Hoshi *et al.,* 1999, Yasui *et al.*, 2000). As different phenotypic pathways are said to exist for each cancer, and as patient characteristics differ, then it would be plausible that there are two distinct genetic pathways.

The wide range of cytogenetic abnormalities found in gastric cancer has been reviewed in the Table 1.1 in chapter 1 which summarises a number of studies. The majority of the studies investigated advanced cancers, and there is general agreement that advanced cancers have more genetic change than early cancers (Kityama *et al.*, 2000, Koo *et al.*, 2000, Kim *et al.*, 2000, Lee *et al.*, 1999, Igasharo *et al.*, 2000, Brito *et al.*, 1994, Uchino *et al.*, 1992). Advanced cancers contain genetic changes that are a result of the inherent instability of the tumour i.e. 'passenger' alterations. These *de-novo* abnormalities arising within the tumour can cause intra-tumoural heterogeneity. Obviously, the most important genetic abnormalities exist early in a cancer, and hence drive the carcinogenic process. These abnormalities would also be present in the pre-malignant stages before cancer is fully established. Cytogenetic abnormalities have already been identified in pre-malignant gastric tissue using patients from the Neath/Swansea locality (Chapter 4). As has been stated in Chapter 1 and Chapter 4,

there is significant variation in the incidence of gastric cancer worldwide. Ideally a comparison should be made between pre-malignant stages and tumour samples from patients in the same area, therefore the gastric cancer resections studied in this chapter were also from patients in the Neath/Swansea area. In fact, the better way to assess whether genetic changes present in pre-malignant tissue have a role in the development of cancer is to examine different tissue stages from the same patient. A longitudinal study like this would probably take several years to accumulate the tissue. Also, because the pre-malignant conditions of atrophic gastritis are so common, a huge population would need to be followed up in the hope of catching some developing cancers. However in this study, different tissue types within the same surgical resection were found and analysed using FISH, which may represent a convenient way to mimic a longitudinal study in individual patients.

Gastric cancers can also be subdivided on the basis of their site into distal and proximal tumours. The more proximal tumours, found at the gastric cardia and gastro-oesophageal junction, are often indistinguishable from tumours of oesophageal origin and in fact may be Barretts associated cancers (Clark *et al.*, 1994). As such they are often managed as oesophageal tumours, where pre-operative chemotherapy now plays an important role (Clark 2001, Walsh *et al.*, 1996). Unlike distal gastric cancers the incidence of proximal tumours is increasing suggesting a different aetiology or pathogenesis from antral tumours (Faraji and Frank 2002, Blot *et al.*, 1991). In Wales, a 3.6% increase in male oesophageal cancer rates per annum has been noted since 1992 (www.wcisu.nhs.uk). The types of genetic changes in proximal and distal cancers have been shown to differ (El Rafai *et al.*, 2002, Beuzen *et al.*, 2000, Nowinski *et al.*, 1990).

Chemotherapy is effective in some human cancers. It can play a role in upper GI cancer as a palliative or curative tool, with or without radiotherapy/surgery (Yosef and Nicolson 2001). Pre-operatively, it can shrink a tumour making successful surgical resection more likely. Agents found to be of use include, 5-fluorouracil, cisplatinum, mitomycin C, doxarubicin, epirubicin and, more recently, etoposide and taxane (Yosef and Nicolson 2001). As chemotherapeutic agents are delivered systemically they have an effect on all cells, although they have a prediliction for those cells that are most actively dividing, and hence cancer cells are selectively targeted. Chemotherapeutic agents have been shown to cause DNA damage, including chromosomal abnormalities, and more specifically aneuploidy (Acar *et al.*, 2001, Silva *et al.*, 2002, Kamguchi and Tateno 2002, Frias *et al.*, 2003). This factor can often make it difficult to assess the genetic abnormalities in tumour tissue after treatment with chemotherapeutic agents.

5.2 Aims

The experiments described in this chapter have looked for specific chromosomal aberrations in ten gastric cancers in the patients from the Neath/Swansea locality using the methodology described in Chapter 4, Interphase Fluorescence In Situ Hybridisation. Hence, it was possible to compare any abnormalities in chromosomes 20, 8, 4 and 17 (p53) found in gastric cancer, with those chromosomal abnormalities already identified in the pre-malignant gastric tissue studied in Chapter 4. In addition, a pseudo-longitudinal study of three patients looked at chromosomal abnormalities in pre-malignant and malignant gastric tissue within three gastric resection specimens. Intra-tumoural heterogeneity was also assessed in

three patients. Finally, the influence of chemotherapy on the chromosomal abnormalities in the gastric tissue of one patient was investigated.

5.3 Materials and Methods

5.3.1. Patients' samples and histological diagnosis.

Surgical resections for gastric cancer were performed at Morriston Hospital Swansea, and samples analysed histologically at the Pathology Department Morrriston Hospital, by a Consultant Pathologist, Dr AP Griffiths. Mucosal brushings were taken from the cancer and surrounding mucosa, as well as from normal gastric or duodenal tissue to act as an internal control. Brushings were taken from freshly opened gastrectomy specimens. After fixation, sections were taken from the brushing sites so that the cytological findings could be correlated with mucosal histology.

Ten patients were included in this study.

5.3.2. Cytology brushings, Interphase cell preparation and FISH analysis.

Within 30 minutes of surgical resection, brushes were taken from the tissue and stored in 90% methanol for transportation and further analysis in the laboratory. The same protocols for Interphase cell preparation, FISH analysis and signal visualisation and scoring, were used as has been described in chapter 2.

The reproducibility of the technique has been described in Chapter 4.

Statistical analysis was determined using the Fisher's exact test.

5.4 Results.

5.4.1 Patient characteristics and histological diagnoses.

Ten patients' surgical resection specimens were analysed. Table 5.1 gives the sex and age of patients, and the histological diagnosis of the tumours surgically resected. Fifty percent of patients were male and the average age was 63.7 years. Male sex is considered a risk factor for gastric cancer and as such it is interesting that 50% of cases in this study were female. Female preponderance was a notable feature in the pre-malignant study described in Chapter 4, with only 33% of the patients studied being male. Therefore, the relative increased representation of men in this gastric cancer cohort as compared to the pre-malignant patient cohort studied earlier, may be important, consistent with the fact that male sex is a risk factor for gastric cancer. Figure 5.1 illustrates the demographics of the patients studied in this chapter.

Nine of the ten gastric tumours were from the distal stomach and one from the proximal stomach. Of the distal cancers, eight were diagnosed histologically as adenocarcinoma, with a variation in the level of cell differentiation found, and only one was noted to be an early gastric cancer i.e. not invading the muscularis propria, patient T7. The other distal lesion consisted of high grade dysplasia / intramucosal carcinoma.

As can be seen from Table 5.1 in three of the distal cancers, T1, T2 and T3, sections were taken at different sites for histological analysis, and correlation made with the brushings taken for chromosomal analysis by FISH. Tissue from all stages of Correa's multi-step pathway of gastric carcinogenesis could be identified in the gastric resection specimens (Correa 1988). Figures 5.2, 5.3 and 5.4 illustrate the

Table 5.1: Patient characteristics and histological diagnoses.

Patient	Age	Sex	Histology and site
T1	45	M	-Adenocarcinoma (poorly differentiated) -Intestinal metaplasia -Normal gastric DISTAL
T2	46	F	-High grade dysplasia -intestinal metaplasia / chronic gastritis DISTAL
T3	72	M	-Intestinal adenocarcinoma (poorly differentiated) -Moderately dysplastic adenoma -Intestinal metaplasia / chronic gastritis DISTAL
T4	83	M	Adenocarcinoma (poorly differentiated) DISTAL
T5	61	F	Adenocarcinoma (poorly differentiated) DISTAL
T6	52	M	Adenocarcinoma (poorly differentiated) DISTAL
T7	70	F	Early intestinal adenocarcinoma DISTAL
T8	74	F	Intestinal adenocarcinoma (poorly differentiated) DISTAL
T9	70	F	Intestinal adenocarcinoma (moderately/poorly differentiated) DISTAL
T10	64	M	Adenocarcinoma post chemotherapy, metastatic adenocarcinoma in nodes PROXIMAL (gastro-oesophageal junction)

Figure 5.1. Gastric surgical resections: age, sex, site of tumour

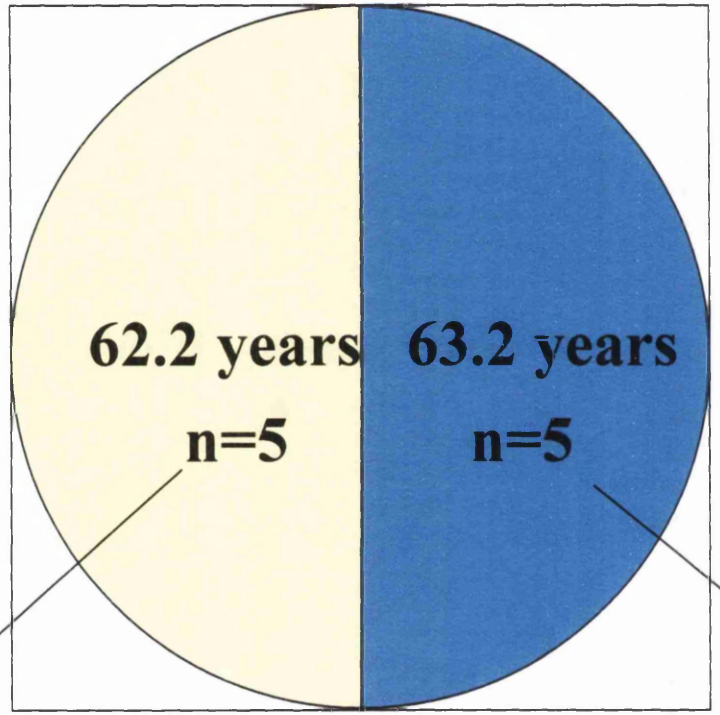


Figure 5.2. Patient T2: histological diagnoses in surgical specimen

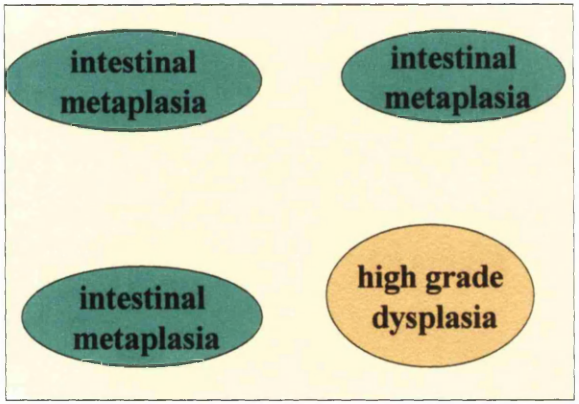


Figure 5.3. Patient T1: histological diagnoses in surgical specimen

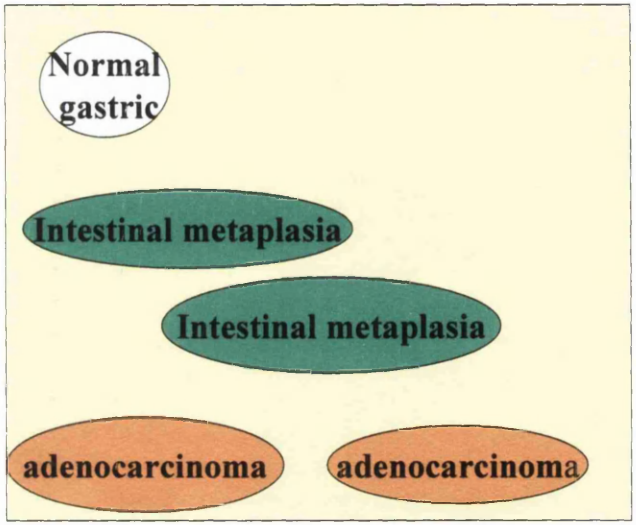
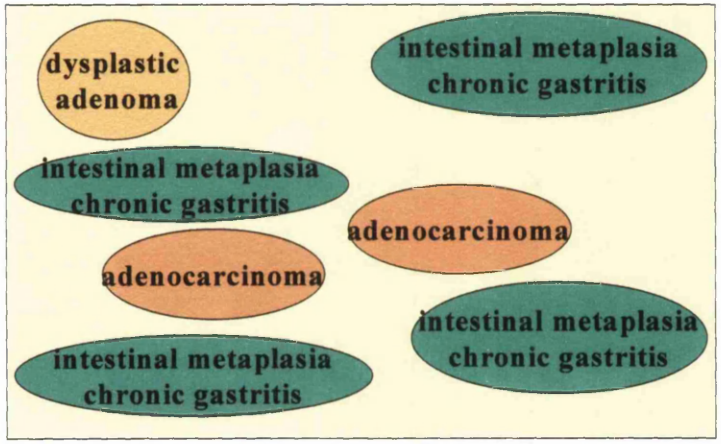


Figure 5.4. Patient T3: histological diagnoses in surgical specimen



different types of tissue found in each resection analysed, and their location with respect to the actual tumour present.

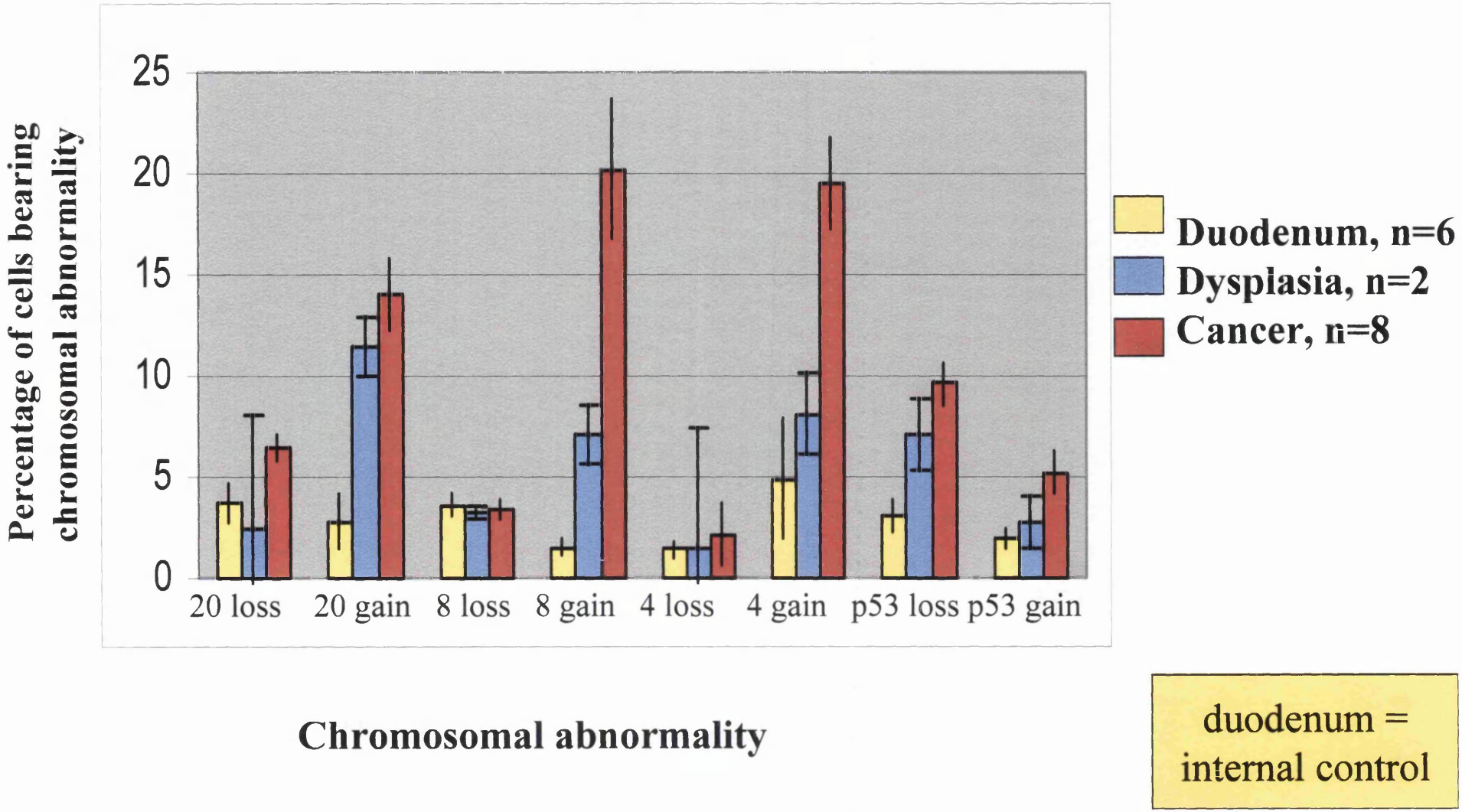
Out of the ten tumour resections examined, one was noted to be at the gastro-oesophageal junction (GOJ), extending proximally into the stomach. This patient, T10, had been managed differently to the other patients, with pre-operative chemotherapy (see appendix 1.5) before resection, and as such the biopsies of the tumour site showed little residual cancer. The surrounding nodes, however, contained metastatic adenocarcinoma. This tumour has therefore been analysed separately.

It is worth commenting that no mucosal site showed the presence of *Helicobacter pylori* infection. *H. pylori* infection is commonly lost in tissues with advanced disease (Graham 2000, You *et al.*, 2000). *H. pylori* may be lost due to intestinal metaplasia, to which it cannot adhere, previous antibiotic therapy or possibly adoption of cocciod morphology.

5.4.2 Chromosomal aberrations in distal gastric cancer.

Abnormalities of chromosomes 20, 8, 4 and 17(p53) were identified in the cells from the gastric tumours studied. Figure 5.5 show the types of specific chromosomal abnormalities present in the cancer and dysplastic cells. Gains of CEN probes 20, 8 and 4 occurred more frequently than losses, showing that amplification of chromosomes, and increase in DNA content was more prevalent in gastric cancer cells and gastric dysplasia. Deletion of the tumour suppressor gene p53 was also common in the cancer cells, which was not surprising, as inactivation of this gene provides a growth advantage in the cell, hence favouring tumourigenesis. Aneuploidy was seen in up to 25% of cancer cells. Compared to the pre-malignant cells with

Figure 5.5. Specific chromosomal abnormalities in distal gastric cancer and dysplastic cells



aneuploidy, the copy number of chromosomes was greater in cancer cells i.e. cancer cells often had greater than 3 copies of chromosomes, see figure 5.6.

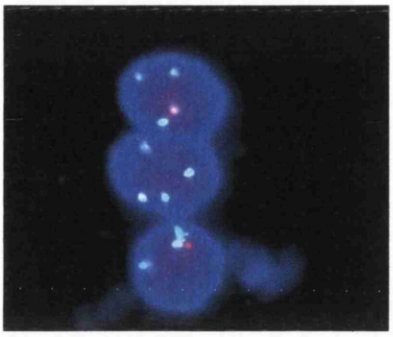
There was only one early gastric cancer found, patient T7. The level of aneuploidy was less in the early cancer as compared to the other more advanced cancers. Amplification of chromosome 4 was seen in 15% of cells in the early cancer compared to 20% in later cancers, and aneuploidy of chromosome 8 also was present in 11.5% of cells in the early cancer compared to 24.7% in the more advanced. No statistical differences have been calculated as numbers in each group were small.

5.4.3 A comparison of the chromosomal abnormalities found in distal gastric cancer cells to those found in pre-malignant gastric cells.

The incidence of gastric cancer, as mentioned earlier, varies depending upon geographical location, and the level of aneuploidy should ideally be compared in cohorts from the same locality, as it was in this study. Figure 5.7 demonstrates the level of aneuploidy found in gastric cancers as compared to that already found in pre-malignant gastric tissue analysed in Chapter 4. There was an obvious increase in the degree of chromosomal instability in the cancer group as compared to the pre-malignant groups. This was visible for each chromosome studied. The trend was stepwise, increasing from normal gastric mucosa, through gastritis, intestinal metaplasia to dysplasia and then to cancer i.e. through the stages of Correa's pathway of gastric carcinogenesis (Correa 1988). The most significant chromosomal abnormalities identified in the pre-malignant tissue studied, were amplification of chromosome 4 and 20, plus deletion of p53. Figures 5.8, 5.9 and 5.10 illustrate the frequency of these chromosomal aberrations in all the tissue types studied in chapters 4 and 5 - normal gastric, gastritis, intestinal metaplasia, dysplasia and cancer. As can

Figure 5.6 Examples of FISH photographs from gastric cancer cells

Amplification of chromosome 4
- 4 copies in one cell
- 3 copies in two cells
with 1 copy of chromosome 20



Amplification of chromosome 4
- 5 copies in one cell, with 2 copies
of chromosome 20

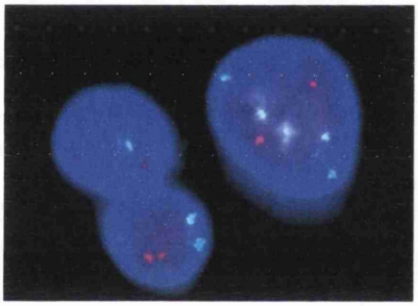


Figure 5.7. Overall chromosomal abnormalities of gastric samples in patients with differing histological diagnoses

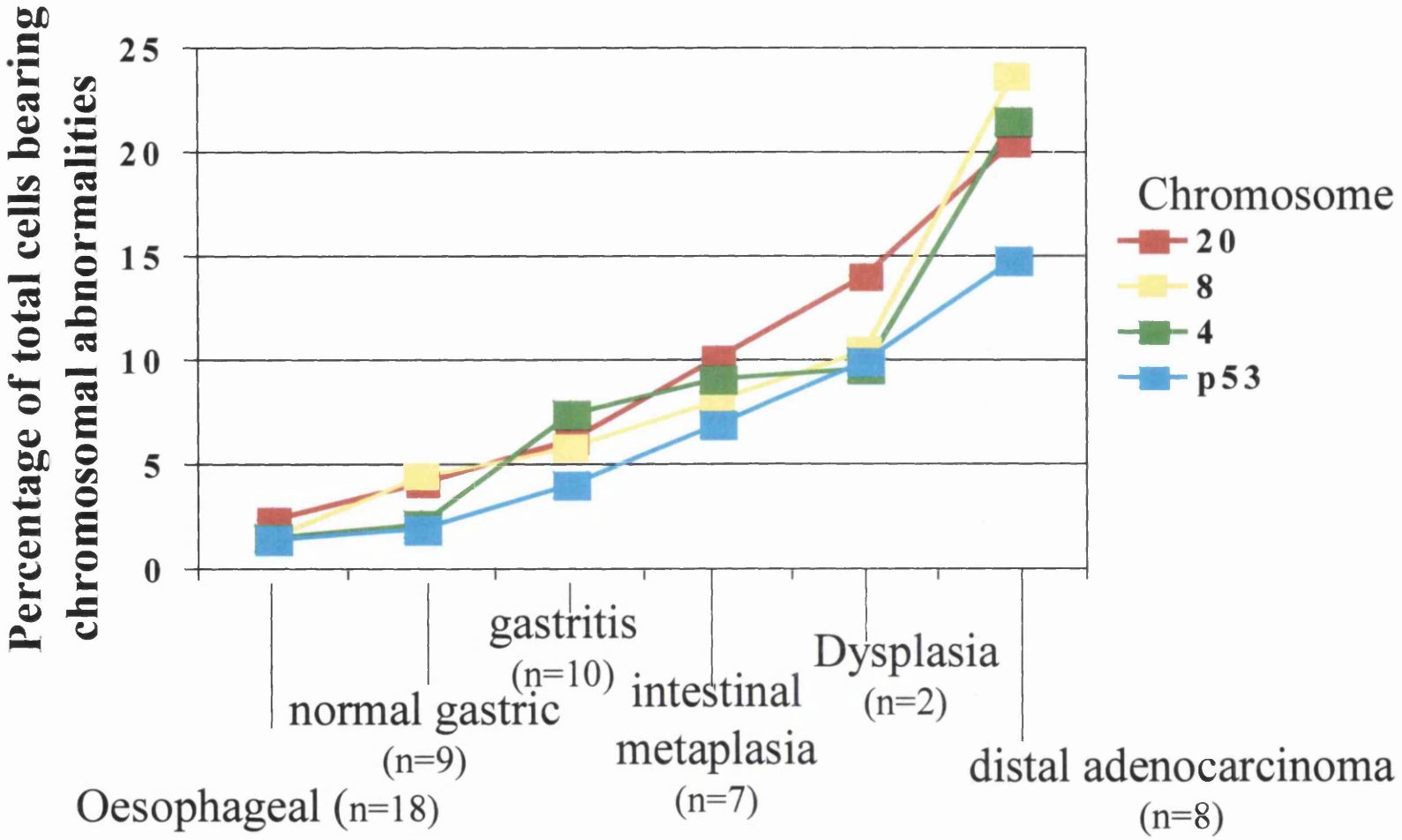


Figure 5.8. Chromosome 4 amplification in pre-malignant gastric and distal gastric cancer tissue

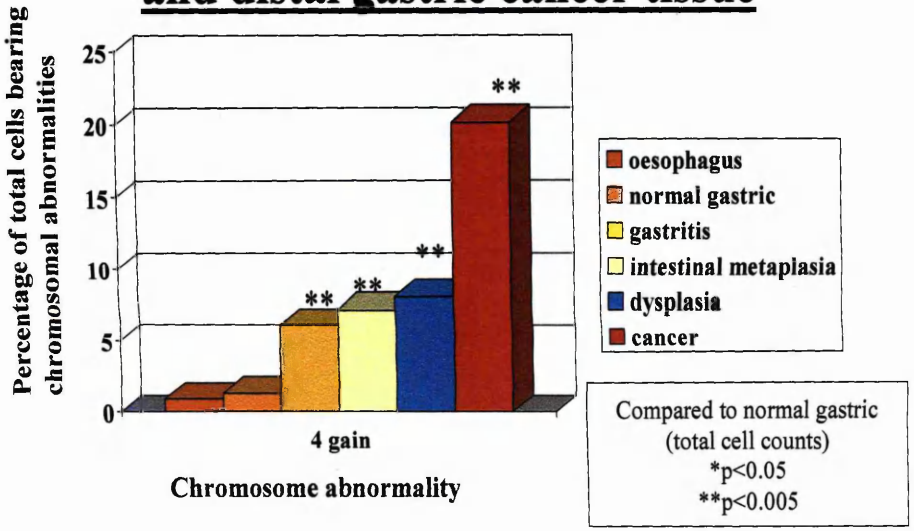


Figure 5.9. p53 deletion in pre-malignant gastric and distal gastric cancer tissue.

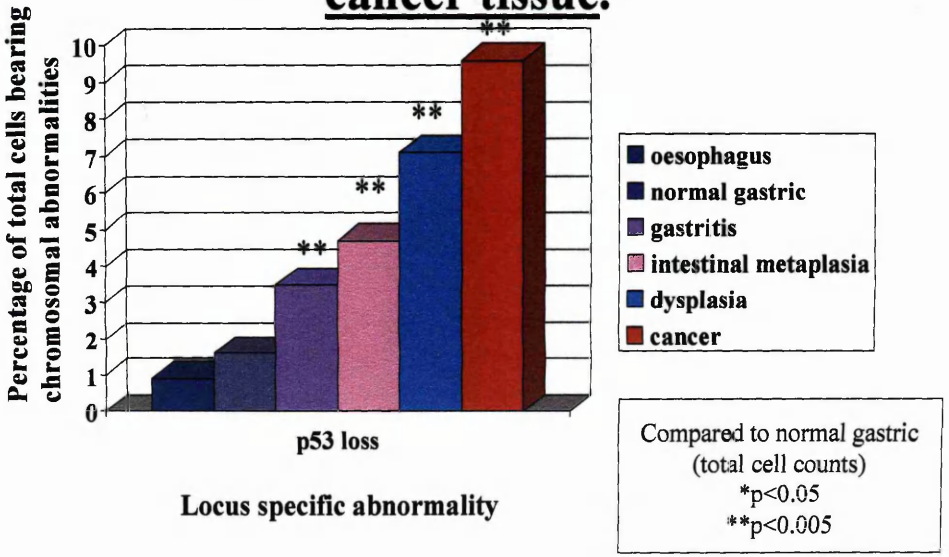
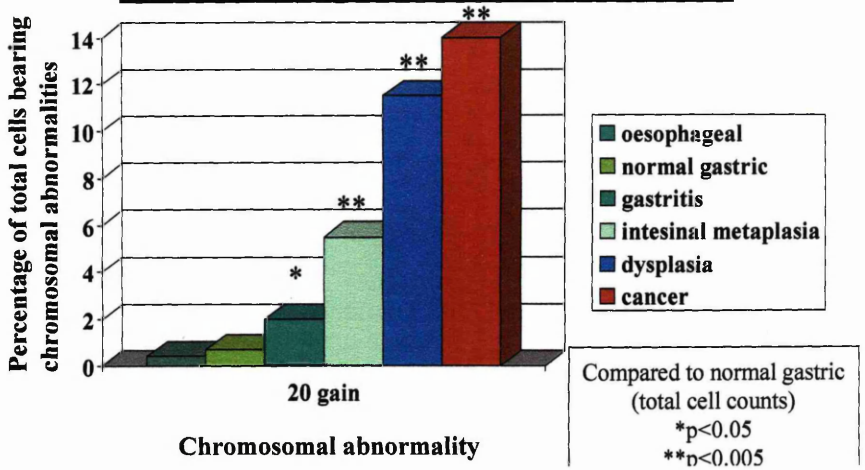


Figure 5.10. Chromosome 20 amplification in pre-malignant gastric and distal gastric cancer tissue



be seen in each graph, there was a stepwise increase in each chromosomal abnormality with histological progression, with up to 20% of cells bearing the abnormality at the malignant stage.

The comparisons of aneuploidy between gastric cancer tissue and pre-malignant tissue described have been made using the pooled data from patient groups. In this chapter, three of the gastric cancer resection specimens contain both pre-malignant and malignant tissue therefore I was able to offer a better comparison of these tissues within an individual. Figure 5.11 depicts the specific chromosomal abnormalities in T1. The most common chromosomal abnormalities in the cancer cells were again the amplifications of chromosomes, and the deletion of p53. Chromosome 4 amplification, which was found to be significantly elevated in the pre-malignant tissue studied in Chapter 4, continued to increase in frequency in this patient with histological progression to cancer, as did chromosomes 8 and 20 amplification. Figure 5.12 looks at the specific chromosomal abnormalities in T2, and again a similar picture existed of increasing abnormalities with histological progression, with chromosomal amplifications and p53 loss predominating. Figure 5.13 illustrates the chromosomal abnormalities in patient T3 and again a similar trend was seen. It was interesting to note that chromosome 4 amplification was often the most frequent chromosomal abnormality seen in the pre-malignant stages in all 3 patients. The frequency of chromosomal 4 amplification continued to increase along the pathway to cancer hence strengthening the hypothesis already suggested that it may have a significant role in gastric carcinogenesis.

Figure 5.11. Chromosomal abnormalities in surgical resection of patient T1

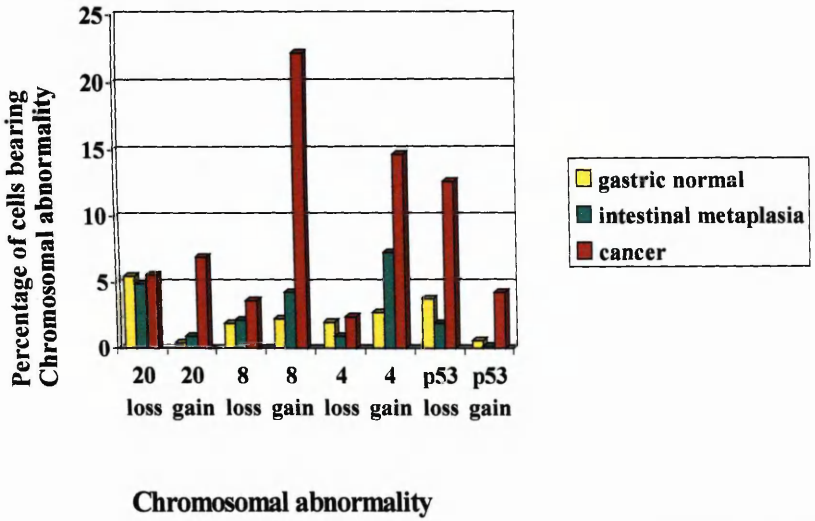


Figure 5.12. Chromosomal abnormalities in surgical resection of patient T2

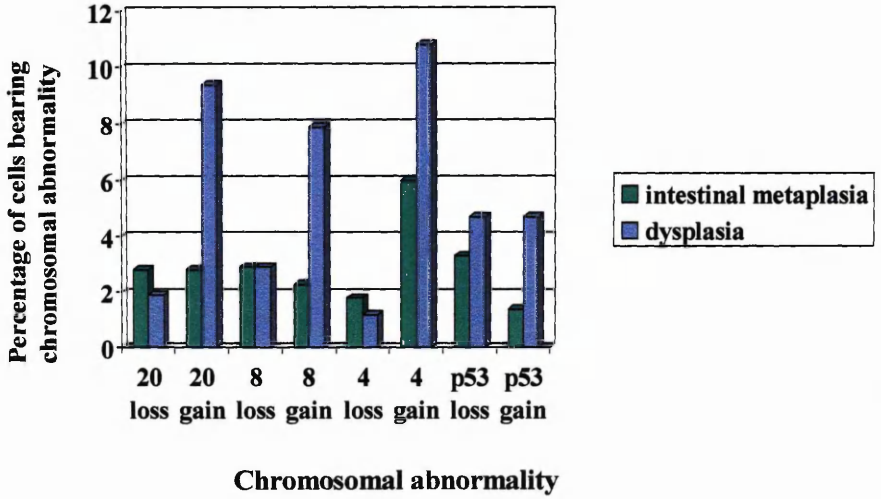
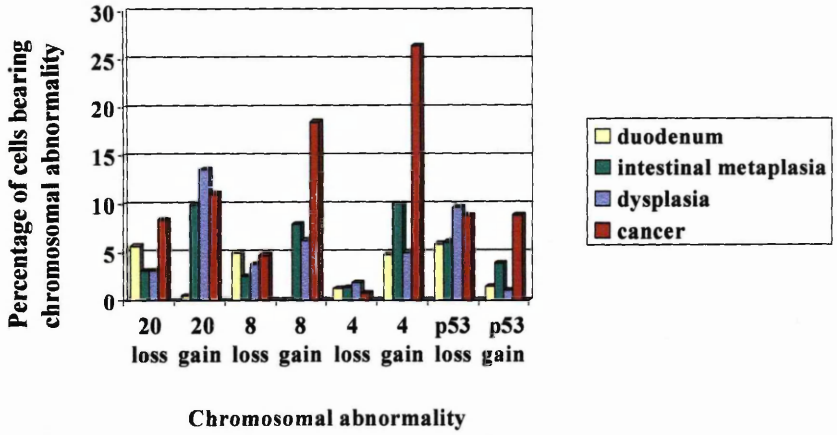


Figure 5.13. Chromosomal abnormalities in surgical resection of patient T3



5.4.3 Intra-tumoural heterogeneity in distal gastric cancer.

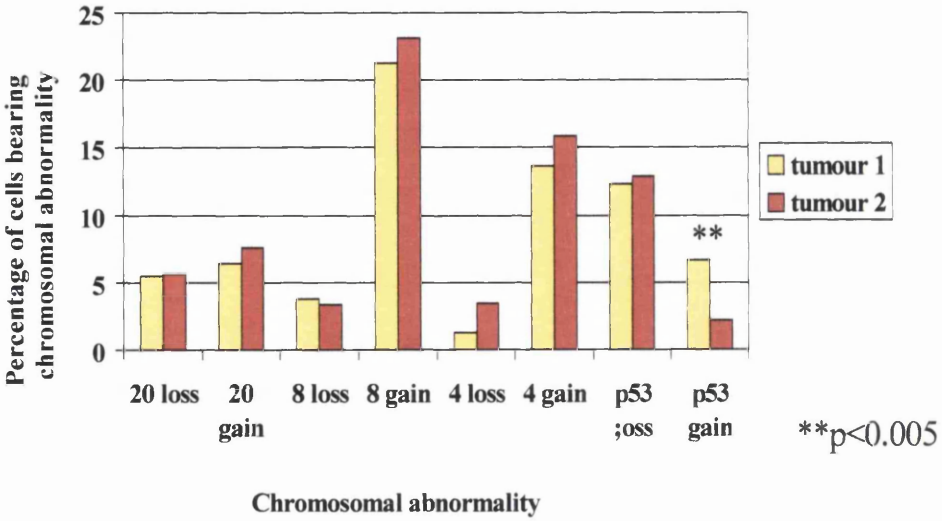
Two of the distal gastric cancer surgical resections analysed had more than one brushing taken from the tumour mass, T1 and T3. Figures 5.14 and 5.15, illustrate the variation of chromosomal aberrations within each tumour. Amplifications and deletions of chromosomes 20, 8, 4 and 17 (p53) are shown. As can be seen T1 had very little diversity within the tumour. A significant difference in the 2 samples was only seen with p53 gain, $p<0.005$. However, T3 showed a greater variation between sites within the tumour, with obvious significant differences occurring in the amplification of chromosomes 20, 8 and 4, $p<0.005$.

5.4.4 Chromosomal aberrations in a gastro-oesophageal cancer, post-chemotherapy.

The resection sample from patient T10 was of a gastro-oesophageal tumour extending into the stomach. Prior to surgery the patient had received chemotherapy (Appendix 1.5) as is the local practice with oesophageal tumours. The tumour mass present at resection was analysed histologically showing little evidence of residual cancer, however metastatic adenocarcinoma was found in associated nodes, suggesting the disease process had not been eradicated fully. Cytology brushes were taken from 4 quadrants of the tumour mass, and from normal gastric mucosa away from the tumour.

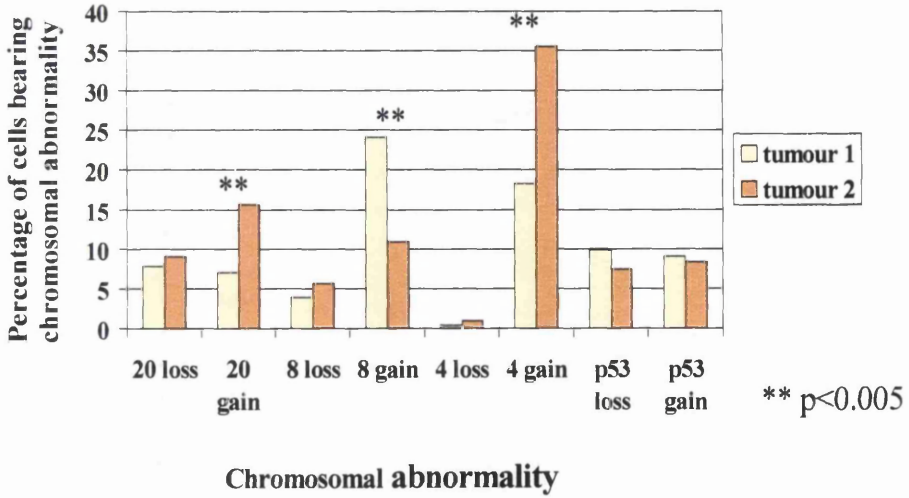
When the tumour cells were compared to normal gastric mucosa in patient T10, significant chromosomal abnormalities were seen in chromosome 20 ($p<0.05$), chromosome 8 ($p<0.005$) and chromosome 4 ($p<0.005$), and in 17(p53) ($p<0.005$), see Figure 5.16. The most frequent abnormalities were as expected chromosome 4 amplification (17%), chromosome 8 amplification (11.6%), p53 deletion (9.4%) and chromosome 20 amplification (7.8%). As few residual cancer cells were found in the

Figure 5.14. Tumour heterogeneity- patient T1



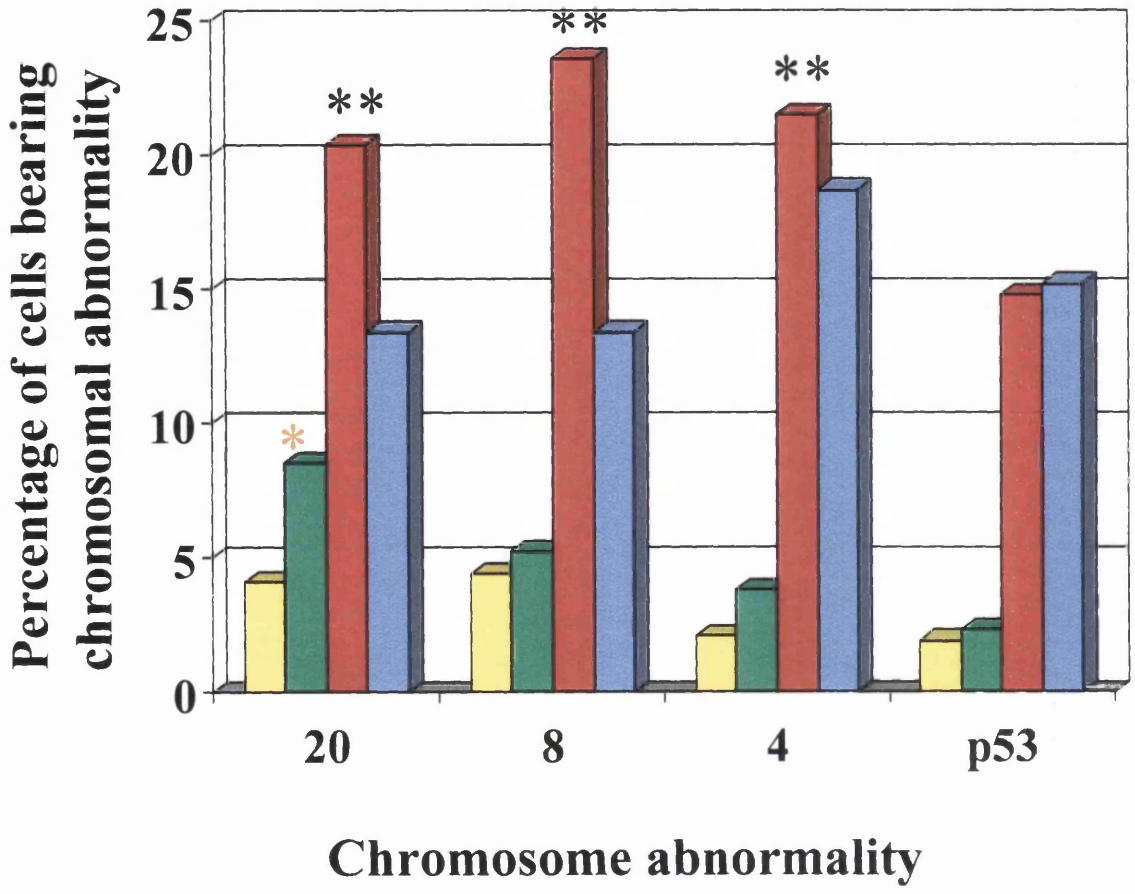
**p<0.005

Figure 5.15. Tumour heterogeneity – patient T3



** p<0.005

Figure 5.16. Comparing chromosomal abnormalities in distal gastric cancer to a gastro-oesophageal junction (GOJ) cancer, post chemotherapy.



**p<0.005 (distal cancer vs GOJ tumour)

*p<0.05 (normal gastric vs GOJ normal gastric)

tumour mass, it was interesting to find such increased levels of chromosomal instability present in the tumour compared to the normal gastric cells. This may possibly reflect the pre-operative chemotherapy the patient had received, and its increased uptake into cancer cells compared to non-malignant cells.

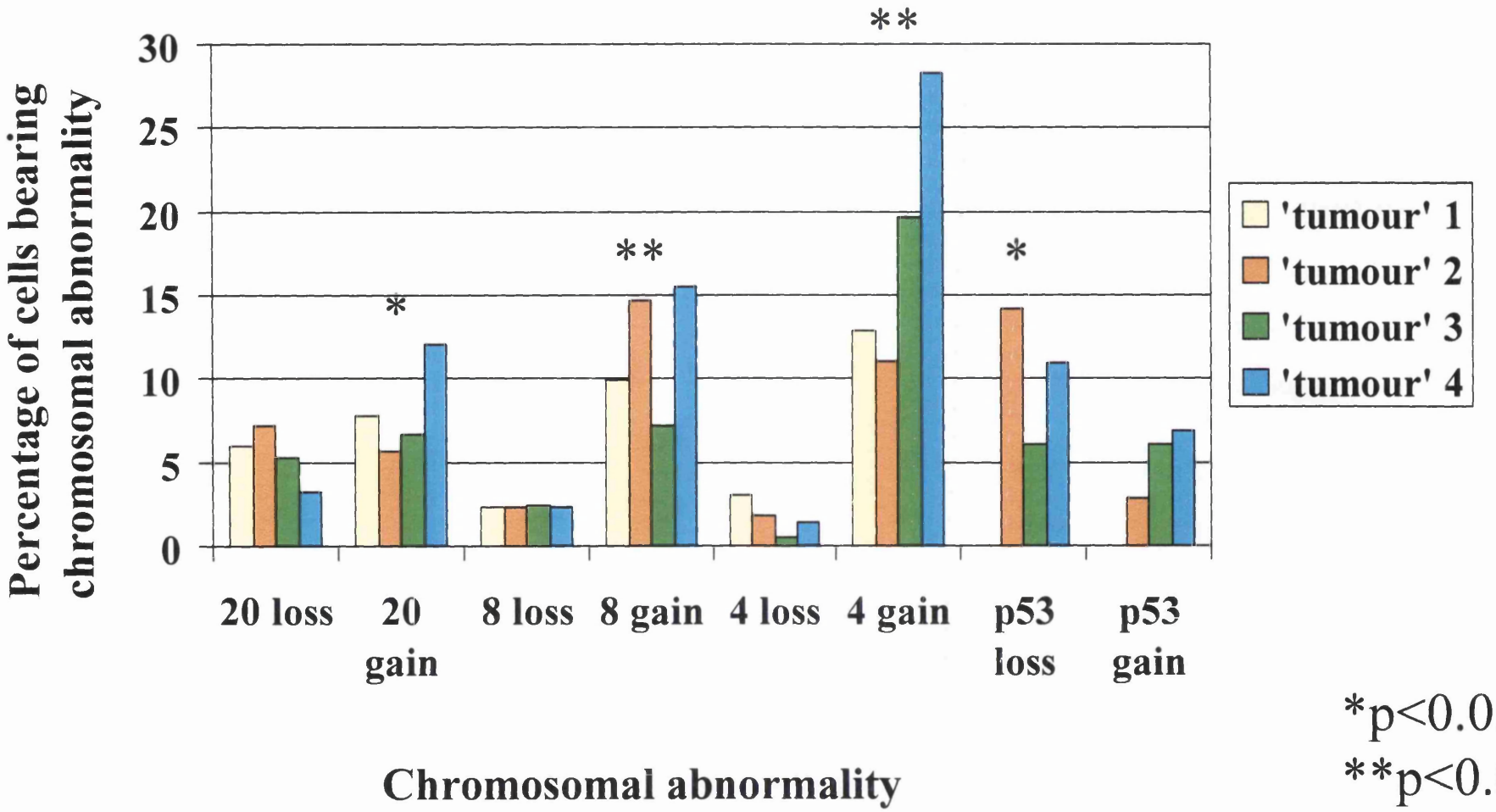
Figure 5.16 also shows a comparison between the chromosomal data from the pooled normal gastric tissue to the normal gastric tissue of T10, away from the tumour. A higher level of chromosomal abnormality was seen in the patient treated with pro-operative chemotherapy, statistical significance was only reached for chromosome 20 abnormalities ($p<0.05$). Again this may well reflect the effects on normal gastric cells of the chemotherapy. The last comparison made was with the data from the pooled distal gastric cancers to the tumour mass at the gastro-oesophageal in T10. As you would expect the distal gastric cancers had more chromosomal abnormalities present, but the order of chromosomal abnormalities was the same, with the most prominent abnormality being amplification of chromosome 4 and deletion of p53.

The level of intra-tumoural heterogeneity was also looked for in this gastro-oesophageal tumour mass. Four quadrantic biopsies were taken and Figure 5.17 shows the results. As can be seen there was a degree of genetic heterogeneity in the tumour mass. A similar trend was observed to that seen for the distal gastric cancer samples already analysed (Figures 5.14 and 5.15) with genetic heterogeneity primarily in chromosomal amplification, $p<0.05$, but also p53 deletion, $p<0.05$.

5.5 Discussion

The aim of the studies described in this chapter was to identify chromosomal abnormalities in gastric cancer resections using Interphase FISH, targeting the

Figure 5.17. Tumour heterogeneity – patient T10.



*p<0.05
**p<0.005

chromosomes found to be abnormal in the pre-malignant tissue studied in Chapter 5 i.e. chromosome 20, 8, 4 and 17 (p53). Aneuploidy of chromosomes 4, 8, 20 and 17(p53) was detected in the gastric cancers studied. Compiling the data from this chapter and Chapter 4, a stepwise elevation of aneuploidy was seen as disease severity progressed from normal gastric mucosa through gastritis and intestinal metaplasia to dysplasia and cancer. Amplification of chromosome 4 and 20, together with deletion of 17(p53) were the most prominent chromosomal abnormalities detected.

This chapter validates the choice of chromosomal probes studied in this thesis, as these chromosomes (20, 8, 4 and 17p53) are indeed progressively involved in gastric cancer. Surprisingly, chromosome 4, which was chosen because of its involvment in the progression of Barretts oesophageal cancer and not gastric cancer, has been shown to be a common event in gastric carcinogenesis

Ten surgical resection samples were studied, and using the brush cytology technique described in chapter 2, FISH was successfully performed. As there are inherent differences between gastro-oesophageal cancers and distal cancers, (as outlined in the introduction), only the nine distal cancers were analysed together. All the cancers were adenocarcinomas as would be expected (Luc 1998). Only one was termed an early cancer, another expected finding, bearing in mind that the bulk of gastric cancers present with symptoms only when the disease is advanced and hence the usual poor prognosis for patients. The majority of cancers in this group were classified as intestinal type gastric cancer. As intestinal type cancers are said to be the endpoint of the Correa pathway of environmental gastric carcinogenesis (Correa 1998, Faraji and Frank 2002), the comparison of the genetic abnormalities found in these tumours to those found in the pre-malignant tissue in chapter 4 was more relevant.

As the chromosomal abnormalities in this patient cohort were compared to the patient group studied in Chapter 4, then ideally a comparison of the patient characteristics should have been made to assess the effect of confounding variables. Unfortunately, in this series of patients only information relating to age and sex was available and not other lifestyle factors, as the patients were not interviewed. The age of the study population was in the 6th decade, generally a decade younger than what would be expected. The mean age for male presentation in Wales is 70.7 years, and 75.2 years in females (www.wcisu.nhs.uk). However it was comparable with the age of patients studied in Chapter 4. The mean age of patients assigned to each histological group i.e. normal gastric tissue, gastritis and intestinal metaplasia, all fell within the 6th decade. Regarding sex, the proportion of women in the study described in this chapter was 50%. Gastric cancer is said to be a predominantly male disease (Faraji and Frank 2002, Sipponen *et al.,* 1998, Grady 2002), and as such this is relatively high percentage. However, the pre-malignant study of patients in the same locality (Chapter 4) only had a male incidence of 33%, therefore the fact the male percentage does increase in the gastric cancer cohort is important. All Wales data shows that Swansea/Neath/Port Talbot area has the highest incidence rate of gastric cancer in women in the principality (www.wcisu.nhs.uk).

Aneuploidy is a common finding in cancer (Lengauer *et al.*, Nardone *et al.*, 1999), and in particular gastric cancer (Beuzen *et al.*, 2000). Specific abnormalities of chromosomes 20, 8 and 4 have been demonstrated using CGH and FISH (see Table 4.1 in Chapter 4). In this study of distal gastric adenocarcinomas in Swansea/Neath, altered copy number of all these chromosomes was found (Figure 5.5). Increased copy number i.e. amplifications, were most often seen, with amplification of chromosome 8 occurring in 20% of the cells studied. As has been discussed in Chapter 4, this

chromosome houses the oncogene c-myc, which is a proliferative transcription factor. c-myc over-expression has been reported in gastric cancers with a varying frequency, 16-67% (Grady 2001), which is comparable to the amplification levels of chromosome 8 in this study. Chromosome 4 amplification was also a significant finding in gastric tumour cells. Again the importance of chromosome 4 amplification has been discussed in Chapter 4, outlining the favourable characteristics this genetic abnormality offers cancer cells with up-regulation of the protein NFκB. The amplification of chromosome 20, which was a prominent abnormality in this study of gastric tumours, has also been documented frequently in a number of cytogenetic studies of gastric cancers (see Table 4.1 in Chapter 4), and the significance of its effect on further aneuploidy development discussed again in Chapter 4. p53 deletion was also noted to be common in the gastric cancers studied here. The action of p53 and its role as an important tumour suppressor gene has also been discussed in Chapter 1 and Chapter 4. The literature reports it be abnormal in 30-58% of intestinal and diffuse gastric tumours (Grady 2001). Therefore, it would be fair to say that the tumours investigated in this study were typical of those seen elsewhere. In addition, as would be expected on the basis of the previous cytogenetic studies discussed in the introduction, the early gastric cancer in this study demonstrated less genetic instability than the more advanced cancers.

A comparison was made of the chromosomal abnormalities found in the pre-malignant tissues in Chapter 4, to the chromosomal abnormalities found in the gastric cancer samples. Figure 5.7 shows that chromosomal abnormalities are more common in cancer cells compared to that seen in its pre-malignant stages. As the multi-step pathway of carcinogenesis has documented the series of phenotypic changes normal gastric cells undergo before becoming cancer cells (Correa 1988), it is important to

investigate and determine the series of genetic changes that occur at each stage. Hopefully, by identifying the genetic changes that occur early in the pathway and persist throughout the process, more information will be available on what drives the carcinogenic process, and hence eventually determine molecular markers of important diagnostic and prognostic value, as well as determine strategies to halt the disease process and prevent cancer. Chapter 4 identified amplification of chromosome 4 and 20, and deletion of p53, as being significantly elevated in pre-malignant gastric tissue. This study (Figures 5.8, 5.9 and 5.10) confirms that these chromosomal abnormalities not only persist but become more common in both dysplastic and cancer cells. This helps to confirm a role for these abnormalities as a driving force in gastric carcinogenesis. When individual patients were assessed for accumulation of genetic abnormalities in their gastric cells as these cells changed from normal to cancer cells, the same result of a stepwise elevation in aneuploidy was seen (Figures 5.11, 5.12 and 5.13).

Intra-tumoural heterogeneity has been a recognised feature of established gastrointestinal cancers, reflecting the general instability of the tumour, and the development of *de-novo* genetic abnormalities in individual cells. Owonikoko *et al.* 2002, has demonstrated this phenomenon in Barretts associated cancers, using a number of genetic markers, whereas Lindforss *et al.* 2000, has looked at the lower GI tract. In this study separate areas within two distal gastric tumours were analysed. The genetic heterogeneity described in these other studies was seen, with statistically significant differences being identified, particularly the levels of chromosomal amplifications ($p<0.005$). The patient with a gastro-oesophageal tumour and treated with chemotherapy also showed genetic heterogeneity and again, chromosomal amplifications varied most, but p53 deletion was also noted to be heterogeneous

within the tumour. If a tumour shows significant intra-tumoural heterogeneity then the likelihood is that at least one of the various clones of cells present will not be as sensitive to chemotherapy as the other clones. Hence these 'chemoresistant' cells will survive, and the cancer will continue to grow.

The gastro-oesophageal tumour mass analysed showed significant abnormalities in the chromosomes studied, in particular the amplification of chromosomes 4 and 8, and deletion of p53. This is interesting as the histology of this tissue showed little residual cancer. However as has been already established, the patient did receive chemotherapy prior to surgery, possibly suggesting this has a causative role for the genetic instability seen. Both clastogenic and aneugenic chromosomal damage has been reported as occurring post chemotherapy (Acar *et al.*, 2001, Silva *et al.*, 2002, Kamguchi and Tateno 2002, Frias *et al.*, 2003). This idea is supported by the fact the normal gastric cells in this patient had a higher level of genetic abnormality as compared to the levels of chromosomal abnormalities already shown in normal gastric mucosa (Chapter 4). Chemotherapeutic agents are taken up by all cells, but cell death is greater in those cells dividing more actively i.e. cancer cells, hence a greater degree of genetic disruption is seen in the tumour. Also, as metastatic adenocarcinoma was found in adjacent nodal tissue, the original disease process was not fully eradicated with the chemotherapeutic treatment prior to surgery, and this may also help to explain the genetic instability seen.

In summary, the prominent chromosomal abnormalities demonstrated in gastric pre-malignant tissue, amplification of chromosomes 4 and 20, plus the deletion of p53, continued to be a feature of dysplasia and cancer. These abnormalities persisted and increased in frequency as the multi-step pathway to gastric carcinogenesis (Correa 1988) progressed. The early appearance of these abnormalities

and their persistence in cancer cells, strengthens the hypothesis that they have an important role in driving gastric cancer, as discussed in Chapter 4. In the future this may help to target patients who are likely to develop cancer, using molecular markers. Identifying the actual mutations that lead to the aneuploidy demonstrated in early gastric carcinogenesis may even help to create potential therapeutic agents capable of halting the carcinogenic process at its earliest stages.

It would also be important to correlate the genetic abnormalities with potential causative agents for this disease, and this has been done to some extent in Chapter 4, and will be developed further in Chapter 6 and correlated with *Helicobacter pylori* subtype.

Chapter 6

The determination of *Helicobacter pylori* infection in gastric biopsies using Polymerase Chain Reaction technology and correlation with histological diagnosis and chromosomal abnormalities.

In this series of experiments *H. pylori* infection in the gastric biopsies collected at endoscopy was determined using PCR technology. All the patients found to be *H. pylori* positive on histological assessment were also detected using PCR. 43% of *H. pylori* infection diagnosed was found to be Cag A positive. Chromosomal abnormalities were greater in those patients with Cag A positive *H. pylori* infection. Amplification of chromosome 4 may be important in *H. pylori* infection.

6.1 Introduction

The bacterium *Helicobacter pylori* has already been introduced in the opening chapters of this thesis, with details of its incidence, association with gastric cancer, its proposed mechanism of action and its effect on aneuploidy in cells discussed.

6.1.1. Detection of *H. pylori* infection.

H. pylori is a known human pathogen capable of causing many diseases, with its most serious association being that with gastric cancer (Blaser 1998). As such the importance of its diagnosis and hence eradication, whereby a stimulus capable of causing serious disease is removed, is obvious. There are a number of methods available to detect *H. pylori* infection and all have differing sensitivities.

Non-invasive methods include the Urea Breath test. This is based upon the fact that the bacterium produces a urease enzyme. An ingested solution of urea, labelled with carbon 13, is rapidly hydrolysed by the *H. pylori* urease enzyme, and the resulting carbon dioxide is absorbed across the gastric mucosa and via the systemic circulation eventually excreted as expired gas. It detects current infection and therefore is a useful follow up investigation to assess eradication. False negatives can occur with antibiotic and proton pump inhibitor therapy. In children a faecal antigen test is also available as an alternative (O'Connor and Sebastian 2003, Dunn *et al.*, 1997, Logan and Walker 2001).

Serological testing relying upon elevated IgG and IgA antibodies is also possible. ELISA (enzyme linked immunosorbent assay) technology is used to detect those antibodies. Different antigen preparations are available in the commercially available kits, giving reasonable sensitivity. Following the successful treatment of *H. pylori* antibody levels can be slow to fall, and hence the test is not a reliable indicator of eradication or re-infection. This method of *H. pylori* detection identifies 'global' infection i.e. is not reliant on detection of the bacteria at the site of infection, and as such it may be a useful screening test, whereby a negative result would confer a low probability of infection, however a positive test would need further evaluation (Dunn *et al.*, 1997). Further technology to develop a 'fingerprick' method would enhance the benefits of this test clinically, although results are not yet reliable (Logan and Walker 2001).

Invasive detection of *H. pylori* infection by the analysis of a gastric biopsy histologically is probably regarded as the gold standard from a clinical perspective, yet newer technology can now questions this. There are several ways to determine infection from a gastric biopsy. The major downside of all these detection methods is

obviously the need to undergo an upper GI endoscopy to obtain the biopsy, a procedure not without risk or discomfort. Sampling error is a major consideration. Up to 14% of infected patients do not have *H. pylori* evident on the biopsy analysed, especially in the case of gastric atrophy, intestinal metaplasia and bile reflux (Logan and Walker 2001). Also after eradication treatment the bacteria levels can be very low making it difficult to diagnose. Proton pump inhibitors also interfere with the gastric pH and as such modify the *H. pylori* habitat again making detection of bacteria harder. Multiple biopsies from multiple sites should minimize sampling error, however laboratory workload is increased. Histological assessment of the gastric biopsies using modified Giemsa staining and immunohistochemistry can give good pick-up rates for *H. pylori* infection, but is heavily reliant upon histopathologist expertise (Dunn *et al.,* 1997). A method of *H. pylori* detection bypassing the need for laboratory analysis would be a CLO test (Delta West LTD., Bentley, Australia), whereby the urease activity of the bacteria causes a colour change detectable in pre-packaged agar gel of phenol red and urea. The test is readable for up to 24 hours in the Endoscopy Unit and as such is now becoming more popular than the histological analysis of biopsies (Dunn *et al.*, 1997, O'Connor and Sebastian 2003). Detection rates however fall in cases of GI bleeding. Culturing the bacterium has always proved to be time consuming, with problems of contamination and overgrowth. This method does however allow determination of the antibiotic sensitivities, and this would help decrease the numbers of resistant infections which is an emerging problem (Logan and Walker, 2001, Owen 2001). Table 6.1 summarises the sensitivity and specificity of the more popular clinical methods of *H. pylori* detection, giving an indication of the cost and availability.

Table 6.1. *H pylori* detection methods. (Logan and Walker 2001)

Test	Sensitivity	Specificity	Available	Cost
Histology	88-95	90-95	++++	++++
Culture	80-90	95-100	++	+++
Clo	90-95	90-95	++++	+
13Carbon	90-95	90-95	++++	+++
Serology	80-95	80-95	+++	+
Stool antigen	90-95	90-95	++	++

The method used to determine *H. pylori* infection in a gastric biopsy studied in this chapter, is the Polymerase Chain Reaction (PCR). This methodology has the advantage of an amplification step enabling a target gene to be amplified more than 10^6 fold (Saiki et *al.*, 1988), and as such even a single bacterium present in the biopsy could be identified making it up to 100% sensitive, excluding sampling error (Clayton *et al.*, 1992, Dunn *et al.*, 1997, Lage *et al.*, 1995). Choosing the appropriate primer to drive the reaction is obviously important, and a number of genes in the *H. pylori* species have been targeted. In this study flagellin primers have been used which target genes involved in the flagella development, and therefore are specific to bacterium such as *H. pylori* that have flagella. The protocol used in this study was initially optimised in a previous study using patients from the same region (Ishmail 2004). The sensitivity was 100% as compared to histological analysis. In fact, *H. pylori* DNA was found in gastric biopsies where *H. pylori* organisms had failed to be identified histologically. This technique also allows the subtyping of the bacterium (Balaratnum *et al.,* 2001, Ismail *et al.*, 2001, Lage *et al.*, 1995), and in this study, the Cag A status of the bacterium was determined by PCR. The major drawback to this technique has to be the technical expertise needed and therefore the cost. Further work by Ishmail and other authors have shown that the technique can be modified and employed successfully on dental plaque, thereby reducing the need for an invasive endoscopy and possibly representing a screening test that could be used nationwide (Banatvala *et al.*, 1994, Dunn *et al.*, 1997, Ishmail 2004).

6.1.2. The Cag A subtype of *H. pylori* infection.

H. pylori has evolved throughout time leading to the emergence of different strains, capable of occurring in a single host; Cag A is one such subtype. Cag A is a

marker for the cag pathogenicity island (35kb), encoding a high molecular weight protein (128 kDa) (Censini *et al.*, 1996). The actual function of the gene is unclear but its effect on intracellular phosphorylation may be the key to its wide range of effects on gastric cells (Catherton 2000). Cag A positive strains are highly immunogenic strains of *H. pylori* and are associated with increased cytokine expression (interleukin-8) (Crabtree *et al.*, 1995), inflammatory cell infiltrate and release of reactive oxygen species in the gastric tissue (Sipponen *et al.*, 1997, Fiocca *et al.*, 1994). Clinical studies have shown significant association between Cag A positive *H. pylori* infection and the activity of gastritis and the presence of gastric atrophy and intestinal metaplasia (Blaser *et al.*, 1995, Fiocca *et al.*, 1994, Kuipers *et al.*, 1995, Sipponen *et al.*, 1998, Yamaoka *et al.*, 1999). Cag A positive *H. pylori* infection is also associated with increased rates of gastric cancer (Basso *et al.*, 1998, Miehlke *et al.*, 2000, Parsonnet *et al.*, 1997, Rugge *et al.*, 1999). Environmental factors and host factors determine how strong the association is between *H. pylori* and gastric disease (Graham 2000). Thus, these factors contribute to the association between Cag A positivity and gastric carcinoma. A Portuguese group showed that polymorphisms of the human interleukin 1 β gene also affect the virulence of Cag A infection (Figueiredo *et al.*, 2002).

The prevalence of *H. pylori* infection varies geographically and so does that of the Cag A subtype. 60% of all *H. pylori* strains in Europe and the USA are said to be Cag A positive (Dunn *et al.*, 1997). A community based Scandinavian study estimated infection rates of Cag A positive *H. pylori,* from antibody levels in the serum, as 20.4% in 1994 which was significantly lower than 20 years before (Perez-Perez *et al.*, 2002). Cag A infection rates also vary between age groups, and again the

Scandinavian study suggested a relatively higher infection rate in the older population studied (Perez-Perez *et al.*, 2002).

6.1.3 Aims

The aims of the studies described in this chapter were two fold.

Firstly, the study compared the sensitivity of PCR technology with histological analysis in diagnosing *Helicobacter pylori* infection in gastric biopsies. At the same time, the frequency of Cag A positive *H. pylori* infection was also determined, using PCR technology again.

Secondly, a correlation was made between *H. pylori* infection, and subtype, in gastric biopsies with the histological diagnosis and the levels of aneuploidy, which have already been determined for each patient in Chapter 4.

Therefore in this chapter I tested the hypothesis that *H. pylori* infection, particularly with the more virulent Cag A strain, would be associated with greater disease severity and greater chromosomal damage.

6.2 Material and Methods

6.2.1 DNA extraction

Gastric biopsies were taken from the antrum of consenting patients enrolled in the study as outlined in Chapter 2. The biopsies were stored at -70°C until analysis, which was between 1 and 12 months. A high salt DNA extraction kit (Stratagene) was used to extract the DNA. The biopsies were sliced using a scalpel onto glass slides, and the diced tissue then added to 700µl of lysis solution in an eppendorf, on ice. 2.5µl of pronase was added to each eppendorf and the solution inverted. They were

placed on a shaking incubator at 37°C for 16 hours. The samples were then chilled on ice for 10 minutes. 250µl of NaCl was added at room temperature and the tubes inverted x3. They were returned to the ice and a white precipitate of protein was formed in each tube. The tubes were centrifuged at –4° C for 15 minutes at 34000rpm to pellet the protein and cell debris. The clear supernatant containing the DNA was extracted into new tubes and 12µl of RNAase added to each tube. They were then left to incubate at 37°C for 15-30 minutes. Two volumes of iced isopropanol were added to precipitate the DNA. The tubes were gently inverted until a 'hairball' appeared in each tube. The tubes were left at -20°C for 1 hour, and then centrifuged at –4°C for 10 minutes at 13000rpm. The supernatant was removed and 2 washes were performed using 70% iced ethanol with centrifugation for 10 minutes at 13000rpm. The pellets were dried at 37°C for 12 hours. 100µl of water was added to each pellet and the DNA was stored at –4° C until further analysis.

6.2.2 DNA quantitation

A mass spectrophotometer (Beckman DU 530) was used to quantitate the DNA extracted from each biopsy. 4µl of the DNA solution was added to 96µl of water to perform x25 dilution. Absorbance at 260nm was measured, and used to calculate the DNA concentration, given that 1OD unit equated to a DNA concentration of 50 µg/ml. To visualise the extracted DNA and check its integrity, agarose gel electrophoresis was performed.

A 1% agarose solution was made using 100ml of 1xTAE buffer and 1g of agarose (Fluorgen). The solution was heated in a microwave until dissolved. After cooling, the solution was poured into a casting stand and allowed to set for 30 minutes. 15µl of the DNA solution was added to 5µl of DNA loading buffer

containing Syber gold (Molecular probes) and the combined solution added to the gel wells. A marker solution of 10µl water, 5µl Syber gold and 2µl of 1kb DNA ladder (Promega) was also run. 900µl of the 1x TAE buffer was used in the electrophoresis tank. The electrophoresis was run at 110V/70 mA for I hour and the DNA visualised under UV light.

6.2.3 PCR to determine *H. pylori* infection and Cag A status

To avoid contamination in PCR reactions, the experiments were performed in designated laminar flow hoods (GRI). Prior to useage the hoods were treated with UV light for 5 minutes, and ethanol wipes were used to reduce contamination with any residual DNA present. Designated PCR eppendorff tubes, pipettes and filtered tips were also used, to again reduce contamination.

The agents needed for PCR were defrosted at room temperature ten minutes before use. A range of PCR primers have been used to detect *H. pylori* in gastric biopsies successfully (Clayton *et al.*, 1992, Lage *et al.*, 1995). In this series *H. pylori* flagellin primers were used to generate a 94 base pair fragment of the flagellin gene (Chaleshtori 1999, Ishmail 2004), designated Fla Reverse (primer sequences ACGGAAGGCTTTCTCTCACA) and Fla forward (primer sequences AAACCAATCGCTGTGAAACC). The CagA primers were synthesised according to Lage *et al.*, 1995. A 'master mix' solution was made allowing 50µl for each sample tested - 5µl Taq polymerase buffer x10 (Promega), 4µl dNTP's (ATP, GTP, CTP, TTP giving a final concentration 100µM), 1.5mM Mg Cl_2, 0.5µl Taq polymerase, 20pM Fla F and Fla R, 3µl DNA, 32µl water. An additional sample was made up to allow a simultaneous 'negative' sample to be analysed, containing no DNA. The master mix solution was centrifuged for 10 seconds to ensure thorough mixing. The

PCR machine (MJ research) was set at 94°C for 2 minutes and then 94°C for 30 seconds, 60°C for 20 seconds and 72°C for 20 seconds, to allow amplification of the DNA sequence recognised by the chosen primers, 30 cycles were performed (Ishmail 2004).

A 6% acrylamide gel was run to visualise the PCR product for each sample. A marker tube was run with each gel. The marker tube contained 5µl of a 100 base pair DNA ladder (Promega), with equal volumes of water and dye. Positive controls for *H pylori* and Cag A positive *H pylori* were obtained from the previous study by Ishmail 2004.

The gels were stained with silver to allow visualisation of the PCR products produced. Each plate was incubated for 5 minutes in silver solution (0.1% $AgNO_3$), and then washed with water, before being incubated for 5 minutes in developing solution (13.5g NaOH, 5ml formaldehyde, 1l water) to develop the colour reaction. Photographs of the gels were then taken using a gel doc 2000 instrument (BioRad). The presence of the *H. pylori* PCR product was visualised as a discrete dark band of 94base pairs and the CagA product of 400 base pairs.

6.2.4 Statistical analysis of chromosomal damage in patients with and without *H. pylori* infection.

The Fisher's exact test was used to assess statistical significance between the degree of chromosomal abnormalities in patients with and without *H. pylori* infection.

6.3 Results

6.3.1 DNA extraction from gastric biopsies and its quantitation.

Gastric biopsies were taken from 19 patients who attended Neath Port Talbot General Hospital for an upper GI endoscopy and were enrolled in the study as outlined in Chapter 2. One biopsy was taken for histological assessment and a second biopsy was taken in close proximity for PCR analysis of *H. pylori* infection. Both biopsies were taken from the gastric antrum. The results of one patient, patient 8, were not included in the analysis as no corresponding histological sample was available.

The amount of DNA extracted from each sample was determined and tabulated in Table 6.2. Agarose gels were run to visualise the DNA product.

6.3.2 PCR to determine *H. pylori* infection and Cag A status in each patients' gastric biopsy.

Duplicate PCRs were performed for each sample, according to the protocol described by Ishmail 2004 using the flagellin primers to determine *H. pylori* infection. Examples of the PCR gels are shown in Figure 6.1, with the discrete dark band representing *H. pylori* DNA.

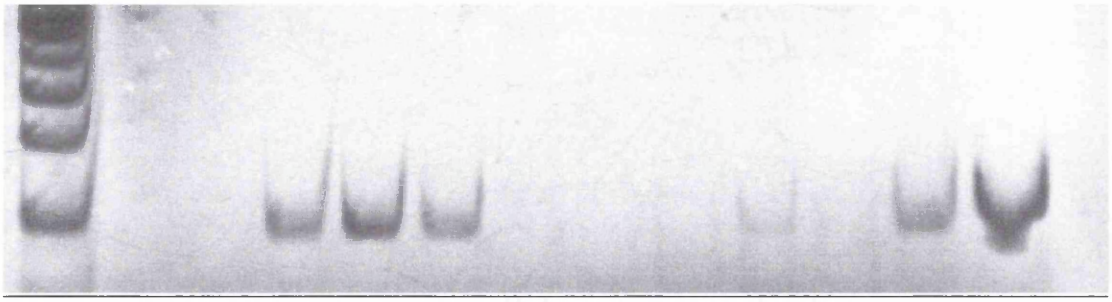
Table 6.3 illustrates those patients with *H. pylori* infection diagnosed using PCR, those with Cag A positive *H. pylori* strains, along with a correlation to histological diagnosis, and *H. pylori* presence on histological slides. All patients with *H. pylori* infection determined using the histological techniques were also identified using the PCR technique. An additional patient, patient 26, had a positive *H. pylori* PCR test. This biopsy contained intestinal metaplasia to which *H. pylori* do not adhere

Table 6.2: DNA values of patient gastric biopsies

Patient	DNA value (μg /ml)
4	25
5	87.5
7	30
9	117.5
10	127.5
11	15
12	22.5
13	67.5
14	35

Patient	DNA value (μg /ml)
15	57.5
16	72.5
17	25
18	30
20	110
22	22.5
23	50
26	50
27	92.5

Figure 6.1: gel photographs to illustrate the *H pylori* infection in gastric biopsies, as determined by PCR (flagellin primers)



Order: marker, 13, 4, 8, 9, patient x, 10, 12, 27, 26, 23, 18, 14

***H pylori* positive – 8, 9, patient x, 26, 18, 14**

(patient x = clinical sample, outside study)

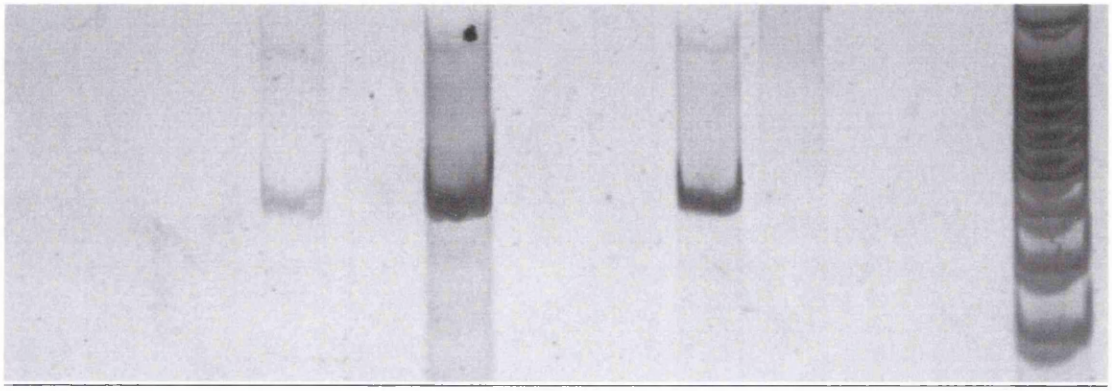
Table 6.3: Correlation of histological diagnosis with *H pylori* status, as determined using PCR with flagellin primers and Cag A primers.

Patient	Histology	*H pylori* histology +	*H pylori* PCR fla +	PCR Cag A +
4	normal	-	-	
5	gastritis	+	+	-
7	gastritis	-	-	
8	no histology	no histology	+	-
9	intestinal metaplasia	+	+	+
10	gastritis	-	-	
11	atrophic gastritis	-	-	
12	normal	-	-	
13	normal	-	-	
14	gastritis	+	+	-
15	normal	-	-	
16	gastritis	+	+	+
17	normal	-	-	
18	gastritis	+	+	-
20	normal	-	-	
22	gastritis	+	+	-
23	gastritis	-	-	
26	intestinal metaplasia	-	+	+
27	intestinal metaplasia	-	-	

(Graham 2000, You *et al.*, 2000). This may well explain why the more sensitive PCR technique demonstrated the *H. pylori* organisms in this biopsy whereas they were not identified on histological assessment. Also *H. pylori* may have been patchily distributed or occur in a coccoid form only recognisable with imunohistochemistry. Seven of the 18 patients who had their gastric biopsies analysed had *H. pylori* infection, 39%. All patients with *H. pylori* infection had abnormal gastric mucosa; gastritis with or without intestinal metaplasia. There was exact correlation between duplicate PCR reactions.

H. pylori organisms with the Cag A pathogenicity island are known to be more virulent, producing more severe pathological infection in humans (Blaser 1998). Again following the protocol described by Ishmail 2004, the Cag A status was determined for each patient with *H. pylori* infection by PCR. Figure 6.2 shows a photograph of a gel identifying those biopsies with Cag A positive *H. pylori* infection. Table 6.3 correlates this result with the histological diagnosis. Three patients were infected with the Cag A strain of *H. pylori*, therefore 43% of the *H. pylori* infections were Cag A positive. Of the three patients found to be Cag A positive two had evidence of the more severe pathological diagnosis associated with *H. pylori*, intestinal metaplasia, with the remaining patient having gastritis. In fact, one of the patients determined Cag A positive, patient 26, was the patient whose *H. pylori* infection was not recognised with histological diagnosis alone, highlighting the importance of using the more sensitive diagnostic tool for *H. pylori* infection.

Figure 6.2: gel photographs to illustrate Cag A positive *H pylori* infections by PCR.



Order: 5, 9, 14, 16, 18, 22, 26

H pylori Cag A positive – 9, 16, 26

6.3.3 Correlation of chromosomal abnormalities with *H. pylori* infection and Cag A status diagnosed using PCR technology.

Figure 6.3 illustrates the differences in the levels of chromosomal damage, as previously determined in Chapter 4 using FISH probes (CEN 20, 8 and 4, and LSI probe p53), between *H. pylori* PCR positive and negative patients. Amplifications of chromosomes 20, 8 and 4 were found more frequently in those infected with *H. pylori*. Amplification of chromosome 4 was found to be significant, $p<0.005$. Deletions of the tumour suppressor locus p53 were also commoner in *H. pylori* infected cells, $p<0.005$, together with deletion of chromosome 20. All of these chromosomal abnormalities have already been suggested to be important in the gastric carcinogenic process, therefore their association with *H. pylori* infection may be important. Chromosome 4 amplification was of particular note.

Figure 6.4 attempts to determine any differences in the levels of aneuploidy for each chromosome studied in those patients infected with the more virulent *H. pylori* infection, Cag A, and those infected with organisms lacking this pathogenicity island. It should be noted that even though numbers were small when the original patient cohort was subdivided this way, three patients versus four patients, a trend could be seen for chromosomal abnormalities to be greater in those infected with the more virulent Cag A strain of *H. pylori* infection. Aneuploidy of chromosomes 8 and 4 were particularly noted, $p<0.005$, with up to 10% of cells infected with CagA positive *H. pylori* being affected. Amplification of chromosome 4, which has already been shown to be prominent in *H. pylori* infected tissue, was again the most frequently observed abnormality in the Cag A patients, affecting 7% of the cells with Cag A infection as compared to 5.9% of Cag A negative *H. pylori* infection (statistical significance was not achieved, $p=0.132$).

Figure 6.3: Chromosomal amplifications and deletions in *H pylori* positive and negative gastric tissue, as detected with PCR Fla primers

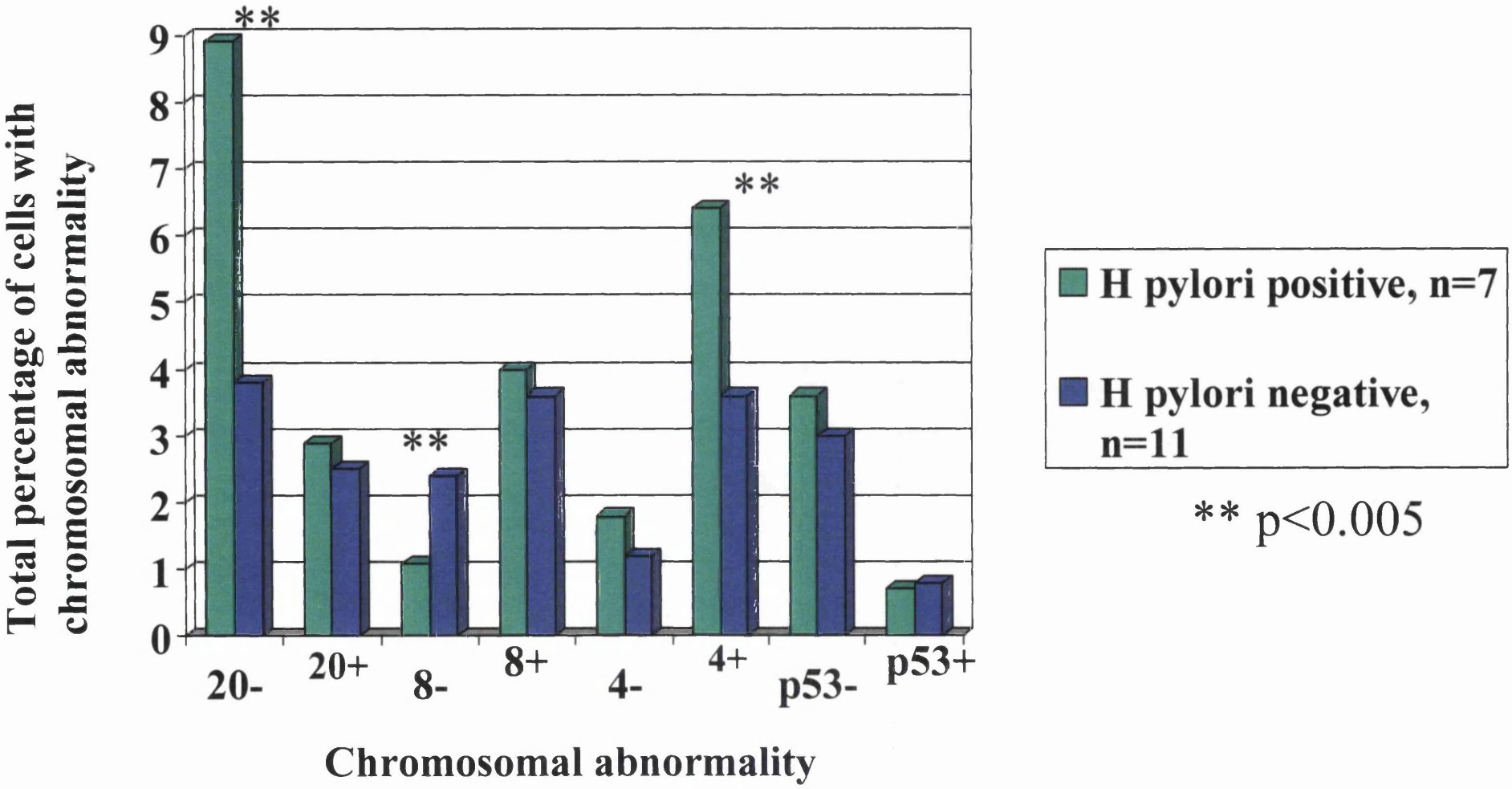
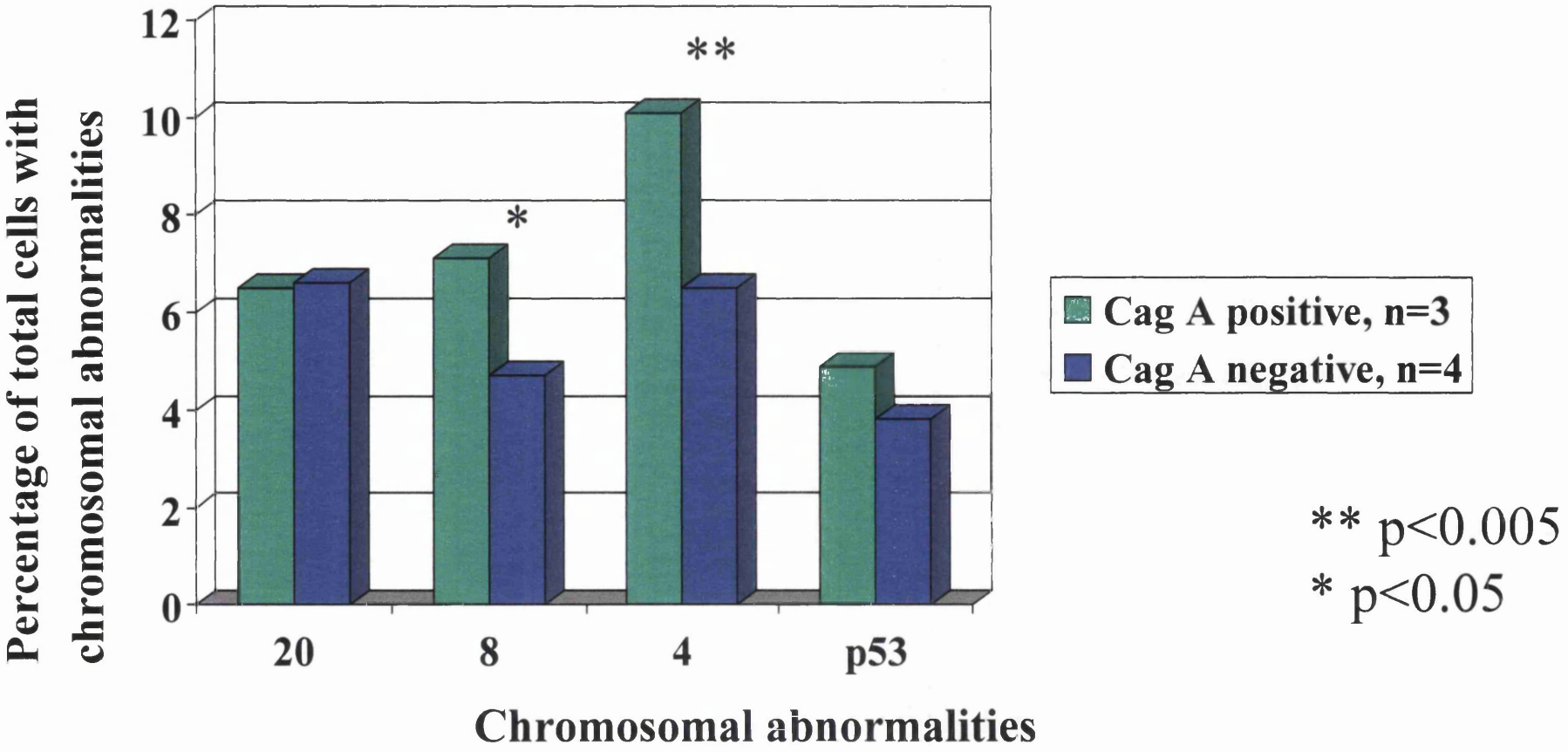


Figure 6.4: Chromosomal abnormalities in PCR determined *H pylori* infected patients, with and without Cag A positivity



** p<0.005

* p<0.05

6.4 Discussion

The experiments in this chapter have shown that PCR detection of *H. pylori* in gastric biopsies is a sensitive method of *H. pylori* detection when compared to the histological analysis of the gastric biopsies. Previous work by a number of groups has supported the accuracy of this method of determination of *H. pylori* infection (Ashton-Key *et al.*, 1996, Clayton *et al.* 1992, Lage *et al.* 1995). Results of this study using gastric biopsies confirms the findings of Ishmail 2004, who previously investigated a different patient cohort in the same locality. In fact, both studies identified patients who had evidence of *H. pylori* DNA in their gastric biopsies hence evidence of bacterial infection, but where the histological analysis had failed to identify the organisms. As already discussed, PCR has the advantage of an amplification step thereby making the detection of very small DNA content possible i.e. a low bacterial load is identifiable.

The clinical relevance of the increased sensitivity of these PCR techniques can be discussed using the information available in this study. The additional patient identified as having *H. pylori* infection in this study was diagnosed as having intestinal metaplasia, a condition known to be a precursor to gastric cancer (Correa 1998). Firstly, *H. pylori* is known to be capable of inducing phenotypic changes in gastric tissue such as intestinal metaplasia (Graham 2000, Kuniyasu *et al.*, 2000, Sipponen *et al.,* 1997), and secondly, it causes genotypic abnormalities (Morgan *et al.*, 2003, Marnett 2004). The mechanism of action of *H. pylori* in inducing cell damage has been discussed in Chapter 1. In this particular patient it would be important to eradicate the bacteria, thereby removing a stimulus for continued DNA damage in a tissue already known to be abnormal and already partway along the multistep path towards gastric cancer (Correa 1998). Previous work has shown that

the successful eradication of *H. pylori* in abnormal gastric tissue can cause a resolution of abnormal phenotypic and genetic changes (Blaser 1998, Nardonne *et al.*, 1999, Walker 2002). Hence, the identification of *H. pylori* in this patient would be clinically important, and may have significant influence on clinical outcome.

As to whether PCR detection of *H. pylori* using gastric biopsies has a practical clinical use would involve a cost analysis and evaluation of the availability of the appropriate expertise. The sensitivity of PCR has been well reported and its reliability in the locality has now been demonstrated in studies performed at different times and with different personnel. A technique worthy of assessment on a larger scale would be *H. pylori* detection using dental plaque. 100% sensitivity has been reported when compared with histological analysis of gastric biopsies (Ismail 2004). As the examination of dental plaque by PCR would be non-invasive, without the need for an upper GI endoscopy, it certainly warrants consideration.

Sampling error has been highlighted in the introduction as a pitfall when relying upon gastric biopsy based techniques to determine *H. pylori* infection. In this study, factors known to be affect sampling error, such as proton pump inhibitor use, were addressed and form part of the exclusion criteria outlined in Chapter 2.2. Patients enrolled in the endoscopic study required 2 sets of cytology brushings and 2 gastric biopsies, as well as whatever was determined appropriate on clinical need. Therefore in an effort not to prolong the endoscopy and put the patients at increased risk of complications I did not take additional biopsies to determine *H. pylori* infection. However, the prevalence of *H. pylori* infection in this cohort of patients undergoing upper GI endoscopy was 39%, and this is comparable to the findings of a similar endoscopy based study from the same locality in which multiple gastric biopsies were analysed for *H. pylori* using PCR. Their prevalence rate of *H. pylori*

infection was 35% (Balaratnam *et al.*, 2001). This infection rate is also comparable to another European study looking at infection rates in patients undergoing endoscopy that found 39% of gastric biopsies had PCR evidence of *H. pylori* infection (Lage *et al.*, 1995). Other studies quote higher rates in patients attending for an endoscopy of 65% (Nardonne *et al.*, 1999) and 72% (Banatvala *et al.*, 1994). Incidence rates vary not only with geography and time difference, as discussed in the introduction, but also with the method used to determine *H. pylori* infection. The incidence rates quoted in the introduction from larger studies tend to be population based and use the non-invasive serological tests.

The utilisation of the PCR technique to detect *H. pylori* organisms in gastric biopsies has also allowed the determination of the *H. pylori* subtype. The identification of the Cag A strain was chosen as it is known to be associated with greater disease severity. The prevalence of the Cag A strain of *H. pylori* in this study was 43%, which is lower than some of the other studies reported but comparable to a local study in which only gastritis patients were analysed, 44% of *H. pylori* infections being Cag A positive (Morgan *et al.*, 2003). Lage *et al.* 1995 documented 80% of the *H. pylori* infections detected in patients undergoing endoscopy were Cag A positive. A Swedish study showed a Cag A positivity rate in their endoscopy patients with *H. pylori* infection of 71% (Maaroos *et al.*, 1999). In 2001 a similar endoscopy study in the Neath Port Talbot area showed that 77% of the *H. pylori* infections were CagA positive (Balaratnum *et al.*, 2001). A significant fall of 25% in the *H. pylori* infection rates in Scandinavia has been reported in the last 20 years (Kosunen *et al.*, 1997), and a similar decline in the frequency of Cag A infection, 26%, has also been reported in larger population based studies, particularly in the younger cohort (Perez-Perez *et al.*, 2002). This could help to explain the 34% difference in prevalence of Cag A infection

seen in the 2 similar studies in Neath Hospital which are separated by 3-4 years. Falls of infection rates in developed countries have been attributed to improved socio-economic conditions and increased antibiotic useage in childhood (Blaser 1999). Reports suggest that the Cag A variant of *H. pylori* is more susceptible to antibiotic treatment than other subtypes (Marais *et al.*, 1998, Van Doornan *et al.,* 1999). This phenomenon, whereby the more virulent bacterial strain is lost first as infection rates decline, has also been observed over time with the Shigella infection in developed countries (Kostrzewski *et al.*, 1968).

In this study all the patients identified with *H. pylori* infection had diseased gastric mucosa, (gastritis or intestinal metaplasia). Three patients had Cag A *H. pylori* infection, two of which had intestinal metaplasia on the gastric biopsy, a more advanced stages of pre-malignant phenotype. As discussed in the introduction Cag A infection is associated with greater inflammation and disease severity. Nardonne *et al.*1999, found that in their patient group high levels of gastric atrophy and intestinal metaplasia were associated with a high prevalence of Cag A *H. pylori* infection. Yamaoka *et al.*, also found an association between the Cag A strain and gastric atrophy and intestinal metaplasia, however they also further subtyped the bacteria and found that different Cag A genotypes conferred different disease severities. Cag A positivity has also been associated with increased rates of gastritis as well as atrophy (Maaroos *et al.*, 1999). Peek *et al.*1999 found that Cag A positive strains of *H. pylori* have a greater effect on the gastric epithelial cell cycle and apoptosis than Cag A negative strains. The effect of the Cag A strain was found to be dependent upon other bacterial characteristics, such as Vac A status, therefore further complicating the association between Cag A infection and disease severity.

Aneuploidy has been shown to exist in gastric tissue, both normal and diseased. Chapters 4 and 5 have shown that levels of chromosomal damage increase with histological progression, peaking as the gastric tissue becomes malignant. Amplifications of chromosomes 4 and 20, together with deletion of p53, have been common abnormalities throughout the gastric carcinogenesis pathway. The frequency of these chromosomal abnormalities has increased stepwise as the phenotype changes from normal gastric mucosa to gastric adencocarcinoma, thereby implicating their importance in driving carcinogenesis. The importance of *H. pylori* as a causative agent for gastric cancer has already been discussed, and in this chapter *H. pylori* infection has been determined using PCR, a more sensitive method of diagnosing *H. pylori* infection, to investigate further the link between *H. pylori* infection and chromosomal abnormalities. For chromosomes 4, 20 and 17(p53) levels of abnormalities were greater in *H. pylori* positive tissue. Abnormalities of p53 have already been identified in *H. pylori* positive infections. Murakami *et al.*1999, has shown that p53 mutations are associated with *H. pylori* infected gastritis tissue. In particular in this study, chromosome 4 amplification was notably increased in infected tissue. The importance of this abnormality has already been discussed in Chapters 4 and 5.

Chapter 4 has already determined that levels of aneuploidy differ between disease severity therefore histology may be a confounding factor in the comparison of aneuploidy in *H. pylori* positive and negative patients. Of the patients found in this chapter to be *H. pylori* positive tissue diagnoses were either gastritis or intestinal metaplasia, whereas half of the patients in the *H. pylori* negative group had normal gastric tissue. Chapter 4 has already established that aneuploidy was a less common finding in normal gastric cells as compared to gastritis/intestinal metaplasia. Chapter 4

analysed gastritis patients only, finding that only abnormalities of chromosome 4 were higher in *H. pylori* infected patients, and that amplification of chromosome 4 in particular was associated with *H. pylori* infection. The link between *H. pylori* infection, an agent known to be causally linked to gastric cancer, and the amplification of chromosome 4 suggests the importance of *H. pylori* as an initiating agent in gastric carcinogenesis, and the amplification of 4 as an early genetic event.

An attempt has been made in this chapter to determine if greater chromosomal damage is seen with infection by the more virulent Cag A positive strain of *H. pylori* infections as compared to Cag A negative infections. Although numbers were small when the data was further subdivided in this way, aneuploidy of chromosomes 4, 8 and 17 (p53) was seen at a higher frequency in those gastric biopsies infected with Cag A positive *H. pylori* infection. Nardonne *et al.*, looked at genetic instability and found that Cag A infection was associated with greater levels of DNA aneuploidy, and p53 and c-myc expression. In this chapter it was possible to highlight amplification of chromosome 4 as a common abnormality associated with Cag A *H. pylori* infection. Chapter 4 has discussed in some detail the potential link between chromosome 4 amplification, NF κB activity, interleukin 8 overexpression and *H. pylori* infection. Cag A infection has already been associated with greater interleukin 8 levels as compared to Cag A negative infection (Crabtree *et al.*, 1995), and therefore the association of chromosomal 4 amplification and Cag A positivity may strengthen the hypothesis that amplification of chromosome 4 is particularly important in gastric carcinogenesis.

In summary, this series of experiments has confirmed that PCR detection of *H. pylori* infection in gastric biopsies is a sensitive and reliable method of *H. pylori* diagnosis, giving the added benefit of enabling the more virulent strain of *H. pylori* to

be identified. The prevalence of Cag A *H. pylori* infection in this patient cohort is in keeping with that recorded in developed countries. The association of increased disease severity and DNA damage with Cag A infection has been shown. The importance of chromosome 4 amplification, which has previously been linked to early gastric carcinogenesis, has also been implicated in *H. pylori* infection especially of the Cag A type.

Chapter 7

General discussion

7.1 A summary of the findings

Helicobacter pylori has been causally linked to gastric cancer (IARC 1994), and much of the evidence to support this statement has been outlined at the start of this thesis. This study has attempted to investigate the hypothesis that *H. pylori* induces oxidative damage, which in turn induces aneuploidy, and that this is important in the initiation and progression of gastric cancer. Initial experiments have shown that Reactive Oxygen Species caused aneuploidy in human cells. The same type of aneuploidy has been demonstrated in pre-malignant gastric tissue and malignant gastric tissue. The level of aneuploidy increased in a stepwise manner as disease severity progressed to gastric cancer. Certain chromosomal abnormalities were notable by their presence in the early stages of carcinogenesis, and continued through to full blown gastric cancer, hence they may play a specific role in the initiation and progression to cancer. Finally, Chapter 6 has confirmed that *H. pylori* can be successfully detected in gastric tissue using PCR technology. This technique has also been employed to distinguish Cag A positive *H. pylori* infection from Cag A negative infection. The detection of this strain is clinically relevant as Cag A *H. pylori* infection is associated with more gastric severe disease (Yamaoka *et al.*, 1999, Sipponen *et al.*, 1998, Fiocca *et al.*, 1994, Blaser *et al.*, 1995, Kuipers *et al.*, 1995), and importantly is also associated with an increased risk of gastric cancer (Parsonnet et al 1998, Miehlke *et al.*, 2000, Rugge *et al.*, 1999, Basso *et al.*, 1998). In this study I wanted to see if patients with Cag A positive *H. pylori* infection had more aneuploidy than Cag A negative infection, but even though the trend was for increased

abnormalities in the Cag A positive group, the numbers analysed were too small to be conclusive.

7.2 Advances in cytogenetic and molecular methodologies

The recent strides in cytogenetic methodologies have meant that chromosomal abnormalities in interphase cells can be investigated in greater detail (Sen 2000), and Fluorescence in Situ Hybridisation (FISH) has been used to detect aneuploidy of specific chromosomes. Table 4.1 in Chapter 4, illustrates a number of studies that have identified a wide range of chromosome abnormalities present in gastric cancers. The determination of chromosomal abnormalities has often proved difficult in solid tumours, but in this study the modification of the technique described by Doak *et al.* 2003 has proved successful at producing a monolayer of interphase gastric cells, which are suitable for FISH analysis. The collection of cells using cytology brushes from both endoscopic tissue and surgical resection tissue, and their preparation with centrifugation/washes and pepsin digestion (as described in Chapter 2), has proved a reliable method of slide preparation. Using this technique it was possible to determine the levels of aneuploidy in gastric, oesophageal and duodenal tissue, and hence this is a useful technique to aid cancer risk assessment.

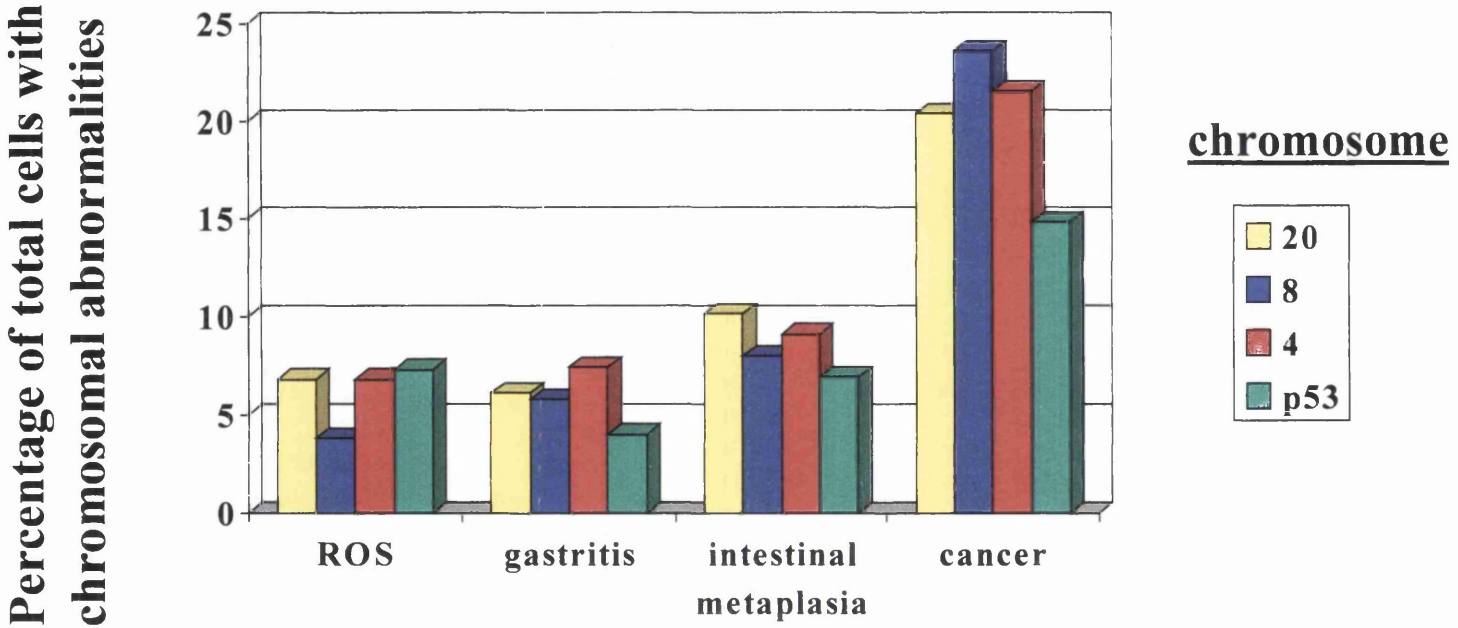
A number of methods are currently available to diagnose *H. pylori* infection, as outlined in chapter 6, and the use of PCR to detect *H. pylori* has been demonstrated in a number of studies (Lage *et al.*, Cayton *et al.*, 1992). Experiments in this study have shown that PCR detection of *H. pylori* infection was at least as sensitive at diagnosing the bacteria as histological assessment. PCR actually detected *H. pylori* DNA in a tissue sample in which was not seen to contain *H. pylori* on histological analysis. This phenomenon has been found in a previous study (Ismail 2004), and

may be due to a low bacterial load, which would not readily be seen on histology but is detectable using PCR due to the amplification step involved (Saiki *et al.*, 1999). Low bacterial load would not be an unexpected finding in the patient in this study who was found to be *H. pylori* positive by PCR alone, as the phenotypic diagnosis was intestinal metaplasia. It is well recognised that *H. pylori* infection is often lost as phenotypic changes occur due to a loss of the optimal habitat for *H. pylori* (Graham 2000, You *et al.*, 2000). The determination of Cag A *H. pylori* infection has been described in previous studies (Lage *et al.*, 1995, Ismail *et al.,* 2001, Balaratnum *et al.*, 2001), and was again successfully performed using the protocol described in Chapter 6. As *H. pylori* is a human carcinogen reliable detection is important, and as more is being learnt about the different strains and their relationship to disease the need to reliably determine bacterial subtype will become more clinically important.

7.3 The importance of ROS and *H. pylori* in gastric disease

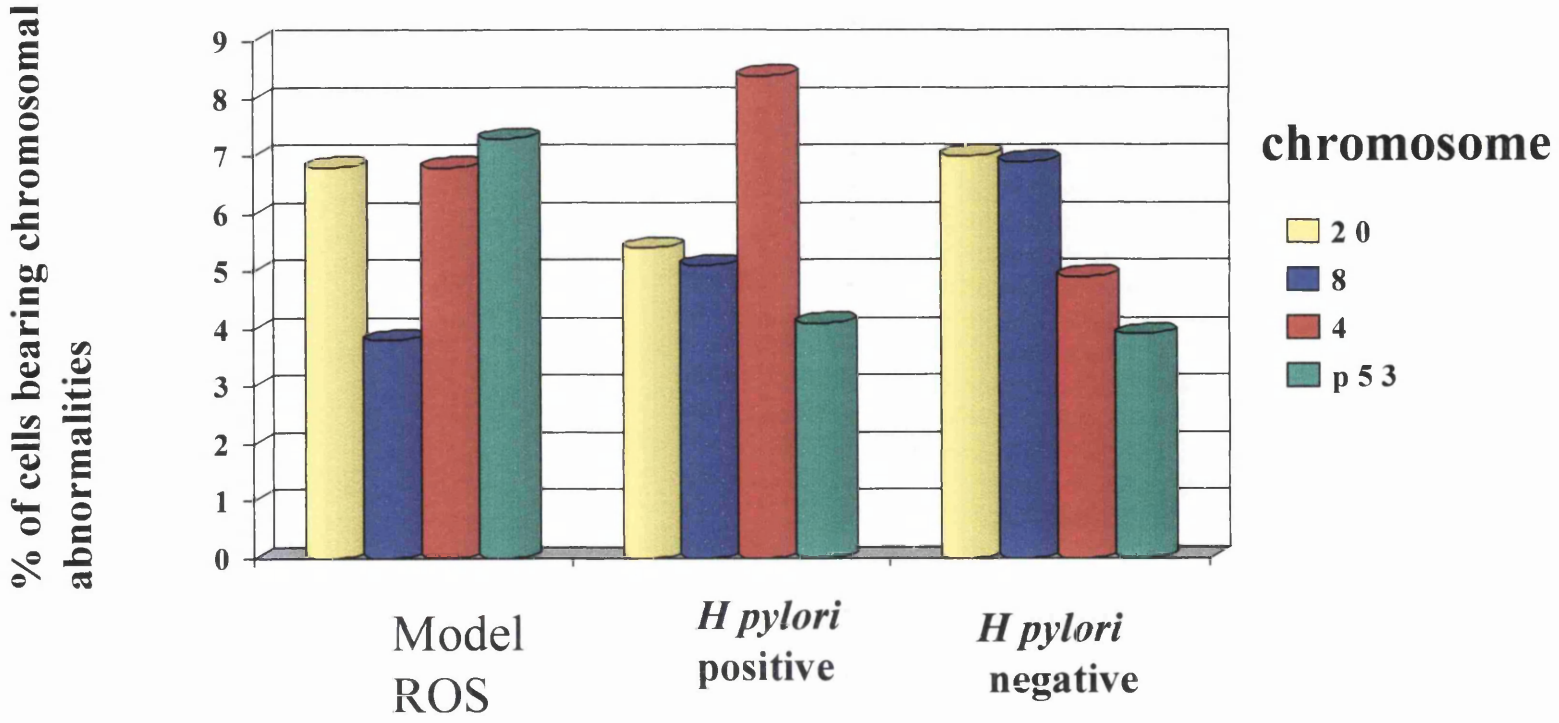
H. pylori is known to cause inflammation and the release of ROS in gastric tissue (Farinatti *et al.*, 1998, Bagchi *et al.,* 1996, Obst *et al.*, 2000). Studies have shown that ROS and oxidative damage are also important factors in gastric cancer development (Correa and Shiao, 1994, Stadtlander *et al.*, 1999). Chapter 3 has described the type of aneuploidy induced by a model ROS, hydrogen peroxide, in a cell line. Hydrogen peroxide was chosen as it has been found *in vivo,* and therefore mimics the actual type of ROS released during tissue inflammation (Fenech *et al.*,1999, Marnett 2000). By comparing the types of chromosomal abnormalities induced by hydrogen peroxide with those found in gastric tissue at different stages of gastric carcinogenesis it was possible to determine any similarities. Figure 7.1 illustrates the type of aneuploidy found in the *in vitro* and *in vivo* studies. As can be

Figure 7.1.A comparison of chromosomal damage in a model ROS study with that in pre-malignant and malignant gastric tissue



seen, chromosomal abnormalities were seen in both groups. The levels of aneuploidy in the model ROS study compared favourably to that seen in gastritis. The actual dose of ROS *in vivo* is unknown, but the dose chosen in this study may represent a chronic inflammatory state as cytotoxicity experiments have shown the dose to be sub-toxic (Jenkins *et al.*, 2001). Gastritis is a highly inflammatory condition possessing high levels of ROS (Morgan 2000, Marnett 2000). Similarities in the levels of aneuploidy of chromosomes 4 and 20 were noted between the model ROS study and the gastritis cells. Interestingly, p53 abnormalities were more predominant in the model ROS. The next step was to compare the chromosomal damage in the model ROS study with that in *H. pylori* positive patients. *H. pylori* infection results in inflammation with a dense infiltrate of neutrophil polymorphs and also high levels of ROS. Figure 7.2 compares the levels of aneuploidy in *H. pylori* gastritis tissue with the model ROS. Generally, the same degree of aneuploidy could be seen in both, however differences were evident. Again, p53 abnormalities were higher in the model ROS study. This difference in levels of p53 abnormality may reflect differences of growth rate in the cell line compared to actual gastric tissue, i.e. cultured cells may select for the p53 abnormality with increased growth rates. The levels of chromosome 4 aneuploidy was similar in both groups but at a higher frequency was seen in gastritis, especially *H. pylori* positive gastritis. Aneuploidy of 20 was also similar between the model ROS study and the *in vivo* study, but this time the similarity was greater between the *H. pylori* negative gastritis cells and the model ROS study. In conclusion, aneuploidy induced by a model ROS is similar to that seen *in vivo* in gastric disease, though not identical. Therefore, the results are inconclusive, and have not proven that *H. pylori* induces oxidative damage through ROS release in gastric disease. This is not surprising when one considers that the ROS released in gastric tissue inflammation

Figure 7.2. Chromosomal abnormalities in *H pylori* positive and negative patients with gastritis, compared to those seen in a model ROS



comprises of a number of different ROS and not merely hydrogen peroxide. Also other toxins, such as pepsin, food and non-*H. pylori* bacteria are in contact with the gastric cells, and hence may also influence chromosome damage.

7.4 Specific cytogenetic abnormalities in gastric carcinogenesis

Certain chromosomal abnormalities have been identified in the experiments with pre-malignant and malignant gastric issue, in particular chromosomes 4 and 20. Figure 4.14 is a modified version of Correa's multistep pathway to gastric cancer, with the addition of chromosomal abnormalities at each stage.

7.4.1 Amplification of chromosome 4

Chromosome 4 was studied in this thesis because of the increasing evidence that has been published demonstrating the importance of chromosome 4 amplification in the progression of another upper GI cancer, Barretts adenocarcinoma (Croft *et al.*, 2002, Doak *et al.*, 2003). The amplification of chromosome 4 was, in this study, the most prevalent abnormality in gastritis and intestinal metaplasia, with significant differences found when compared to normal gastric tissue (Figure 4.6). The amplification persisted with increasing frequency to gastric adenocarcinoma, suggesting it may have an important link with the initiation and development of gastric cancer. Chromosome 4 abnormalities were also highlighted in *H. pylori* patients (Figure 6.4) In Chapter 4, when *H. pylori* positive and negative gastritis patients were compared, chromosome 4 aneuploidy was the only abnormality to be significantly increased in *H. pylori* positive patients. In fact, Figure 4.13 shows that the predominant abnormality was again amplification of chromosome 4. Chapter 4 has discussed in some detail the potential effects amplification of chromosome 4

could have on a cell. Jenkins *et al.* 2003, have linked the amplification of chromosome 4 to the protein Nuclear Factor κB (NFκB). NFκB, present on chromosome 4, has also been linked to gastric cancer (Rayet and Gelinas 1999). NFκB acts as an anti-apoptotic factor and influences the pro-inflammatory action of interleukin-8 (IL-8) (Rayet and Gelinas 1999). The increased copy number of NFκB in a cell could allow continued cell survival with the avoidance of the usual cell regulators, hence favouring tumourigenesis. Selection for the abnormality that causes the increased level of this protein could result in the continued presence of these abnormal cells throughout the disease process. The selection of chromosome 4 amplification in cells has been found in the analysis of aneuploidy in pre-malignant and malignant tissue (Chapter 4), with the increasing presence of chromosome 4 amplification occurring as carcinogenesis progresses. *H. pylori* infection has also been linked to NFκB, in that infected gastric cells have been shown to have an upregulated expression of IL-8, and we know that IL-8 is a transcription factor of NFκB (Jenkins *et al.*, 2003). Hence, *H. pylori* infection may act as a trigger to activate NFκB (Maeda *et al.*, 2001). This would support a mechanistic role for *H. pylori* in gastric carcinogenesis.

7.4.2 Aneuploidy of chromosome 20

Chromosome 20 aneuploidy has been a frequent finding at all stages of gastric carcinogenesis, with amplification of the chromosome being the predominant finding. As both amplification and deletion are common this could therefore be representative of general instability of the tissue, and be a marker of aneuploidy, due to increased cell proliferation. As discussed earlier, Aurora kinase A has been mapped to chromosome 20, and this has been found to be amplified in cancer and cancer cell

lines (Carmen and Earnshaw 2003, Zhou *et al.*, 1988). This protein is one of a family of serine/threonine kinases that have important roles in the regulation of cell division. Aurora A is associated with centrosomes and nearby microtubules, exerting an effect on the separation of centrosomes and microtubules as well as on spindle assembly (Carmena and Earnshaw 2003). Hence, the abnormalities of chromosome 20 may reflect abnormalities of this protein kinase, whose altered function may then affect the development of further aneuploidy.

7.4.3 Deletion of chromosome 17(p53)

p53 deletion was the other significant abnormality that emerged during this thesis. p53 is involved in the control of cell cycle progression and apoptosis in response to DNA damage, and hence loss of its function would lead to cell instability (Grady 2001).This tumour suppressor gene is the most frequently involved gene in human cancers (Levine *et al.*, 1991) and loss of p53 is a frequent finding in gastric cancer (Grady 2001). In this study, loss of p53 was shown to be a frequent abnormality in pre-malignant tissue. Many studies have demonstrated abnormalities of p53 in gastritis (Morgan *et al.*, 2003, Stemmerman *et al.*, 1994), and intestinal metaplasia (Uchino *et al.*, 1993, Shaio *et al.*, 1994) as well as gastric cancer. p53 abnormalities have also been linked to *H. pylori* infection (Murakami *et al.*, 1999). In this study p53 was abnormal slightly more often in *H. pylori* positive patients than in patients without the infection, Figure 4.13 and Figure 6.4.

7.5 Expanding this work – future studies

It would be possible to build on the work described in this study in a number of ways. Firstly, it would be extremely interesting to perform a follow up study in 5

years time and review individual patients found to have high levels of aneuploidy, and assess any phenotypic deterioration or progression of aneuploidy. This would allow assessment of aneuploidy as a predictor of gastric cancer. Also, the patients with Cag A *H. pylori* infection could be assessed to determine if successful eradication of *H. pylori* has occurred, and whether there was a regression of the degree of aneuploidy. Resolution of some genotypic abnormalities has been reported when eradication of *H. pylori* has been successful (Blaser 1998, Nardonne *et al.*, 1999, Walker 2002). Another idea would be to use the remaining gastric DNA from the patients enrolled at endoscopy, after receiving ethical approval of course, and consider investigation into the potential causes for the aneuploidy demonstrated in these patients. Mutations of the mad and bub genes, introduced in Chapter 1 as a potential reason for aneuploidy, may be worthy of investigation. Also further investigation into Aurora A kinase, which has already been discussed in relation to chromosome 20 amplification and aneuploidy development, would be worthwhile. As chromosome 4 amplification was consistently found to be a significant event in gastric carcinogenesis and *H. pylori* infection, the further analysis of the protein NFκB may prove fruitful.

It would also be interesting to expand the *in vitro* study, and attempt to assess the level of aneuploidy induced by exposure to activated neutrophils, hence improve the in vitro modelling of carcinogenesis. It may be possible to identify the types of ROS present.

In the *in vivo* pre-malignant study described in Chapter 4, chromosomal abnormalities were found in normal gastric tissue. If the study was expanded to include a larger patient cohort an age related effect may be seen. Other risk factors were considered in this study but no significant association found, hence for example a more detailed dietary history may be useful. A high female prevalence in gastric

cancer patients has been reported in the Neath/Swansea/Port Talbot area (www.wcisu.nhs.uk). This study has also demonstrated a high percentage of women in the gastric cancers studied which is unexpected as male sex is a known risk factor to gastric cancer, and interestingly female sex was also common in the pre-malignant patients examined in Chapter 4. It would be possible to arrange a data collection study, GP/Endoscopy Units, to determine the numbers of women with *H. pylori* infection, and those who have pre-malignant gastric disease.

The intra-tumoural heterogeneity of some cancers was revealed in this study. As the success of chemotherapy may be reliant on tumours having little clonal variation with fewer surviving resistant clones, a study to assess the effect chemotherapy has on tumours with different levels of heterogeneity may be interesting. Also, the effect specific chromosomal abnormalities have on success of chemotherapy may prove interesting. The techniques in this study to assess aneuploidy could be used for this investigation.

Background levels of aneuploidy have been demonstrated in stomach, and these were not apparent in squamous oesophageal mucosa. It could be that these are related to the microenvironment of the stomach. It would be interesting to analyse tissue from other GI sites, such as duodenal, jejunal, ileal, colonic and rectal to see whether a similar phenomenon exists or if it is peculiar to the stomach. This could be achieved if brushings were taken of fresh surgical resections.

Finally, as the detection of *H. pylori* infection using PCR has again been proved effective, it may be possible to assess the cost effectiveness of undertaking a change in clinical practice in this area. Initially, a study would be required, and possibly it would be more sensible to use a less invasive technique which would not require endoscopy such as PCR of dental plaque, as described by Ishmail 2004.

7.6 Concluding remarks

In conclusion, aneuploidy is important in gastric carcinogenesis. The identification of chromosome 4 amplification and chromosome 20 amplification, in pre-malignant and malignant gastric tissue, may have highlighted specific mechanisms in gastric cancer progression. The development of these findings into clinically useful molecular markers of gastric cancer may come with future studies.

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Appendix 1.1

Research Project looking at the cause of gastric disease.

[Fluorescence in situ (FISH) analysis of *Helicobacter pylori* induced chromosomal aberrations in gastric tissue.]

Lisa Williams, Research Registrar Gastroenterology
Day Ward, Neath General Hospital, Neath
01639 762304

Patient information leaflet
24th October 2001

When you come to the Day Ward for the endoscopy that has been arranged for you, you will be invited to take part in a research study. Before you agree to this, please read this information leaflet and discuss it with friends or family. If anything is unclear and you need to ask any questions please contact us before, or when you attend for the appointment. Please take time to decide if you wish to participate.

Why are we doing this research?
A bacterium is often found in the stomach, that can cause different diseases in the stomach. In our study, we want to look for this bacterium and then, very closely at the cells lining the stomach. Hopefully this will help explain what triggers disease, and improve our ability to diagnose and treat gastric diseases.

All patients attending the Day Ward for this test are being asked to read this leaflet.

It is up to you to decide whether or not you wish to take part in the study. If you do agree then you will be asked questions, by a doctor when you arrive in the hospital, and then asked to sign a consent form and participate. If you are taking certain tablets or have had surgery on your stomach, then we may not ask you to participate. If you do not wish to take part, the endoscopy that has been arranged for you will take place as planned, and your future care will not be affected. If you agree to take part you may change your mind at any time.

If you agree to take part, then it will not involve any additional hospital visits.

When you come to the Day Ward we will need to collect certain details from you. Your age, gender, family history, eating and drinking habits, smoking habits and medication history. All the information we collect will remain confidential. This will take no longer than ten minutes.

During the endoscopy we often need to take small samples of tissue to help us make a diagnosis. This we do with a biopsy, whereby we take very small bites of tissue, and a small brush, which allows us to trap the cells in the bristles. Both procedures take a minute or two and you will be unable to feel them. Taking biopsies carries a very small risk. For this study we will need extra biopsies and brushings, which we can look at closely in the laboratory in University of Wales Swansea. We may also need to gently scrape the inside of your mouth and collect some cells. This again takes only a minute and is not painful. Any samples we take out of the hospital will have had your name removed and you will not be recognised from them.

If you agree to participate then we hope that the information we get will help us understand better the reason why people suffer with stomach diseases, and may help in the treatment of future patients.

There are no special compensation arrangements to anyone taking part in this research project. You are a patient of Bro Morgannwg NHS Trust and as such the NHS complaints mechanisms are available to you if needed.

When the research study stops all the information will be analysed and the results used to improve our knowledge of gastric disease. They may be published in the scientific literature, but you will not be identified.

After the tissue samples have been analysed for this research project they will be incinerated, following the guidelines at the University.

The research study is under the supervision of Professor J Williams and Professor J Parry. Professor Williams is a consultant in Gastroenterology at Neath General hospital and supervises endoscopy lists at the hospital. I am employed as a research registrar by Bro Morgannwg NHS Trust to perform these tests, and to undertake this research project. Professor J Parry is a molecular biologist in University of Wales Swansea and is responsible for the supervision of my work in the laboratory, in accordance with the University guidelines.

This research project has been approved by the Local Research Ethics Committee.

If you need any further information please contact me at the Day Ward, Neath General Hospital, 01639 762304.

Thank you for taking the time to read this leaflet and I look forward to meeting you on the Day Ward when you attend for the test.

Lisa Williams

Appendix 1.2

Fluorescence in situ hybridisation (FISH) analysis of *Helicobacter pylori* induced chromosomal aberrations in gastric tissue

Lisa Williams, Specialist Registrar Gastroenterology

Patient consent form

I have read the patient information leaflet and received a verbal explanation of the proposed research project.

I consent to a buccal scrape and the taking of additional biopsies and brushings during the endoscopic procedure for research purposes.

I agree for information about me to be held but I understand that the information I give will not be communicated to anyone outside the research team.

Patient name:

Patient Signature: Date:

I have supplied the written information on this research project, given a verbal explanation and answered any questions posed.

Investigator name:

Investigator signature: Date:

Appendix 1.3

Fluorescence in situ hybridisation (FISH) analysis of *Helicobacter pylori* induced chromosomal aberrations in gastric tissue

Lisa Williams, Specialist Registrar Gastroenterology

Patient profile

Name:

Date of birth: Sex: Race:

Family history of GI cancer:

Family history peptic ulcer disease:

Smoking history:

Alcohol history:

Dietary habits (salt, fruit, vegetables, meat):

Drug history:

Employment history:

Endoscopic diagnosis (+ location):

Histological diagnosis:

Helicobacter pylori status: [1] histological [2] PCR

Helicobacter pylori strain:

Appendix 1.4

FISH raw data from gastric and oesophageal brushings, chapters 5 and 6.

Brushings from patients enrolled at endoscopy

Patient 1 – very few cells therefore not suitable for FISH

Patient 2

-gastric

Probe	2 signal	1 signal	3 signals
20	123	20	1
8	112	9	5

-oesophageal

Probe	2 signal	1 signal	3 signals
20	127	7	6
8	118	4	2

Patient 3

-gastric

Probe	2 signal	1 signal	3 signal
20	51	7	3
8	55	4	3

-oesophageal

Probe	2 signal	1 signal	3 signal
20	123	13	1
8	123	1	3

Patient 4

-gastric

Probe	2 signal	1 signal	3 signal
20	203	7	0
8	195	3	2

-oesophageal

Probe	2 signal	1 signal	3 signal
20	115	5	0
8	117	1	0

Patient 5

-gastric

Probe	2 signal	1 signal	3 signal
20	212	21	3
8	401	10	18
4	200	1	6
p53	213	7	1

-oesophageal

Probe	2 signal	1 signal	3 signal
20	400	12	0
8	400	5	3
4	280	2	5
p53	220	2	1

Patient 6

-gastric

Probe	2 signal	1 signal	3 signal
20	115	1	0
8	115	2	0

-oesophageal

Probe	2 signal	1 signal	3 signal
20	400	4	1
8	400	1	3

Patient 7

-gastric

Probe	2 signal	1 signal	3 signal
20	330	6	1
8	337	6	2

-oesophageal

Probe	2 signal	1 signal	3 signal
20	336	7	0
8	327	2	1
4	393	2	1
p53	399	4	1

Patient 8

-gastric

Probe	2 signal	1signal	3 signal
20	291	6	3
8	341	11	8
4	400	6	10
p53	400	7	1

-oesophageal

Probe	2 signal	1signal	3 signal
20	280	4	1
8	283	2	4
4	306	2	3
p53	306	2	0

Patient 9

-gastric

Probe	2 signal	1 signal	3 signal	4 signal
20	293	18	0	
8	270	2	4	
4	290	6	8	2
p53	320	5	1	

-oesophageal

Probe	2 signal	1 signal	3 signal
20	198	6	0
8	203	0	1
4	429	0	5
p53	429	2	4

Patient 10

-gastric

Probe	2 signal	1 signal	3 signal	4 signal
20	294	4	13	1
8	290	3	21	1

-oesophageal

Probe	2 signal	1 signal	3 signal
20	100	4	1
8	100	0	4

Patient 11

-gastric

Probe	2 signal	1 signal	3 signal	4 signal
20	246	16	6	
8	323	11	16	
4	403	4	6	1
p53	236	10	0	

-oesophageal

Probe	2 signal	1 signal	3 signal
20	76	4	0
8	131	2	2
4	260	2	0
p53	260	0	1

Patient 12

-gastric

Probe	2 signal	1 signal	3 signal
20	239	10	2
8	248	3	6

-oesophageal

Probe	2 signal	1 signal	3 signal
20	239	5	2
8	248	1	4
4	251	2	1
p53	251	1	1

Patient 13

-gastric

Probe	2 signal	1 signal	3 signal
20	330	19	0
8	392	0	19

-oesophageal

Probe	2 signal	1 signal	3 signal
20	50	0	0
8	50	0	0

Patient 14

-gastric

Probe	2 signal	1 signal	3 signal	3 signal
20	267	9	12	
8	356	0	21	7
4	408	3	18	2
p53	410	10	3	

-oesophageal

Probe	2 signal	1 signal	3 signal
20	196	4	0
8	215	1	4
4	200	1	1
p53	210	1	1

Patient 15

-gastric

Probe	2 signal	1 signal	3 signal	4 signal
20	354	6	7	
8	358	4	19	6
4	380	1	4	
p53	387	2	2	

-oesophageal

Probe	2 signal	1 signal	3 signal
20	320	0	1
8	318	0	6
4	300	1	0
p53	300	2	2

Patient 16

-gastric

Probe	2 signal	1 signal	3 signal	4+ signal
20	403	5	1	
8	396	3	11	13
4	400	0	39	2
p53	400	19	1	

-oesophageal

Probe	2 signal	1 signal	3 signal
20	271	4	1
8	271	2	2
4	100	1	0

Patient 17

-gastric

Probe	2 signal	1 signal	3 signal	4 signal
20	281	10	0	
8	385	8	10	2
4	413	4	6	
p53	290	3	1	

-oesophageal

Probe	2 signal	1 signal	3 signal
20	366	19	2
8	366	5	3
4	216	4	4
p53	200	2	0

Patient 18

-gastric

Probe	2 signal	1 signal	3 signal	4 signal
20	437	13	2	
8	443	2	3	
4	385	0	26	2
p53	411	18	0	

-oesophageal

Probe	2 signal	1 signal	3 signal
20	400	3	10
8	400	1	4
4	405	0	9
p53	375	6	0

Patient 19

-gastric

Probe	2 signal	1 signal	3 signal
20	96	10	1
8	101	2	0

-oesophageal

Probe	2 signal	1 signal	3 signal
20	400	10	0
8	400	3	1

Patient 20

-gastric

Probe	2 signal	1 signal	3 signal
20	407	7	3
8	407	5	8
4	345	2	1
p53	345	4	0

-oesophageal

Probe	2 signal	1 signal	3 signal
20	75	1	0
8	73	0	0
4	200	1	1
p53	200	2	0

Patient 21

Patient 22

-gastric

Probe	2 signal	1 signal	3signal	4 signal
20	185	11	9	
8	231	6	4	
4	180	3	17	3
p53	194	7	3	

-oesophageal

Probe	2 signal	1 signal	3signal
20	196	0	0
8	208	1	3
4	400	1	2
p53	210	3	0

Patient 23

-gastric

Probe	2 signal	1 signal	3 signal	4 signal
20	203	15	5	
8	271	5	6	3
4	202	0	17	3
p53	262	8	3	

-oesophageal

Probe	2 signal	1 signal	3 signal
20	190	3	0
8	202	1	2
4	215	1	1
p53	197	3	0

Patient 24

-gastric

Probe	2 signal	1 signal	3 signal	4 signal
20	195	1	3	
8	174	13	11	1
4	218	8	14	
p53	189	22	5	

Patient 25

Patient 26

-gastric (repeat)

Probe	2 signal	1 signal	3 signal	4 signal
20	297	34	11	
8	214(218)	5(5)	16(19)	1
4	300	8	20	4
p53	216(202)	16(16)	3(6)	2

-oesophageal

Probe	2 signal	1 signal	3 signal	4 signal
20	251	4	0	
8	345	3	1	
4	251	1	1	1
p53	215	3	1	0

Patient 27

-gastric

Probe	2 signal	1 signal	3 signal	4 signal
20	265	20	44	2
8	336	18	8	7
4	297	9	22	3
p53	338	16	8	

- oesophageal

Probe	2 signal	1 signal	3 signal
20	255	5	2
8	300	0	5
4	255	4	7
p53	300	2	5

<u>Surgically resected patients</u>

Patient T1

(See pictures 6.3)

-gastric a1 - cancer

Probe	2 signal	1 signal(0)	3 signal	4 signal
20	193	12	11	3
8	215	11	48	13
4	192	3	21	10
p53	230	33	16	3

-gastric a2 - cancer

Probe	2 signal	1 signal	3 signal	4 signal
20	183	12	10	6
8	239	11	73	2
4	183	8	31	5
p53	276	42	7	

-gastric a4 – intestinal metaplasia

Probe	2 signal	1 signal	3 signal	4 signal
20	191	4	2	
8	239	5	10	4
4	186	2	15	3
p53	191	16	2	

-gastric a6 – intestinal metaplasia

Probe	2 signal	1 signal	3 signal	4 signal
20	213	17	2	
8	307	7	9	2
4	218	2	10	4
p53	213	6	1	

-gastric a9 =- intestinal metaplasia

Probe	2 signal	1 signal	3 signal	4 signal
20	238	14	3	
8	310	6	7	
4	243	5	6	1
p53	300	12	2	

Patient T2

(See pictures – 6.1)

-gastric b2 - dysplasia

Probe	2 signal	1 signal	3 signal	4 signal
20	188	4	17	3
8	214	7	16	3
4	228	3	25	3
p53	195	10	3	7

-gastric b3 – intestinal metaplasia

Probe	2 signal	1 signal	3 signal	4 signal
20	195	7	5	
8	200	8	5	
4	191	1	11	
p53	198	5	4	

-gastric b4 – intestinal metaplasia

Probe	2 signal	1 signal	3 signal	4 signal
20	208	5	6	1
8	128	2	3	
4	208	7	13	2
p53	200	9	2	

Patient T3

(See pictures 6.4)

-gastric c1 - cancer

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	195	18	13	3	
8	203	11	43	16	9
4	182	1	33	8	
p53	213	26	18	6	

-gastric c2 – intestinal metaplasia

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	203	9	13		
8	199	4	20	5	3
4	185	2	39		
p53	227	13	8	2	

-gastric c3 - cancer

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	149	18	21	8	2
8	175	12	4	19	
4	127	2	53	14	4
p53	170	15	14	3	

-gastric c4 – intestinal metaplasia

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	176	2	24	1	
8	197	8	17	2	3
4	179	4	17	3	
p53	197	14	8	2	3

-c5 - duodenum

Probe	2 signal	1 signal	3 signal	4 signal
20	263	16	1	
8	208	11	0	
4	235	3	12	
p53	206	13	3	

-gastric c6 – intestinal metaplasia

Probe	2 signal	1 signal	3 signal	4 signal
20	188	9	18	
8	218	5	1	
4	202	1	12	
p53	215	17	7	

-gastric c7 – intestinal metaplasia

Probe	2 signal	1 signal	3 signal	4 signal
20	186	7	28	2
8	189	5	19	1
4	207	4	10	1
p53	201	13	6	

-gastric c8 – mod dysplastic adenoma

Probe	2 signal	1 signal	3 signal	4 signal
20	186	7	28	2
8	215	9	15	
4	207	4	10	1
p53	186	20	2	

Patient T4

-tumour

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	169	8	50	3	1
8	137	4	54	54	5 1
4	172	0	39	14	2
p53	185	26	4		

-duodenum

Probe	2 signal	1 signal	3 signal
20	230	2	5
8	241	11	3
4	227	3	5
p53	268	3	1

Patient T5

-tumour

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	170	11	30	8	
8	248	11	23	4	1
4	199	4	26	10	
p53	192	21	4	1	

-gastric

Probe	2 signal	1 signal	3 signal	4 signal
20	214	14	6	
8	170	10	10	1
4	205	1	21	5
p53	172	7	4	1

Patient T6

-tumour

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	187	10	34	6	
8	162	13	30	16	10
4	169	9	45	18	2
p53	173	24	7	1	

-duodenum

Probe	2 signal	1 signal	3 signal	4 signal
20	114	9	10	2
8	194	9	5	1
4	103	1	23	4
p53	191	8	5	

Patient T7

-tumour (repeat)

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	169	12	20	1	1
8	185	2	17	4	1
4	108(101)	2(4)	28(16)	2	
p53					

-duodenum

Probe	2 signal	1 signal	3 signal
20	200	3	2
8	196	3	3
4	200	3	5
p53	196	3	5

Patient T8

-tumour

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	212	27	29	13	4
8	208	4	25	6	2
4	200	7	20	13	4
p53	203	16	6	1	

-duodenum

probe	2 signal	1 signal	3 signal
20	222	11	5
8	212	5	3
4	284	8	4
p53	222	11	5

Patient T9

-tumour

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	159	16	16	14	7
8	184	5	20	11	2
4	162	7	28	5	3
p53	191	13	17	3	

-duodenum

Probe	2 signal	1 signal	3 signal	4 signal
20	187	7	7	4
8	198	9	3	2
4	124	0	8	1
p53	200	3	6	2

Patient T10

-normal gastric

Probe	2 signal	1 signal	3 signal
20	194	11	7
8	255	5	9
4	205	3	5
p53	214	3	2

-tumour(2)

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	200	14	12	6	
8	227	6	14	4	
4	169	6	16	9	1

-tumour (3)

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	182	15	12		
8	175	5	20	12	1
4	191	4	18	5	1
p53	88	15	3		

-tumour (4)

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	198	12	9	6	
8	212	6	17	6	1
4	158	1	23	15	1
p53	216	15	14	1	

-tumour (5)

Probe	2 signal	1 signal	3 signal	4 signal	5 signal
20	133	5	19		
8	181	5	23	6	3
4	102	2	25	14	2
p53	181	24	12	3	

Appendix 1.5

Welsh Cancer Trial for operable carcinoma of oesophagus – chemotherapeutic regime (abbreviated version)

Induction chemotherapy (weeks 0-6)

Paclitaxel 125mg/m^2 3 hours day 1
Cisplatin 60mg/m^2 days 1
5FU 200mg/m^2/day 1-21

(pre-medication – dexamethasone, chlorpheniramine)
(Antiemetics)
(Hydration)

repeated every 3 weeks for 2 cycles

Concurrent chemotherapy (weeks 7-11)

Paclitaxel 40mg/m^2 day1
Cisplatin 30mg/m^2 day 1
5FU 200mg/m/day/m^2 days 1-35

(pre-medication / antiemetics / hydration)

repeated every 1week for 5 cycles

Radiotherapy – 45Gy/25F/5 weeks

Radical oesophagectomy (weeks 16-18)

Appendix 1.6

Publications/abstracts from this work to date

Fluoresence in situ hybridisation (FISH) of *Helicobacter pylori* induced chromosomal abnormalities in gastric tissue.
L Williams, JG Williams, E Parry, JM Parry. Mutagenesis 2002; 17(6): 565.

Interphase Fluoresence in situ hybridisation (FISH) to detect chromosomal abnormalities in gastric cancer progression.
L Williams, JG Williams, AP Griffiths, T Brown, S Doak, GSJ Jenkins, E Parry, JM Parry. Gut 2003; 52: A 50.