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**Fluorescence *in situ* Hybridisation (FISH) analysis of
Chromosome 1 and gene expression levels of MAD2
and BUB1 levels in premalignant stages of gastric
tissue**

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**A thesis submitted in
partial fulfilment of the requirements for the degree of MD
Institute of Life sciences, School of Medicine, University of
Swansea.**

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Summary

Gastric cancer is a common cause of cancer death in the world. The mortality rate from gastric cancer is high in UK as it often presents late, often with local or distant metastasis. This makes the treatment options limited. The pathogenesis of gastric cancer occurs in a multistep pathway with pre cancerous conditions leading to cancer eventually. It is important to understand this carcinogenic process (aneuploidy and abnormal gene expression levels) and the driving forces (eg. *Helicobacter Pylori* infection) which will enable us to alter the disease outcome.

This series of experiment included cytogenetic investigation which involved obtaining gastric cells using brush cytology and using Fluorescent *insitu* hybridisation technique to look for aneuploidy levels of chromosome 1 and 4. These two chromosomes were chosen as chromosome 1 has been recently shown to be abnormal in early in premalignant stages of gastric cancer. Chromosome 4 was chosen as hyperploidy of chromosome 4 was the predominant chromosomal aberration in Barrett's oesophagus. This study has shown that the aneuploidy level of chromosome 1 progressively increased with the progression of the histological stages according to the Correa's premalignant gastric cancer pathway. Significant increase in aneuploidy levels of chromosome 1 was seen in *H. Pylori* associated gastritis, implying that *H. Pylori* play a very important role in the progression of the disease.

Aneuploidy can occur due to various genetic defects that may potentially occur during mitosis. Spindle cell check points play a vital role in preventing the cells from proceeding to the anaphase stage if there is any defect in the kinetochore attachment. Certain genes like MAD2 and BUB1 are thought to be instrumental in controlling the spindle cell check points and it is believed a steady state of genes like MAD2 and BUB1 are required for this. In the second part of this study, the MAD2 and BUB1 expression levels were measured and correlated to the aneuploidy stages. There was no significant difference in their expression levels in patients with significant aneuploidy level. MAD2 levels were increased in *H.Pylori* associated gastritis, which implies that *H. Pylori* plays an important role in the pathogenesis of gastric cancer.

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List of abbreviations

APC- Adenomas Polyposis Coli
APC/C- Anaphase promoting complex/cyclosome
BUB- Budding Uninhibited Benzimidazole
cDNA – Complementary DNA
CDC20 – Cell division cycle protein 20
CDK1 – Cyclin dependent kinase1
CENP – Centromere protein E
CIN- Chromosomal instability
CLO – Campylobacter like organism
CML –Chronic myeloid leukemia
DAPI - 4'6- diamidino-2- phenylindole
DNA – Deoxynucleic acid
DNMT- DNA methyl transferase
EDTA – Ethylenediaminetetraacetic acid
ELISA – Enzyme linked immunosorbent assay
FISH – Fluorescent *insitu* hybridization
HNPCC- Hereditary non polyposis colorectal cancer
HRT- Hormone replacement therapy
IARC- International association of research council
ISH- *Insitu* hybridization
INCENP- Inner centromere protein
MALT- Mucosal associated lymphoid tissue
MAD- Mitotic arrest deficient
mRNA- Messenger RNA
NSAID- Non steroidal anti-inflammatory drugs

PPI- Protein pump inhibitor

RT- Reverse transcriptase

RT-PCR- Reverse transcriptase polymerase chain reaction

RNP-Ribonuclear protein

RNA- Ribonucleic acid

CHAPTER 1

GENERAL INTRODUCTION

1.1 Gastric cancer

Gastric cancer is one of the leading causes of cancer-related deaths worldwide (Parkin et al 2005) and is the fourth most common cause of death in Europe (Ferlay et al 2010) as it is often diagnosed at an advanced stage when patients present with symptoms like abdominal pain, vomiting, weight loss or gastrointestinal bleeding or anaemia. When diagnosed at an advanced stage, it is usually incurable. The effectiveness of treatment regimen like surgery, chemotherapy and radiotherapy are limited by advanced local disease and metastatic spread. In fact, only 55-65% of gastric cancers are surgically amenable to resection at diagnosis (Keighley 2003). While the 5 year survival of patients with advanced gastric cancer is only approximately 20%, early tumour resection can achieve 5 year survival rates of 90% (Karpeh et al 2001). In the United Kingdom, the 5 year survival rate from gastric cancer has tripled but is low at 15%. In the United Kingdom, most patients are diagnosed with advanced disease often with lymph node metastasis; their survival rate is less than 5% (Cancer Research UK 2010). Other countries in Europe have similar 5 year survival rates, but the rates have increased in Spain and Austria (Keighley 2003). However, very good results can be achieved if the disease is diagnosed at an earlier stage (small tumours of less than 5cms, no serosal invasion or lymph node metastasis). The 5 year survival rate of patients diagnosed with earlier disease is greater than 80% and therefore the challenge is to increase the patients diagnosed with early disease (Cancer Research UK 2010).

In Japan, there is high disease prevalence and also a surveillance program to detect early cancers, their 5 year survival rate is more than 90% (Bowles, Benjamin 2001).

1.2 Cellular composition of gastric and oesophageal mucosa

1.2.1 Oesophagus – Anatomy and Histology

The oesophagus acts as a conduit for the transport of food from the mouth to the stomach. It is 18-26cms muscular hollow tube with an inner skin like lining of stratified squamous epithelium. Structurally, it consists of innermost mucosa, submucosa, muscularis propria and outermost adventia. On Endoscopy, the mucosa appears smooth and pink. The oesophagogastric junction can be recognized by the presence of an irregular Z line, demarcating the difference between the light oesophageal mucosa and the dark red gastric mucosa. This multilayered epithelium consists of three functionally distinct layers: stratum corneum, stratum spinosum, and stratum germinativum. The most lumen oriented stratum corneum acts as a permeability barrier between the lumen content and blood. The middle layer of stratum spinosum consists mainly of metabolically active spiny cells. This spiny shape is due to the numerous desmosomes connecting cells throughout the layer. This desmosomal network maintains the structural integrity of the tissue. The basal layers of stratum germinatum contain cuboidal cells that occupy 10% to 15% of the epithelium's thickness and are uniquely capable of replication. The oesophageal epithelium contains a number of other cell types including enteroendocrine cells, melanocytes, lymphocytes, Langerhans cells (macrophages), and eosinophils.

Below the epithelium is the lamina propria, a loose network of connective tissue within which blood vessels and scattered lymphocytes, macrophages, and plasma cells are present. The lamina propria protrudes into the epithelium to form dermal papillae. Normally, this protrudes to less than 50% of the thickness of the epithelium; when greater, it is a recognized marker of gastro oesophageal reflux. The muscularis mucosa is a thin layer of smooth muscle that separates the lamina propria above from the submucosa.

The submucosa comprises dense network of connective tissue within which are blood vessels, lymphatic channels, Meissner's plexus, and oesophageal glands. The oesophageal glands produce and secrete a lubricant, mucus, and factors such as

bicarbonate and epidermal growth factor that are important for epithelial defense and repair.

The muscularis propria is responsible for carrying out transport function. The upper 5% to 33% are composed of exclusively of skeletal muscle and the distal 33% are composed of smooth muscle. In between there is a mixture of both skeletal and smooth muscle fibres (fig 1.1).

Figure 1.1 Histology of the oesophagus

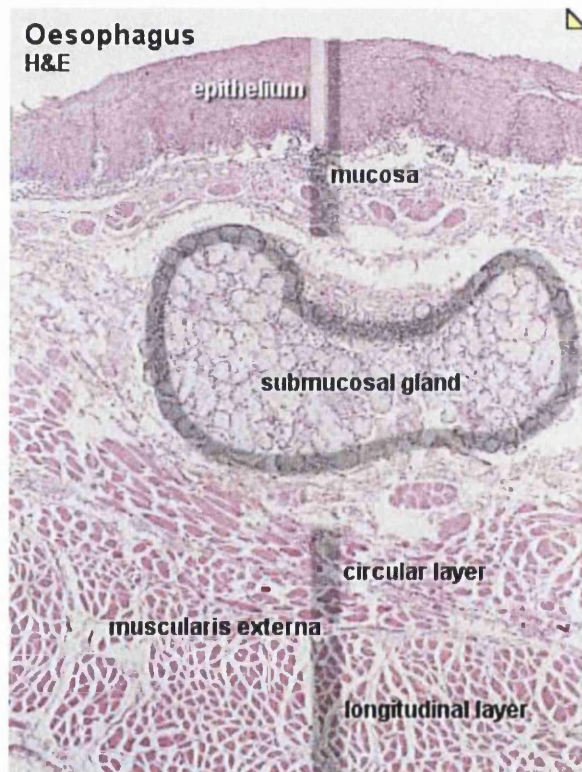


Figure 1.1. shows the innermost lining of the oesophagus, which is made up of squamous epithelium. Below this are mucosa, submucosa and the muscular layer. The muscular layer consists of circular and longitudinal muscle fibres.

www.lecannbiculteur.free.fr

1.2.2 Stomach – Anatomy and cellular composition

The stomach is a J shaped most distensible organ in the body. It receives food mixed with saliva and softened by saliva and acts as a reservoir to store large quantities of recently ingested food. The stomach volume ranges from about 30mL in the newborn to 1.5 to 2.0L in adults. The stomach is divided into four regions, which can be defined anatomical or histological landmarks. The cardia of the stomach is the small, ill defined area of the stomach immediately adjacent to the gastro oesophageal. The cardia is the most fixed region of the stomach. The fundus projects upward, above the cardia and the gastro oesophageal junction. The dome shaped area of the stomach is the most superior portion of the stomach. The body, or the corpus, is located immediately below and is continuous with the fundus. The incisura angularis, a fixed sharp indentation two thirds of the distance down the lesser curve, marks the lower end of the gastric body. The gastric antrum extends from the indistinct border with the body to the junction of pylorus with the duodenum. The pylorus is a tubular structure joining the duodenum to the stomach and contains circular muscle fibres, the pyloric sphincter.

The luminal surface of the gastric wall forms thick, longitudinally oriented folds or rugae, which flatten on distension. The gastric wall mucosa has four layers: mucosa, submucosa, muscularis mucosa, and serosa. Mucosa lines the gastric lumen as a smooth velvety lining. The mucosa of the cardia, antrum, and pylorus are somewhat paler than that of the fundus and the body. The functional secretory elements are located within the mucosal layer. The submucosa, which is located just beneath the mucosa contains the collagen and elastic fibres which forms the connective tissue skeleton. The submucosal layer also contains lymphocytes, plasma cells, arterioles, venules and lymphatics. The third layer is muscularis mucosa consists of: inner oblique muscle, middle circular fibres, and outer longitudinal fibres. The final layer of the stomach is the transparent serosa, a continuation of the viseral peritoneum (Figure 1.2)

Figure 1.2 Anatomy of the stomach

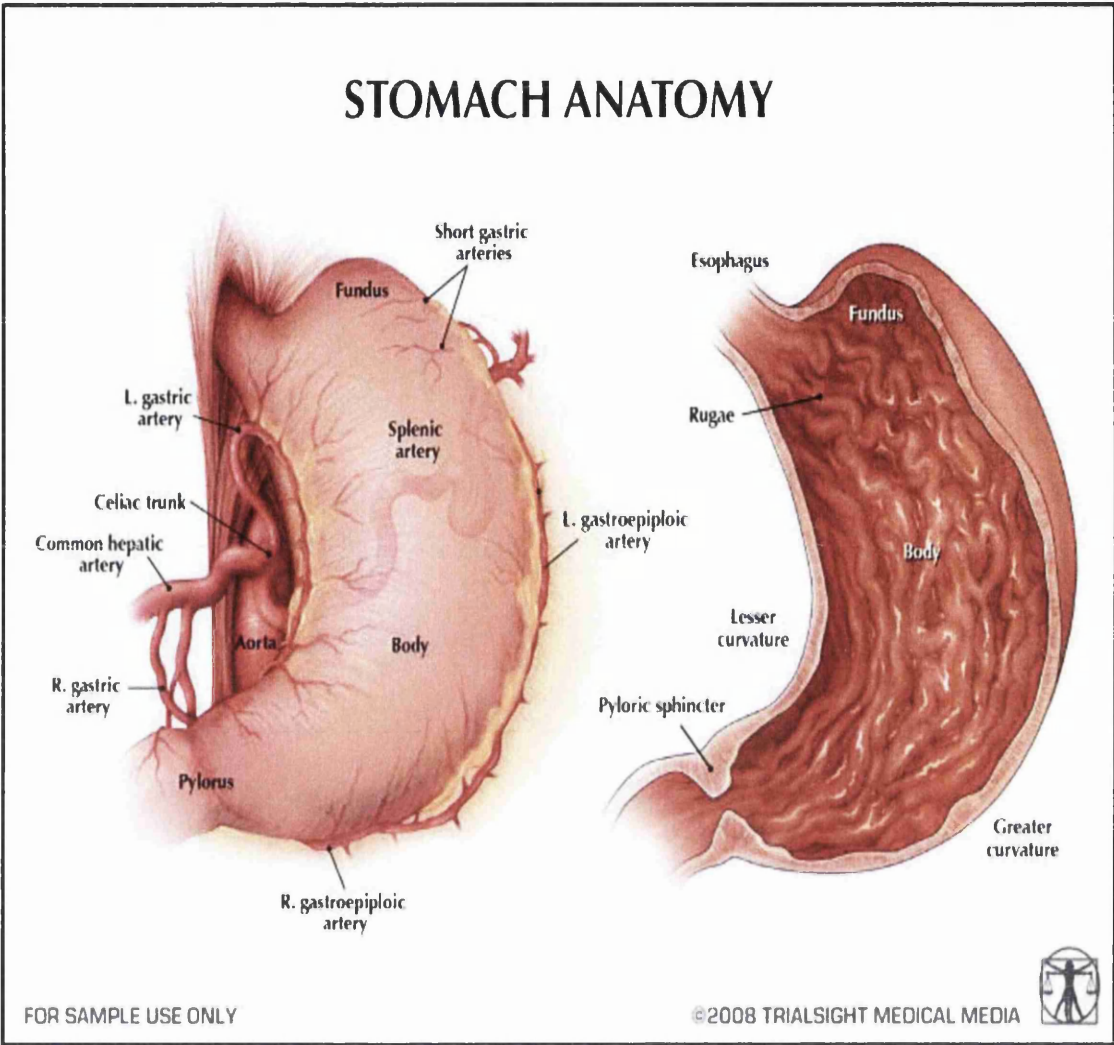


Figure 1.2 shows the internal and external appearance of the stomach along with its blood supply. www.trialsightmedia.com

1.3. Incidence of Gastric cancer

Gastric cancer is regarded as the fourth most common cancer worldwide and there are 934,000 (8.4% of all cancers) incident cases worldwide in the world each year (Parkin et al. 2005). The incidence of gastric varies depending on the geographical location. Almost two thirds of the cases occur in developing world. In China, gastric cancer is the commonest cancer diagnosed and account for 38 % of gastric cancer worldwide (Parkin et al 2005). It is characterised by wide international variation – the high risk areas are East Asia (China/Japan), Eastern Europe, part of South and Central America and the low risk areas are Southern Asia, North America, North and East Africa. In the United Kingdom, the crude incidence rate per 100,000 Population is 12.7% (Cancer Research UK 2010). In the United Kingdom, the incidence of gastric cancers increases with age with less than 8% of cases detected before 55years but the incidence increases steeply with age.

Gastric cancer is one of the main causes of death associated with cancers worldwide and is responsible for 699,000 deaths (Parkin et al 2005).In the United Kingdom, gastric cancers are the seventh most common cause of cancer deaths and the mortality rates have fallen by around 70% over the last 30 years. Throughout the world the gastric cancer mortality has been falling over the last few decades. This has happened at different time period in different countries.

In the United Kingdom and the USA, the mortality rate started to decline in 1930's while in Japan the rates were continuing to rise until 1950's and then began to decline. In Poland and other European countries the peak was reached in 1960's and into the 1970's in Portugal. This happened without any significant improvement in the diagnosis or the treatment. This is thought to be the modification of effect of the risk factors involved with the pathogenesis of gastric cancers.

1.4. Aetiology of gastric cancer

The aetiology of gastric cancer is multi factorial. Host genetics and environmental factors have been implicated in the pathogenesis of gastric cancers.

1.4.1 Family history

Family history is associated with increased risk of gastric cancers (Nagase et al 1996, Correa 1988, Bernini et al 2006) and with increased risk of precancerous abnormalities (Carneiro et al 2004). A recent study has highlighted the increased risk of gastric cancer among siblings with gastric cancer (Bakir et al 2000). A recent study has looked at the incidence of gastric cancers in patients with relatives with digestive system cancers and reported an increase in the risk of both cardiac and non cardiac type gastric cancers (Dhillon et al 2001) . A recent study from Sweden has shown an increased risk in the sibling of gastric cancer patients (Altieri, Hemminki 2007) .

El- Omar et al (2000) have shown that the relatives of patients with gastric cancers have increase prevalence of precancerous gastric lesions, although this was more common in patients with *Helicobacter pylori* infection. The family clustering of gastric cancer may be due to a combination of genetic and also due to other environmental factors such as *H. pylori* infection and diet.

1.4.2 Helicobacter pylori

Helicobacter pylori is a Gram negative bacilli, which is known to inhabit the human host (Fig 1.3) In 1984, Warren and Marshall described this spiral bacterium that is similar to campylobacter, in the mucosa of patients with chronic gastritis. *Helicobacter pylori* was declared a class 1 carcinogen in 1994 due to its association with gastric cancer (IARC 1994) and, because of the available epidemiological and experimental evidence is considered to be a major epidemiological factor in gastric carcinogenesis. Other bacteriae have been implicated in the pathogenesis of gastric cancer for e g., *Helicobacter heilmannii* and *Mycoplasma* infection but their correlation is

Figure 1.3 *H. pylori*



Figure 1.3 shows the electron microscopic picture of *H. pylori*, which is a spiral like bacterium with multiple flagellae. www.fallingpixel.com

weak (Stadtländer, Waterbor 1999). There are studies which looked at other infectious cause (viral, fungal and parasitic) for gastric cancer. Epstein-Barr viral infection, which is a recognised human carcinogen implicated in the development of other cancers like Hodgkins's disease and Naso sinal carcinoma (IARC 1997), is one of the infections which has been implicated in the gastric carcinogenesis (Stadtländer, Waterbor 1999).

The prevalence of *H. pylori* worldwide has been estimated at 50% but they vary depending on the geographic location (Nepomnayshy, Birkett 2000). A recent review has shown the variation in the prevalence of *H. pylori* with the prevalence rate in Japan (4%) and an African village (82%) (Bruce, Maaros 2008). *H. pylori* is transmitted through faecal oral route and the high rates of infectivity could be secondary to large family units and poor hygiene. Infection occurs in early childhood and re infection is rare after eradication in adults, although this is not always the case in the developing world (Logan, Walker 2001).

Asymptomatic *H. pylori* infection is common and only 20% of the infected individuals develop clinical disease (Uemura et al. 1997). *H. pylori* is associated with superficial gastritis, chronic gastritis and intestinal metaplasia (Bornschein et al 2010) and all these changes are found in the premalignant stages of gastric carcinogenesis (Correa 1988). The site of the infection plays an important role in determining the type of disease. Infection of the corpus can lead to acute pan gastritis and result in mild gastritis with no upset in gastric acid secretion and the patient will remain asymptomatic. On the other hand, chronic infection with *H. pylori* causes loss of acid secreting cells, hypochlorhydria, bacterial overgrowth and increased risk of gastric ulcer and gastric cancer. A similar effect is seen in patients with autoimmune pangastritis (pernicious anaemia) resulting in hypochlorhydria and resulting in the increase in the risk of gastric cancer (Faraji, Frank 2002). *H. pylori* infection of the antrum leads to antral gastritis with intact acid secreting corpus and results in increased parietal cell mass leading to increased acid secretion due to dysregulation of feedback pathway controlled by gastrin produced in the antrum. This leads to high incidence of duodenal pyloric metaplasia, which are colonised by *H. pylori* and may result in duodenal ulceration (Logan, Walker 2001, Faraji, Frank 2002).

Chronic infection with *H. pylori* triggers host immune response, which may not be strong enough to clear the infection. Similarly, concurrent infection with different strains of *H. pylori* result in DNA exchange between the strains and result in more virulent strains (Logan, Walker 2001). A number of virulence factors are described in *H. pylori* and can be divided into colonisation factors and disease associated factor. Almost all strains express colonisation factors, which help them in colonising the gastric mucosa but only certain strains of *H. pylori* has disease associated factors. The two major virulence factors are the vacuolating cytotoxin (Vac A) and the cytotoxin associated protein (Cag A) (Dundon et al 2001)

The majority of gastric adenocarcinoma is thought to be the result of chronic *H. pylori* infection. The development of adenocarcinoma is a result of the multistep process of mucosal alterations varying from gastritis, intestinal metaplasia and eventually invasive carcinoma is well recognised.

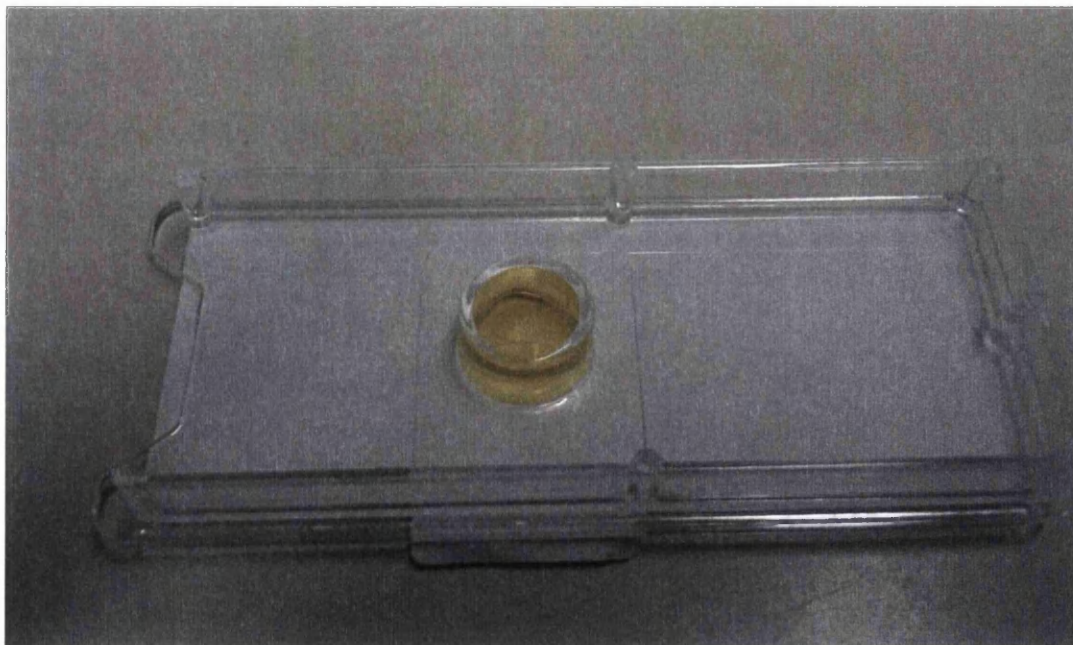
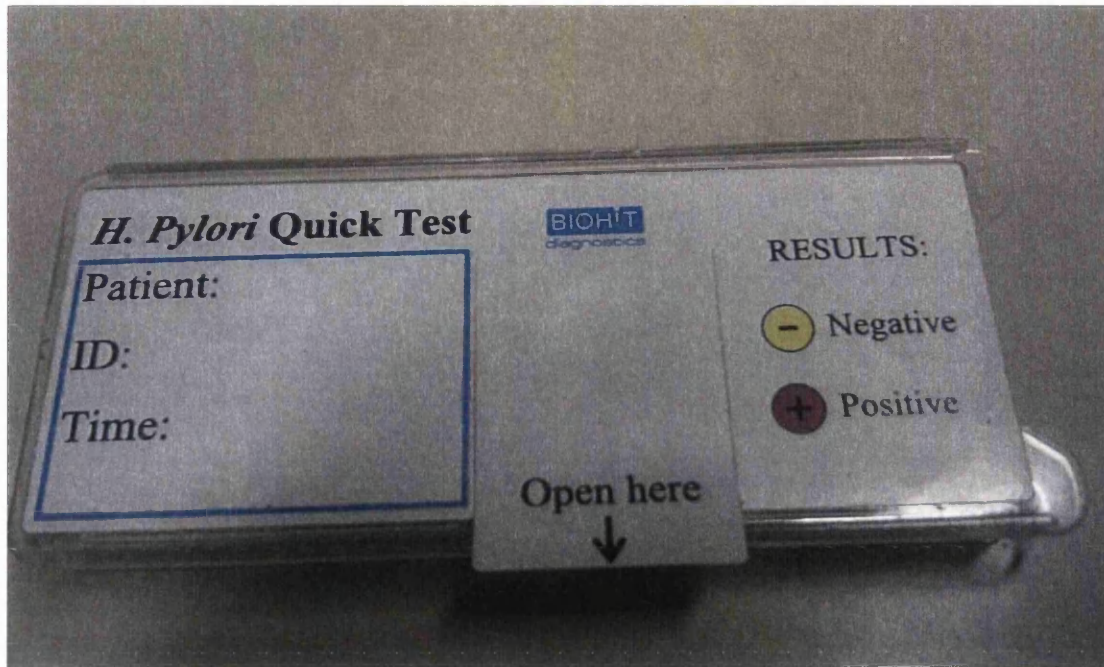
Recent studies focus on detecting the “point of no return”, which is defined as alterations that are no longer reversed by treating and eradicating the *H. pylori* infection and thereby the progression to gastric cancer is not altered. *H. pylori* infection not only alters the immune response but also induces genetic alterations. It is important to identify these genetic alterations as this would help us in identifying the risk factors involved in the development of gastric cancer. The detection of high risk individuals who are at risk of developing gastric cancers would help us in designing appropriate preventative and treatment strategies (Bornshein J et al 2010).

There are number of invasive and non invasive methods to determine the infection of *H. pylori*. The most commonly used method is the Urea Breath test. This is based on the fact that the bacteria produces 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, which is absorbed through the gastric mucosa and into the systemic circulation. This is then released in the expired gas. It detects current infection and is useful in assessing the eradication of *H. pylori* after treatment. Faecal antigen test is available to detect *H. pylori* is useful in detecting infection present after treatment

(Manes et al. 2001, Vaira et al. 2000). Serological testing of *H. pylori* antibodies relies upon detecting IgG and IgA antibodies and ELISA is used to detect this. This method is reasonable sensitivity and is used for global screening purpose. The antibody level is known to decrease slowly after the treatment and is not a reliable indicator of eradication or re-infection. Invasive detection of *H. pylori* involves detection of the organism in gastric biopsy. This is considered to be a gold standard test for the detection of *H. pylori*. But the major disadvantage with this is that it requires an upper GI endoscopy, which is a test associated with complication such as perforation and bleeding. Again it is noted that previous treatment with proton pump inhibitors results in alteration of gastric pH and decrease the detection rate of *H. pylori*. Sampling error is common and up to 14% of infected patients do not have *H. pylori* on the biopsy analysed especially in patients with gastric atrophy, intestinal metaplasia and bile reflux (Logan, Walker 2001). Multiple biopsies and multiple sites of gastric biopsies would result in decrease in the sampling error but this is in time and labour intensive. A method of *H. pylori* detection by passing the need for histological detection would be a CLO test (Delta West Ltd., Bentley, Australia), whereby the urease activity of *H. pylori* is detected by a change in colour in a pre packaged agar gel of phenol red and urea. This is readable in the endoscopy unit and is widely available (O'connor, Sebastian S 2003). The sensitivity of CLO test decreases in bleeding (Figure 1.4)

Treatment of *H. pylori* involves a combination of an acid suppressor (proton pump inhibitor, H₂ receptor antagonist or bismuth) and two antibiotics (amoxicillin/clarithromycin, metronidazole/clarithromycin or amoxicillin/metronidazole) is a popular and effective therapy, with eradication rate of 90% (Unge 1998). Quadruple therapy using bismuth is recommended if the first line treatment (Hojo et al. 2001). Successful eradication of *H. pylori* leads to peptic ulcer healing and results in decrease in ulcer recurrence. Eradication is recommended in first degree relatives of patients of gastric cancers, patients with atrophic gastritis, patients with gastro duodenal diseases with peptic ulcer, low grade dysplasia, mucosa associated with lymphoid tissue [MALT], iron deficiency anaemia and chronic idiopathic thrombocytopenia purpura (Malfertheiner, 2007)

Figure 1.4 CLO test for *H. pylori* detection



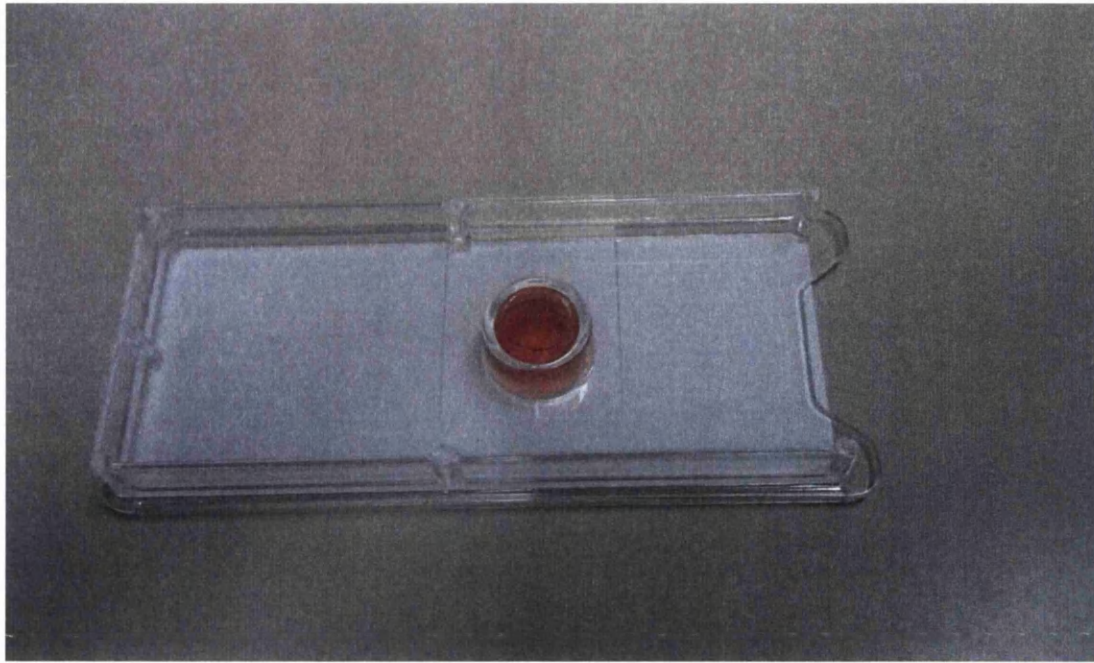


Figure 1.4 A Sample of CLO test, the kit is opened and a gastric biopsy specimen is placed in a well filled with an agarose gel and urea. The second picture shows the CLO test kit before the gastric biopsy is placed and the pink well demonstrates that the specimen is CLO positive, implying that the patient is infected with *H. pylori*.

1.4.3 Smoking

Smoking increases the risk of developing gastric cancers. Smoking is particularly a strong risk factor in men (Chung et al. 2010, Aragonés et al. 2009). It has also been noted that the effect of smoking in gastric cancer development is dose related and high incidence of gastric cancers in heavy smokers (Koizumi et al. 2004).

1.4.4 Socio economic factors

Both socioeconomic and geographic differences influence the incidence of gastric cancers. Gastric cancer incidence is high in developing world when compared with the developed world (Stadländer, Waterbor 1999, Parkin 2005). There is difference in the incidence within the same country (Aragonés et al. 2009).

1.4.5 Diet

Epidemiological and experimental studies have shown that certain food can be protective against gastric carcinogenesis, and include fruits (especially containing ascorbic acid) and vegetables. Nitrosamines and salt, alcohol are implicated with increase in the rates of gastric cancers (Tsugane, Sasazuki 2007, Wang et al. 2009, Moy et al 2010). The countries with high incidence of gastric cancers have high salt intake and restriction of salt intake is advocated as a prevention strategy (Tsugane, Sasazuki 2007).

1.4.6 Sex difference

The incidence of gastric cancer increases in men after the third decade, but the incidence in females only starts to increase after the sixth decade (Sipponen et al 2002). The incidence of gastric cancer in males to females is 2:1 and this cannot be entirely attributed to the environmental risk factors. It is implicated that oestrogen may have a protective role in the pathogenesis of gastric cancer. Those with longer fertility and on hormone replacement are found to have lower gastric cancer incidence, whereas use of

tamoxifen is associated with increased gastric cancer incidence. Similarly men with prostate cancers treated with oestrogen are found to have lower incidence of gastric cancer (Chandanos et al 2008).

There is a significant overlap between the various aetiological factors discussed above in determining the effect of individual risk factors in the development of gastric cancer.

1.5. Classification of gastric cancer

Gastric cancers can be classified by different methods. Anatomically, they can be classified depending on the site of the cancer. Distal gastric cancers used to be the most common form of gastric cancer but their incidence has been slowly diminishing in the Western countries and increasing incidence of proximal gastric cancers are noted (Grady 2001). The decrease in the incidence may reflect the improvements in lifestyle and the socio – economic conditions in western population. Proximal gastric cancers of the cardia are not always distinguishable from the oesophageal adenocarcinomas and the presentation is usually at an advanced stage of the disease and a collective term of gastro –oesophageal cancer is used.

Gastric cancers can be classified based on the cell of origin and the histological pattern. Ninety percent of the gastric tumours are adenocarcinomas, lymphomas, carcinoid tumours and leiomyosarcomas accounting for the rest.

Adenocarcinomas are classified into two main types based on their histological pattern- better differentiated intestinal-type adenocarcinoma and the poorly differentiated diffuse –type (Lauren 1965). A more recent classification is based on mucin expression and classifies gastric cancer in to 4 different sub groups: the gastric or foveolar type (G type), the intestinal type (I- type), the gastric and intestinal mixed type (GI – type) and the neither gastric or foveolar type (N–type) (Tatematsu et al. 1990) .Distinct genetic changes appear to be associated with distinct phenotypes. In I–type gastric cancer, p53 mutations and allelic deletions of the adenomatous polyposis coli (APC) gene are observed frequently than in G type adenocarcinomas. Microsatellite instability (MSI) is

found more often in G – type adenocarcinoma than in I-type adenocarcinoma (Endoh et al. 2000, Shibata et al. 2003).

1.6. Familial gastric cancers

Hereditary diffuse gastric cancers account for approximately 1-3% of the gastric cancers and they are caused by a germ line mutation of CDH1 gene, which encodes E-cadherin, a molecule central in the process of development, cell differentiation and maintenance of epithelial architecture (Grunwald 1993). Gastric cancers in its hereditary form can be caused by germ line mutation of TP- 53 tumour suppressor gene which occurs in Li- Fraumeni syndrome (Oliviera et al 2004, 2009). BRCA2 gene mutations are associated with not only breast carcinomas but also gastric, ovarian, pancreatic and pancreatic cancers (The Breast Cancer Link –age Consortium 1999, Jakubowska et al 2002). A proportion of hereditary on polyposis colorectal (HNPCC) is also associated with high frequency of extra colonic carcinomas, most commonly associated with gastric and endometrium cancers (Lynch et al. 1996) and has microsatellite instability(Peltomäki et al 1993). Thirty to forty percent of all HDGC families carry CDH1 gene mutations (Oliveira et al. 2009). The gastric mucosa in CH1 germ line mutation carriers is normal until the second CDH1 allele is inactivated in multiple cells in the gastric mucosa, accounting for the multifocal tumour lesion (Carneiro et al 2004). It is not exactly clear of the role *H. pylori* infection plays in the pathogenesis of the diffuse gastric cancer in patients with CDH1 mutation. It is possible that the infection with *H. pylori* as well as the dietary and other environmental factors influences the disease risk of the susceptible individuals (McColl et al 2002).

1.7 Multistep pathway of gastric carcinogenesis

The exact pathway leading to gastric cancer from normal gastric mucosa is unknown. Correa proposed a multistep pathway to intestinal cancer, including the precancerous stages and the possible environmental insults that could contribute in the pathogenesis of cancer (see figure 1.5)

Figure 1.5 Correa hypothesis of gastric cancer aetiology (Correa 1988)

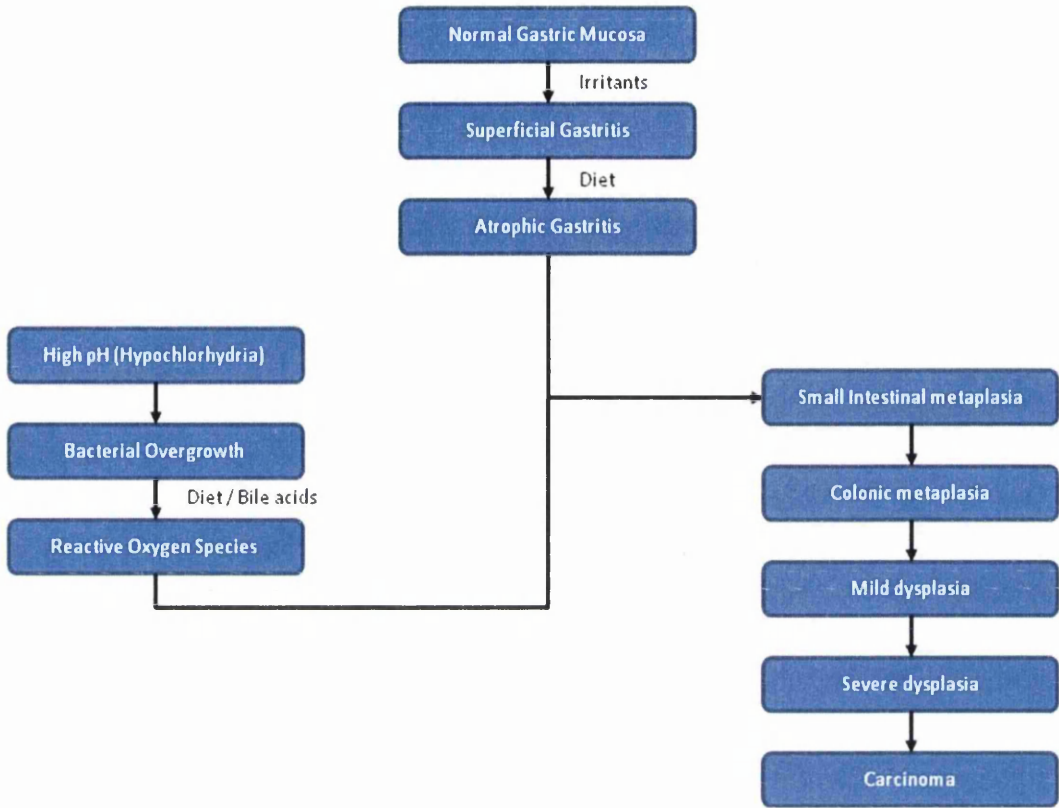


Fig 1.5. The premalignant stages of gastric cancer and its potential contributing factors as described by Correa

Gastritis is an inflammation of the gastric tissue and this could be acute or chronic. Gastric atrophy occurs as a result of prolonged ulceration or inflammatory process. Intestinal metaplasia represents a non- neoplastic change in the cell and is usually due to the sustained adverse environment, and is caused by alteration of the stem cell lineage or epigenetic changes. This can be associated with increased cancer risk (Morson et al. 1980). Intestinal metaplasia is common but gastric cancer is not. Intestinal metaplasia may progress from small bowel metaplasia to colonic metaplasia and can be divided into 3 types – complete (small intestine type containing goblet, Paneth, endocrine and brush bordered cells -enterocytes), incomplete – (goblet cell metaplasia) containing goblet and mucous cell, no enterocytes, plus sialomucins, incomplete (colonic type) – containing goblet and mucous cells, no enterocytes, plus sulphomucins(Correa 1988). Dysplasia is the term used to describe cells that has the potential to progress to cancer (Grunwald 1993, Morson et al. 1980). Dysplastic cells have enlarged hyperchromatic nuclei with coarse chromatin and irregular nucleoli. These features suggest that there is failure of cells to mature as they migrate from the stem cell compartment.

1.8 Cancer initiation

Cells are continuously exposed to potential mutagenic agents through their exposure to environmental carcinogens or due to their normal metabolism. This results in constant genetic alterations within the cells, but stringent monitoring within the cells ensures that this damage is repaired before it is transmitted to the daughter cells. If the genetic alteration is beyond repair, it results in the cell death (apoptosis). The first step in the cancer pathogenesis is the interaction between the cell DNA and the carcinogen, which results in a cell with altered cell genome- this process is described as cancer initiation. The genetic alteration could occur as a result of exogenous stimulus for Eg., chemical carcinogens, oncogenic viruses, exposure to radiation or due to endogenous stimulus Eg., oxygen free radicals formed as a result of normal metabolism.

1.9 Cancer Progression

Carcinogenesis is multistep progress that involves accumulation of genetic changes that progressively transform normal cells (Vogelstein et al 1993). Once the cancer initiation occurs, the subsequent cells resulting from the mitosis becomes increasingly abnormal and develop into cancer. This is cancer progression. Hanahan and Weinberg (2000) suggested six possible mechanisms in cell physiology that could result in cancer progression

1. Self sufficiency in growth signals (production of their growth signals allows these cells to dissociate from their normal micro environmental control mechanisms)
2. Insensitivity to growth inhibitory signals.
3. Evasion of apoptosis
4. Maintenance of telomere length which results in limitless replicative potential
5. Sustained angiogenesis (new vessel formation ensures oxygen supply and nutrients to the growing tumour)
6. Capacity to invade tissue resulting in metastasis, responsible for distant spread.

1.10 Clonal evolution

During cancer initiation, a genetic alteration in a single cell gives this cell a significant growth advantage, which allows it to proliferate and overgrow its neighbours producing a homologous clone. Further mutations occur within this clone as a result of genetic instability. Sporadically, one cell may develop additional survival advantage and result in a dominant sub –population with the original clone. In 1976, Nowell showed that the neoplastic progression occurs as a result of sequential selection of sub-clones with increasingly dysregulated growth controls. This results in transformation of the nature of growth from a benign to a growth with malignant potential, which allows these strains to invade and undergo metastasis.

1.11 Genetic instability of cancer

There are two opposing theories thought to be responsible for the genetic instability of cancer and they are somatic gene mutation and the aneuploidy hypothesis.

1.11.1 Somatic gene mutation hypothesis

Cancer inducing mutation could occur in normal spontaneous mutation in somatic cells, but numerous further alterations are needed for the progression of the cancer. The normal mutation rate is thought to be insufficient to accumulate the amount of genetic alteration required to result in a cancer. It is therefore, thought that the mutation occurs in a gene that is responsible for DNA fidelity. Dysfunctional oncogenes, tumour suppressor genes or DNA repair genes are thought to be important causes for this as it may result in the absence of important checkpoints that ensures DNA fidelity.

1.11.2 Aneuploidy Hypothesis

Chromosomal instabilities manifest as aneuploidy and the exact mechanism which triggers and drives this is unknown. There are different possibilities which could cause aneuploidy for Eg.sister chromatid cohesion, abnormal kinetochore structure or disruption of spindle check points.

Mitosis is a process which results in two identical daughter cells and this requires precision. The mechanism which ensures this has been a challenge for the scientists ever since this was shown by Theodor Boveri nearly 100 years ago. Any defects in the mitosis in the germ line results in embryonic lethality apart from certain chromosomal abnormalities which results in birth defects eg. Trisomy 21 results in Down's syndrome. Most solid tumours demonstrate chromosomal instability and Boveri was the one who postulated that these changes could be the cause of tumour development.

There are different types of genetic alterations in tumours:-

1. Subtle sequence changes: due to base substitution or deletion of few nucleotide and these changes are not detectable by cytogenetic analysis e g., defects in mismatch repair is seen sporadic non familial CRC, defects in nucleoside excision repair is responsible for Xeroderma pigmentosa.
2. Aneuploidy: alterations in the chromosome numbers due to loss or gain of whole chromosome e g., loss of chromosome 10 results in glioblastoma, gain of chromosome 9 results in papillary renal cell carcinoma.
3. Chromosomal translocation: can be detected cytogenetically as fusions of different chromosome or of normally non contiguous segments of a single chromosome. For e g., Translocation of chromosome 9 and 22 is seen in CML.
4. Gene amplifications: seen cytogenetically as homogenously stained regions .At molecular level, multiple copies of DNA megabases are seen and are different from the duplication of much larger chromosomes, which results in translocation or aneuploidy. Although it is well known that solid tumours have genetic instability, it is not their existence but the rate at which this occurs is of significant importance.

Aneuploidy is a state of abnormal chromosomal number and content. Some cancers may have stable chromosomal alteration as a result of chromosomal redistribution at some stage during the pathogenesis of the disease conferring it some proliferative advantage. But in majority of cell lines these indicate chromosomal instability and they are demonstrated well in the colorectal cancers (Lengauer et al 1997).

Aneuploidy can occur in several ways:

1. Aberrant mitotic division caused by polyploidisation, due to cytokinesis defects, cell-cell fusion or cell skipping mitosis together result in cells that enter the subsequent mitosis with multipolar spindle (Storchova et al 2004). It can also be

caused by defects in duplication, maturation or segregation of chromosomes (Nigg 2001).

2. Chromosome cohesion defects might also result in aneuploidy in human cancer cells. Separation of sister chromatids depends on separase and this is inhibited by securin. Human cancer cells in which securin is inhibited show high levels of chromosomal instability (Jallepalli et al. 2001)
3. Aneuploidy can arise from improper attachment of microtubules from each pole to the one kinetochore (Cimini et al 2001). Inhibition of attachment –error – correction mechanism by aurora kinase B, borealin, survivin and inner centromere protein (INCENP) (Gassmann et al 2004).
4. Aneuploidy results from defects in mitotic check points that prevent cells from entering into anaphase if all the chromosomes are not attached by microtubules. There are three checkpoints which are documented: DNA damage checkpoint, which is able to block cells in G1/ S, G 2 or even in mitosis (Hoeijmakers 2001), DNA replication check point that monitors progression through S phase, and the spindle check point, which monitors attachment of chromosomes to functional spindle microtubule and delays exit from mitosis until all chromosomes have bipolar attachment and thereby ensuring equal distribution of genomic material to the daughter cells. Spindle check point is discussed in detail in the further section in this chapter. Figure 1.6 illustrates the different components in mitosis that may lead to chromosomal misaggregation and hence aneuploidy (Pihan, Doxsey 1999).

Figure 1.6 Mitotic Machinery and their functions

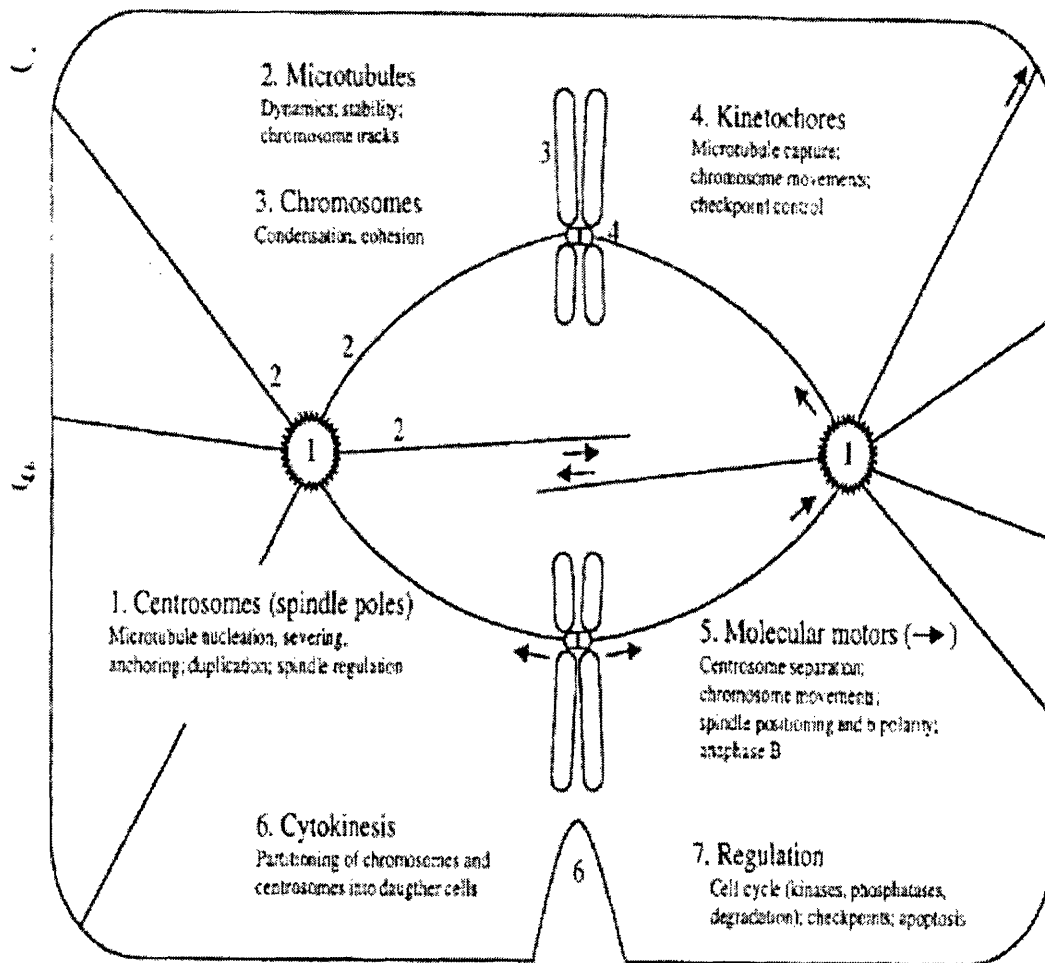


Figure 1.6 Components of the mitotic machinery and their functions. Defects in several mitotic functions have the potential to contribute to chromosome missegregation, aneuploidy and tumorigenesis (Pihan, Doxsey 1999).

1.12 Aneuploidy and cancer

Aneuploidy is commonly seen involving one or more chromosomes in human cancers (Sen et al 2001) and is the most prevalent genetic abnormality seen in solid tumours (Hein, Mittleman 1986). The association of cancer and aneuploidy is undisputed but the exact mechanism of the induction of aneuploidy and its role in the pathogenesis of cancer remains unknown.

It has been suggested that chromosomal instability (CIN: rate) results in aneuploidy (the state) is a dynamic chromosome mutation event and is a distinct form of genetic instability in cancer. This may lead to phenotypic alteration during the cell progression (Bialy et al 1998). Cancer cells may undergo structural and functional phenotypic changes. These include altered morphology, proliferative capacity and metastatic potential. They also develop traits needed to survive E.g., antigen resistance, immune resistance.

The identification of aneuploidy earlier in cancer development has suggested that this may play an important role in the development and progression of cancer (Barrett et al. 1999, De Angelis et al. 1999). Further support to the aneuploidy as cause for carcinogenesis is the fact that non – genotoxic carcinogens such as asbestos and mitotic spindle blockers (e.g., colcemid) induces chromosomal instability leading to aneuploidy but they do not cause mutations.

The association of aneuploidy and gastrointestinal cancer is strong. It is common in colorectal, oesophageal and gastric adenocarcinoma. Tumours with aneuploidy tend to behave more aggressively (Doak 2008, Bondi 2009). In gastric cancer higher level of aneuploidy is associated with advanced tumours (Sugai et al 1999) and metastatic spread of gastric cancer (Sasaki et al 1999). Recurrent chromosomal abnormalities have been noted in gastric cancers but no particular aberration has been established as a specific cause for gastric cancer development. Chromosomes 1, 7, 8, 9, 17, 20, X and Y were the most commonly involved chromosomes. It has been observed that the genetic abnormalities seen in advanced gastric cancers are also seen in the early stages of

gastric cancers implying that these changes are the most likely driving force in the development of the cancers (Hamamoto et al 1997). Beuzen et al showed that the same chromosomal abnormalities increased progressively with advancing pathological stages. In 2000, Kobayashi et al looked at the intestinal metaplasia and adenocarcinoma samples from the same patients and demonstrated that they share the same genetic abnormality but the abnormality was severe in cancer. They also showed that the loss of heterozygosity was more common in cancer when compared with intestinal metaplasia.

1.13 Methods of looking for aneuploidy

1.13.1 In situ hybridisation (ISH)

In *situ* hybridisation (ISH) involves binding of a labelled probe to the cellular DNA and is used to analyse the numerical and structural abnormalities in the chromosomes. This technique was developed in 1969 (John et al. 1969, Gall and Pardue 1969). The probes used were radio isotope probes and were associated with long exposure times with radioactive materials and high background noise. This led to new techniques involving non isotope probes, which were considered safe and user friendly.

1.13.2 Fluorescence in situ hybridisation

Various methods using direct and indirect labelling were tried to overcome the disadvantages of isotope probes. Indirect labelling involved three basic steps – a) preparation of the specimen in a slide b) introduction of a labelled probe which attaches to a specific homologous sequence on the DNA c) detection of this targeted area by another process. The labelled probe could be a fluorochrome linked to an antibody or a fluorescently labelled DNA. Fluorochromes are chemical groups which emit a specific wavelength of fluorescent light after excitation light of a characteristic and longer wavelength. In this study, fluorescently labelled DNA probes were used. Fluorescence labelled signal is highly sensitive and allows detection of several different sequences using different coloured sequences simultaneously.

Direct labelling with fluorescent probes established this technique as a widely used technique in cytogenetics. This involves directly labelling of the fluorochrome to a DNA molecule and has advantage over the indirect labelling in that this method is both simple and quick to do. Another advantage of this method is that the probes can be removed relatively easily when compared to the indirect labelling and fresh probe can be used. The disadvantage with this is that the signal produced by the direct labelling cannot be amplified whereas the signal produced by the indirect method can be amplified.

1.13.3 Advantages of FISH

Conventional cytogenetic analysis involved using cultured cells to obtain metaphase preparations. FISH can be applied to inter phase cells and therefore enables us get the cytogenetic data from the inter phase cells, which was not possible in the past. The ability to get this data from the inter phase cells is likely to give us data that is more representative of the specimen as the data gathered from the cell culture could be influenced by the genetic composition of cells cultured through the selection of favourable growth characteristics.

Another advantage of these probes is that they are commercially available and several probes can be used in a single experiment, which conserves both labour and time. FISH is technically simple and speedy to analyse thereby enabling us to study loss/gain of chromosomes as well as deletions/amplification of chromosomes. Conventional molecular methods (like comparative genomic hybridisation) rely on bulk analysis and therefore has the potential to miss rare chromosomal abnormalities but FISH can easily distinguish and score many cells allowing detection of rare chromosomal abnormalities.

1.13.4 Limitations of FISH

FISH is used to study either the whole genome or specific genomic loci. This depends on the probes used. This can be broadly divided into three subtypes, each with different range of applications (Kearney 2001) – Whole chromosome painting probes; repetitive sequence probes and locus specific probes. Repetitive sequence probes hybridize specific chromosomal regions or structures that contain short sequences which are present in many thousand copies (Kerney 2001, Gozzetti 2000) Eg., centromeric probes that target the alpha and beta satellite sequences, which flank the centromeres of human chromosome. Centromeric probes are particularly suitable for the detection of monosomy, trisomy and other aneuploidies in solid tumors and leukemias (Gozzetti 2000). Numbers of signals from these probes are supposed to be identical to numbers of homologous chromosomes per interphase nucleus but however this is not the case (Lourov et al 2006, Lourov et al 2007, Lourov et al 2009, Lierhr et al 2009). This is a main disadvantage of interphase FISH with centromeric probes. However this can be overcome with the use of site specific DNA probes (locus specific probes) (Lierhr et al 2009) Differences of hybridisation efficiency complicate simultaneous application of different set probes (Lourov et al 2006). DNA replication during the S phase of the cell cycle is another major problem of interphase FISH applications (Lourov et al 2006, Soloviev et al 1995) FISH analysis can be hindered by replicative signal appearance. This is mainly related to site specific probes but can also be seen in centromeric probes (Lourov et al 2006, Lourov et al 2007, Lourov et al 2009, Soloviev et al 1995, Yrov 2005).

The sensitivity and the resolution needed for the FISH experiment depends on the sensitivity and the resolution and that is directly attributed to the technical limits of the fluorescence microscopy. Sensitivity depends on the light gathering ability of the particular microscope. Resolution depends on the ability to distinguish between two points along the length of a chromosome. With technical difficulties in mind, we also need to consider the conformation of the DNA within the chromosome. Metaphase chromosomes are thousands of times more compacted than interphase chromosomes, which in turn are 10 times more compacted than the naked chromosomes. This gives rise

to resolution in the range of tens of thousands of kilobases for interphase chromosomes (O' Connor 2008).

1.13.5 Brush cytology and FISH

Brush cytology using exfoliated gastric cells obtained during routine upper GI endoscopy was used in this study. This was chosen as it had been successfully used in the study of aneuploidy in Barrett's oesophagus (Doak et al. 2003) and premalignant gastric tissue (Williams et al. 2005) by our group. The brushes were pushed through the endoscope channel and scraped along the gastric mucosa and collected the surface epithelial cells and did not penetrate into the stromal tissue (as in gastric biopsies). This method proved to be successful and subsequently used for this study.

1.13.6 Detection of abnormalities in chromosome1 using FISH

Fringes et al showed that the human gastric cancer appear in two genomic groups that can be reliably diagnosed by FISH on routine biopsy specimens and that the numerical aberrations of chromosomes 1,3,10 and 17 are independent of histological subtypes and polysomic copy number abnormalities of chromosome 1 and 17 correlate to intra gastric tumour site and are highest in gastric cardia cancers, suggesting high tumour instability at this particular location. In 2000, Kitayama et al showed that the earliest chromosomal abnormalities that occur early in gastric carcinogenesis, involves aberrations of chromosome 1 and 2 and that chromosomal aberrations expand in a stepwise manner with cancer progression. In a recent study involving 51 gastric cancer patients, it was shown that aberrations of chromosomes 1, 8, 17, 20 and x were frequent regardless of the histological type of cancer (Kitayama et al. 2003). In this study, FISH was used to study the aberrations of chromosome 1 in the premalignant gastric tissues.

In this study, aberration of chromosome 1 was studied through the varying histological stages of gastric cancer development as outlined by Correa (Correa 1988). Premalignant lesions occur early during the neoplastic progression and they possess an increased risk for cancer development but only a certain proportion of the premalignant

lesions are actually committed to neoplastic progression. Occasionally, premalignant lesions develop into tumours through a series of defined morphological events that can be staged by histology according to the disordered cell growth and loss of differentiation present. Such lesions have proven ideal human models for investigations into tumourogenesis, as the analysis of each stage allows the sequence of genetic events that eventually cause malignant transformation to be established. Understanding the genetic factors involved in the multistep progression of certain cancers could ultimately lead to streamlining the strategies involved in the surveillance, management and prevention of cancers. Genetic biomarkers may be identified that prove to have higher sensitivity and specificity, which will eventually lead to early detection of gastric cancers by stratifying the surveillance of these cancers and will also help in facilitating cancer prevention by early medical intervention. This could also lead to conserving the health resources and improving the cost effectiveness by relaxing or omitting the surveillance for patients with low risk of developing cancers.

1.14 The mitotic spindle checkpoint

The mitotic spindle checkpoint is a highly regulated feedback mechanism that plays an important role in the maintenance of genetic stability and integrity. This checkpoint ensures the correct attachment of chromosomes to the spindle microtubules during mitosis. Prevention of premature entry to anaphase is induced until all the chromosomes are correctly attached to the spindles. The spindle checkpoint therefore blocks chromosome segregation until the spindle has been correctly assembled and the bipolar attachment of the sister chromatid is achieved, ensuring that they are precisely divided into two daughter cells.

The spindle checkpoint acts as a surveillance system by monitoring the status of kinetochores – a protein complex that is present on the centromeres of every chromosome during mitosis. The spindle microtubule attaches to the kintechores in the chromosomes during mitosis. A single unattached kinetochore provides sufficient signal to activate the spindle check point, which triggers a signal cascade that induces the anaphase inhibitors (Nicklas et al. 1995, Chan et al 2005). Defects in spindle assembly,

kinetochores, the structure/number of centromeres and the alignment/attachment of the chromosomes on the spindle triggers the mitotic spindle check point and delays the progression into anaphase until the defects are rectified. Alternatively, adaptation occurs, where a prolonged cell cycle arrest induces apoptosis or a return to the interphase occurs by overriding the spindle checkpoint mechanism (Gorbsky et al. 1998)

1.14.1 The mitotic checkpoint components and its mechanics

The components of the mitotic spindle cell checkpoints were originally identified in budding yeast by screening agents that induced de polymerisation of the microtubule[(Hoyt et al 1991,Li and Murray 1991,Weiss and Winey 1996).

Mitotic progression and sister chromatid segregation is controlled by the anaphase promoting complex/ cyclosome. APC is a large protein complex that induces degradation of cohesion complex, which itself comprises of atleast four different subunits (allowing chromatid separation) and the mitotic cyclins responsible for maintaining mitosis. APC/C function is regulated by a) phosphorylation and b) association of activator protein Cdc20.Cdc20 has been implicated in the regulation of APC/C dependant proteolysis and is essential for chromosome segregation (Visintin et al. 1997). After ubiquitination APC/C initiates degradation of securin. Securase is inhibited by securin, a protein involved in the control of transition between the metaphase to anaphase, throughout the cell cycle. But when securin is degraded, separase is not inhibited and this leads to cleavage of protein subunit Scc1.The cleavage of Scc1 is irreversible, therefore very closely controlled. The cleavage of Scc1 leads to dysjunction of sister chromatids and rapid transition from metaphase to anaphase. APC/C also leads to degradation of other protein called cyclin B1 and this leads to inactivation of CDK1 (cyclin dependant kinase1) and initiates mitotic exit.

These events are controlled by the mitotic check point, and prevents premature advance into anaphase. The mitotic check point is activated immediately after the entry of cells into mitosis or meiosis. The signal for this is provided by unattached

kinetochores. These trigger check point components which prevent the cdc20 dependant degradation of securin and cyclin B by activation of APC/C complex(Cleveland et al. 2003).Experiments have shown that even one unattached kinetochore can delay anaphase. The other theory is that the lack of tension in the unattached kinetochores delays anaphase. Experiments in the insect spermatocytes suggested that it is the application of tension in the unattached kinetochore that allows anaphase onset (Nicklas et al 1995). It is a matter of controversy which exactly triggers the delay in the anaphase onset. In fact it has been difficult to distinguish between these as attachment of microtubule to kinetochore produces tension and this promotes attachment of additional tubules (King, Nicklas2000). Both unattached kinetochore and the lack of tension results in delayed anaphase signal through different check point components.

Initial studies involving the use of drugs to disrupt the mitotic spindle demonstrated two genetic factors in budding yeast that cause failure to arrest in mitosis and therefore were linked to a defective spindle check point. The mutants were named Mitotic Arrest Deficit (MAD 1, 2 and 3) and Budding uninhibited by Benzimidazole (BUB1, 2, and 3)(Hoyt et al. 1991, Li, Murray 1991). Homologues of BUB and Mad genes are identified in all higher eukaryotes suggesting the mechanism has been conserved throughout evolution. Only exception is that while yeast and plants have conserved Mad3 protein that lacks kinase domain, *Drosophila* and mammals have a protein that shares similarly to Mad3 at its N-terminus but also has a C terminal serine. The protein kinase domain, that is highly homologous to BUB1 and was therefore named BUBR1 and probably represents homolog of Mad3 (Chan et al 1999). The mitotic checkpoint requires BUBR1kinase (a hybrid yeast MAD2 and BUB1, which is encoded by BUB1B gene), the ZW10-ROD-Zwilch complex, CENPE (microtubule motor protein centromere protein E) and mitogen activated protein kinase (MAPK) to regulate the signalling pathway.

The mitotic checkpoints are rapidly bound by and released by unattached kinetochores (Shah et al. 2004, Howell et al. 2004). After the nuclear envelope breakdown, the checkpoint proteins are recruited to the outer kinetochore surface of all the unattached kinetochores.

MAD1-MAD2, BUB1-BUB3 and BUB3-MAD2B/BUBR1 complexes along with the MPS1 protein are recruited to unattached kinetochores forming a large multiprotein complex. Fig 1.7 represents the role played by MPS1. The exact mechanism of this is unknown. It is indicated that BUB1 and BUB3 proteins are involved in recruiting the other checkpoint proteins to the kinetochore and this process activates and releases the MAD2 in a modified state. MAD2 forms a complex with CDC20-APC, inactivating it and halting the cell cycle progression. (Musacchio, Hardwick 2002, Millband et al. 2002). MPS1 is known to phosphorylate MAD1 during checkpoint activation (Hardwick et al. 1996)- this can re-release MAD2 but does not activate MAD2. MAD2 occurs in two forms-a dimer and a tetramer. Although the dimer and the tetramer binds the CDC20, the tetramer is believed to be active form involved in the inhibition of APC (Fang et al. 1998).

Figure 1.7 Inhibitory effect of protein MPS in the event of unattached kinetochore

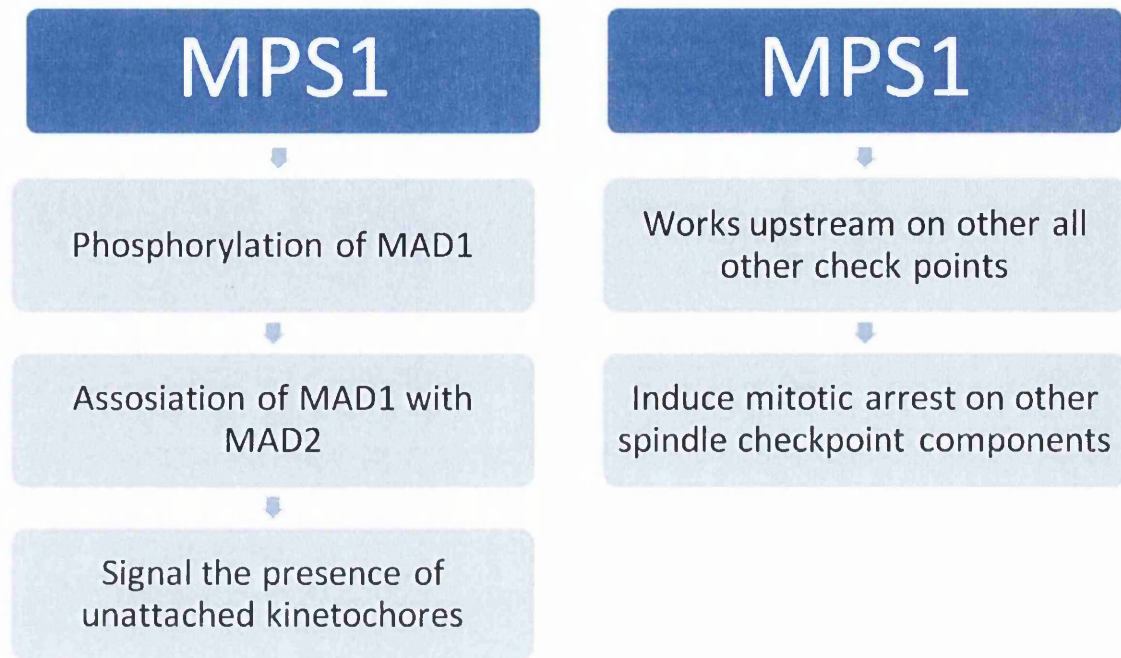


Figure1.7. Represents the inhibitory effect of protein MPS in the event of unattached kinetochore that eventually leads to mitotic arrest.

Direct binding of the protein CENPE (the microtubule motor protein centromere Protein E) to its binding partner BUBR1 activates the BUBR1 kinase activity (Mao et al. 2003). BUBR1 kinase activity is required for the recruitment of a stable MAD1-MAD2 heterodimer and this in combination forms the active MAD2 (Chan et al. 1999, Mao et al. 2003, Shah et al. 2004). Activated MAD2 and or BUBR1 in a complex with BUB3 is tightly associated with CDC20 and prevents it from activating the APC/C and thereby inhibiting the ubiquitination of securin, a protein involved in the control of metaphase – anaphase transition and cyclin B1 (Fang et al. 1998)

The exact mechanism involved in the mitotic spindle checkpoint is not known, but the final pathway is the inactivation of the CDC20-APC complex (to prevent anaphase entry) in the presence of chromosomes unattached to the spindle. Dissociation of certain checkpoints from the kinetochore region and the CDC20-APC complex occurs after the bipolar attachment of the chromosomes and results in terminating the metaphase arrest (Figure 1.8).

Figure 1.8 Events triggered by phosphorylation of protein CDC20

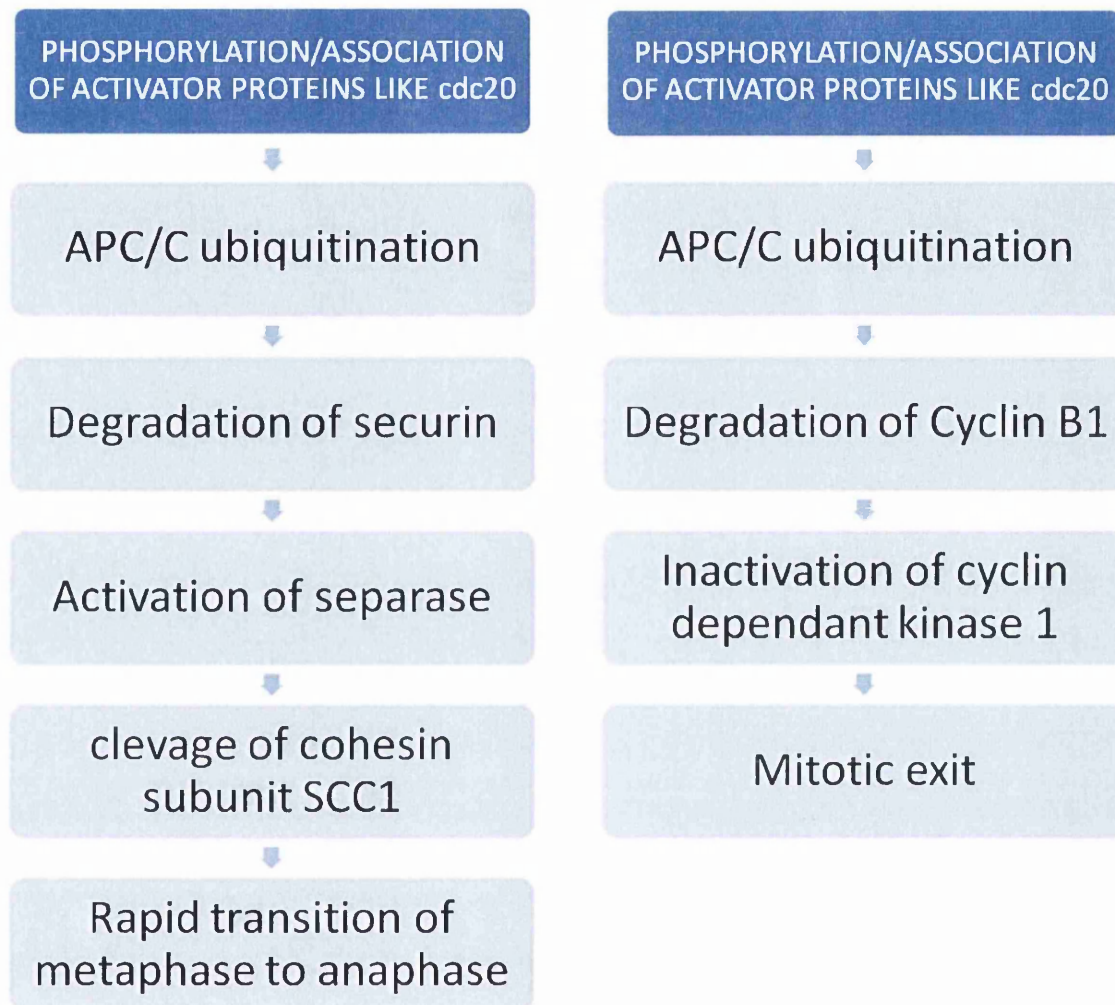


Figure 1.8 shows the cascade of events triggered by phosphorylation of protein CDC20 which leads to the progression of cells from metaphase to anaphase.

1.14.2 Mitotic checkpoint loss and aneuploidy

The components of mitotic spindle checkpoints were identified in studies that showed that the isolated mutated yeast strains were unable to activate mitotic arrest in response to the microtubule- damaging agents such as nocodazole and benomyl (Hoyt et al. 1991, Li, Murray 1991). This demonstrated that the disruption of the spindle checkpoint results in aneuploidy by enabling the premature exit from mitosis. This results in two daughter cells with unequal number of chromosomes.

As the defect in the spindle checkpoint plays an important role in the prevention of aneuploidy, there has been lot of interest in identifying the possible mitotic checkpoint defects in human malignancies that exhibit chromosomal instability.

MAD2 and BUB1 genes are frequently studied genes as they play an important role in the mitotic checkpoint. Mutations of the BUB1 gene have been identified in several cancers although at a low frequency. BUB1 mutations have been observed in lung(Gemma et al. 2001), thyroid(Ouyang et al. 2002) and colorectal cancers(Shichiri et al. 2002).Both increased and decreased levels of BUB1 expression levels have been observed in breast cancer cell lines (Myrie et al. 2000, Yuan et al. 2006) and colorectal cancers (Shichiri et al. 2002). A recent study has shown that BUB1 expression levels were significantly greater in the diffuse gastric cancer subtype when compared with intestinal type gastric cancer (Grabsch et al. 2004). It has also been shown that over expression of BUB1, BUBR1, BUB3 is associated with increased proliferation in gastric cancer (Grabsch et al. 2003). The regulation of gene expression of these mitotic checkpoints may therefore be an important factor that facilitates carcinogenesis.

Studies involving MAD2 genes are rare and the mutations of MAD2 are seldom found. MAD2 sequence variation has been found in breast cancer cell lines (Percy et al. 2000). It has also been shown that reduced MAD2 gene expression levels have been associated with dysfunctional mitotic check point in breast, nasopharyngeal and ovarian cancer cell line (Li,Benezra 1996, Wang et al. 2000,Wang et al. 2002). Over expression of MAD2 levels have also been observed in gastric cancers (Tanaka et al. 2001) but the levels of this over expression did not correlate with aneuploidy, clinical or pathological

characteristics . Mutation has been observed in the MAD2 gene in gastric cancer suggesting these mutations could lead to the development and progression of gastric cancers. Wang et al (2009) have shown that the expression of MAD2 is related to the histological differentiation and lymph node metastasis of gastric cancer. The higher expression level of MAD2 was correlated with more poorly differentiated gastric cancer and lymph node metastasis. It was also shown that MAD2 is expressed mainly in the nucleus in gastric cancers whereas they are expressed mainly in the cytoplasm in gastric tissue. This shift was thought to be due to tumorigenesis. They suggested that MAD2 might be a crucial marker of prognosis in gastric cancer and could be a target in the search for an effective treatment against gastric cancers.

These reports suggest that the steady – state levels of MAD2 may be important in regulating the mitotic checkpoint. Therefore, inactivation of any of the components of the mitotic checkpoint may play an important role in the pathogenesis of cancer. The status of these genes and their expression levels in human cancers will help us understand the complex nature of the spindle check points and its role in carcinogenesis.

1.15 Gene expression

Only a small proportion of human genes are expressed at any given time and this depends on the cell type function and differentiation. Some genes are expressed in all the cells all the time and are called housekeeping genes and are essential for the very basic cellular function. Gene expression is regulated by alteration of the transcription and translation of the genes. All genes are surrounded by DNA sequences that control their expression and this done by producing proteins called transcription factors. These proteins can switch the gene on and off, thereby regulating it. These transcriptional factors are produced by a different gene, which is again regulated by a different set of transcriptional protein. There is a strict hierarchy in the regulation of gene expression. However, if a single gene is inappropriately expressed, serious dosage imbalances may be induced, particularly if the gene product is a key regulator of pathways vital for cell function or survival.

Gene expression occurs in two major stages. The first stage is transcription, by which the gene is copied to produce a primary transcript (mRNA molecule) with essentially the same sequence as the gene. Most human genes are divided into exons and introns and only the exons carry information required for protein synthesis. The primary transcripts are processed by splicing the introns and leaving just the exons to form a mature transcript or messenger (mRNA) that contains only exon sequences. The second stage is protein synthesis and this is also called translation.

Abnormal expression of genes involved in cell growth, differentiation and division have a potential to result in cancer; up regulation of the proto-oncogenes *c-erbB-2*, *c-myc* and *cyclin D1* have been associated with bladder, breast and gastric cancers respectively (Bièche et al 1999, Oda et al 1999, Underwood et al 1996). The steady-state mRNA levels within the cells under differing conditions can provide us with information of how a cell copes and adapts to a changing environment. The investigation of transcriptional levels in cells at differing stages of neoplastic pathway will provide us with valuable information regarding the gene expression patterns responsible for driving tumorigenesis. Such analysis relies on accurate quantification of mRNA concentration as a measure of gene expression and the most common method used is reverse transcription-polymerase chain reaction (RT-PCR) (Bustin 2000).

1.16. RT-PCR

RT-PCR is a sensitive technique used for the quantification of steady-state mRNA levels especially in samples with limiting RNA quantities. Reverse transcriptase is used to convert the mRNA to cDNA (complementary DNA) as the *taq* polymerase used in the PCR cannot use mRNA as a template. The reverse transcriptase enzyme catalyses the first strand of cDNA synthesis from mRNA in the presence of gene specific/non specific primers. Gene specific primers are used when analysing rare messages and it involves a single step so the chance of contamination is very low but the disadvantage with this primer is that separate RT reaction is needed for each gene studied. The use of non specific primers (e.g. oligo dT or random decamers) gives a cDNA pool which can be used for several subsequent reactions using a number of

different gene specific primers. This is particularly advantageous if RNA sample is limited. PCR technique is used in amplifying the signal and is quantified

The PCR reaction involves an exponential phase (exact doubling of products occurs and is very specific), a linear phase (the reaction is slowing down and the products are starting to degrade) and endpoint phase (the reaction has stopped, no more products are formed and if left long enough degradation of the products occurs).

Therefore accurate detection and quantification of the resultant amplified product is paramount in studying the gene expression levels and the reliability of the data gathered using conventional RT PCR methods is poor(Bustin2002).

Conventional methods involved end-point analysis, which is a gel based method involving electrophoresis of the amplified products after completion of the PCR reaction. Subsequent quantification is achieved by using image analysis software. A lot of variables could affect the result – small pipetting errors, errors in loading the gel and buffer could result in unreliable data and the image analysis method is subject to inaccuracies. It is also labour intensive.

Real time PCR uses the data gathered during the whole course of the PCR reaction and does not use any post PCR data. The quantification of a fluorescent reported that accumulates during the course of the PCR reaction is directly proportional to the amplicon generation. Therefore this eliminates the post –PCR processing for quantification of the amount of PCR produced. There are different types of fluorescent probes that could be used in the real time RT PCR reaction – hybridisation probes, molecular beacons, and SYBR green. SYBR green probes were used in this study. This is an intercalating dye that binds to double stranded DNA via the minor grooves and as a result of this interaction, releases a fluorescent signal. The advantage of using this is that it binds to all double stranded DNA eliminating the need to optimise specific probes. As the product of the PCR reaction accumulates with each cycle, the fluorescent signal emitted from the chosen probe/dye increases in direct proportion. Real time PCR has greatly improved the reliability, reproducibility and the accuracy of the RT –PCR. Real time RT –PCR is used in various fields – it is used in viral detection, detection of

gene expression levels, genotyping, drug therapy efficacy, DNA damage measurement and pathogen detection. Real time PCR is also considered to be more sensitive than endpoint analysis and as a result is increasingly used for mRNA quantification (Mok et al 2001, Sgroi et al 1999).

1.16.1 The analysis of real time PCR

The basis of the real time PCR is that as the double stranded DNA accumulates during the PCR reaction, the fluorescent signal released increases in direct proportion and therefore this can be detected and quantified. The software on automated thermal cyclers (e.g. BIO RAD *icycler*) commercially available performs the data analysis. The increase in the fluorescence is plotted against the cycle number to determine the threshold cycle (Ct) of the sample – the point at which the fluorescent signal can be detected above the background fluorescence. This is then plotted against the automatically calculated standard curve (from a reference series of samples) and from this the initial template quantity within the sample is calculated. The sensitivity of fluorescence detection and the high sensitivity of PCR in amplification of a single template allow detection of mRNA from even single, laser micro-dissected cells (Bustin et al 2002).

1.17 Aims and Hypothesis

1.17.1 Hypothesis

1. Recent studies have shown that aneuploidy in chromosome 1 and 4 are common in gastric cancer (Kitiyama 2000, Fringes 2000) and aneuploidy in chromosome occurs early in gastric cancer (Kitiyama 2003). It is hypothesised that if the aberrations in chromosome 1 and 4 are common and seen in early gastric cancer, then it is likely that they are common and are significant in the premalignant stages of gastric cancers

2. Family history, smoking habits and *H.pylori* infection play an important role in gastric cancer pathogenesis and this could be due to facilitation of chromosomal instability in the premalignant stages of the gastric cancer.
3. MAD2 and BUB1 are widely studied genes that control spindle cell check points and alteration of gene expression levels may lead to increased aneuploidy levels of chromosome 1 and 4.
4. Family history of gastric cancers, smoking, and *H. pylori* play an important role in gastric cancer pathogenesis and they may contribute to the alteration of the gene expression levels of MAD2 and BUB1.

1.17.2 Aims

1. To study chromosomal aberrations in chromosomes 1 and 4 in all premalignant stages of gastric cancer.
2. To correlate the known risk factors of gastric cancer like family of gastric cancer, smoking and *H.pylori* infection with the aneuploidy levels of chromosome 1 and 4.
3. To study the gene expression levels of MAD2 and BUB1 in the premalignant stages of gastric cancer.
4. To correlate the MAD2 and BUB1 gene expression levels with the aneuploidy levels of chromosome 1 and 4.
5. To correlate the MAD2 and BUB1 gene expression levels with the known risk factors of gastric cancers like family history of gastric cancers, smoking and *H. pylori* infection.

1.18 Summary of experimental approach

Patients were recruited from an open access endoscopy list after obtaining their consent, a brief questionnaire were filled in to assess their risk factors for the development of gastric cancer. At endoscopy, gastric cells were obtained using gastric cytology brush. These cells were used to analyse the aneuploidy levels of chromosome 1 and 4. Gastric biopsies were obtained during the procedure and depending on the patient

tolerance, upto three samples were taken. These samples were used for *H. pylori* detection, histological staging, and obtaining RNA, which was used in the gene expression levels of MAD2 and BUB1 levels.

The following chapter deals with the materials and methods used in this study. Chapter 3 details the results of the levels of aneuploidy using fluorescent insitu hybridisation technique. Chapter 4 investigated the levels of MAD2 and BUB1 levels using RT-PCR technique on the RNA extracted from the gastric biopsy specimen obtained at different stages of premalignant gastric cancer stages.

CHAPTER 2

Materials and methods

This chapter outlines the process involved in patient recruitment and data collection in those patients enrolled in the study in chapter 3 and in chapter 4. The method of cell collection from the endoscopic samples is described and also the Fluorescent *in situ* hybridisation used to detect the chromosomal aberrations. This chapter also explains the method adopted to get the tissue samples, the method used to extract the RNA from the gastric biopsies, the statistical methods used to analyse the results of the interphase FISH, the gene expression levels of MAD2 and BUB1 levels, as well as the process whereby the histological diagnosis of gastric biopsies was made.

2.1 Patient enrolment into the study

The study enrolled samples from the Neath Port Talbot Hospital and the Royal Glamorgan Hospital, South Wales. The Neath Port Talbot hospital is situated in the mixed rural and urban area of West Wales and serves a population of approximately 140,000. The Royal Glamorgan Hospital serves the area of Rhonda Valley. These two areas represent similar population demographics and characteristics in that they both are ex mining areas and are. Prior to the start of the study Ethical Approval was obtained from Dyfed ethics committee, October 2007. The study was performed between October 2007 and January 2010.

Initially the patients were enrolled from the Royal Glamorgan Hospital as a similar study was being conducted in that hospital. I not only learnt the method used to enrol patients for the study, the use of gastric cytology brush to obtain the gastric cells, transferring the cells to a transport media and the method used in the lab to obtain the cells on to the slide. This helped me immensely in enrolling the patients independently at the Neath Port Talbot Hospital.

Patients enrolled in this study were identified during routine endoscopy waiting lists at Neath Port Talbot Hospital and also from the Royal Glamorgan Hospital, Llantrisant. Only the patients attending my lists at Neath Port Talbot Hospital and the patients attending the lists of the gastroenterologists at the Royal Glamorgan Hospital were invited to participate in the study. Patients who were enrolled in this study were above 18 years of age, were not on any anti coagulants or medications prolonging their clotting time, and were enrolled from an open access list from their primary physician. An informed consent was obtained from these patients prior to the upper GI endoscopy. The samples were collected and transferred to the university to analyse the samples, whenever possible on the same day. Two or three samples were collected during an endoscopy list due to the time constraints on a busy endoscopy schedule.

Exclusion to the study were made when it was felt that the patients could not adequately consent (<18 years, unable to give informed consent). Patients who were taking drugs, which could prolong the bleeding For Example., warfarin were not included in this study. In an effort to achieve informed consent an information leaflet (Appendix -1) were sent out to all patients together with their appointment letter and a discussion of the leaflet was made prior to the consent (Appendix -2). A short clinical questionnaire was given to the patient to fill in before their endoscopy and information regarding age, sex, diet, smoking, alcohol consumption and drug intake were gathered (Appendix -3). This information was the only source of information and I did not corroborate this information by reviewing their medical records.

2.2 Endoscopic cytology brushings

During the upper GI endoscopic examination, endoscopic cytology brushes (gastric cytology brushes, Premier endoscopy) were used to exfoliate the cells from the gastric and oesophageal mucosa of various histological stages. The endoscopic procedure was prolonged by 2-3 minutes to collect the brushings and the biopsies needed for the study. Figure 2.1 shows an upper GI endoscope. Gastric brushings were performed first and then the biopsies were taken from the same site Figure 2.2 shows a cytology brush and Figure 2.3 shows a cytology brush coming out of a biopsy channel

from the upper GI endoscope. The brushings were performed first as taking the biopsies initially would have contaminated the cytology with red blood cells. If the area was normal the brush/biopsy were taken from the gastric antrum but otherwise they were taken from the abnormal area. This methodology has been described and successfully used by Doak S *et el* in our laboratory. Patients with bleeding tendencies, patients on anticoagulants and those with obvious bleeding lesions at the time of procedure were not included in the study. No patients reported any immediate complications following their endoscopic procedure. The patients were observed between two to four hours after their procedure before their discharge and were advised to seek medical attention if they noticed any bleeding.

Figure 2.1 Upper GI Endoscope

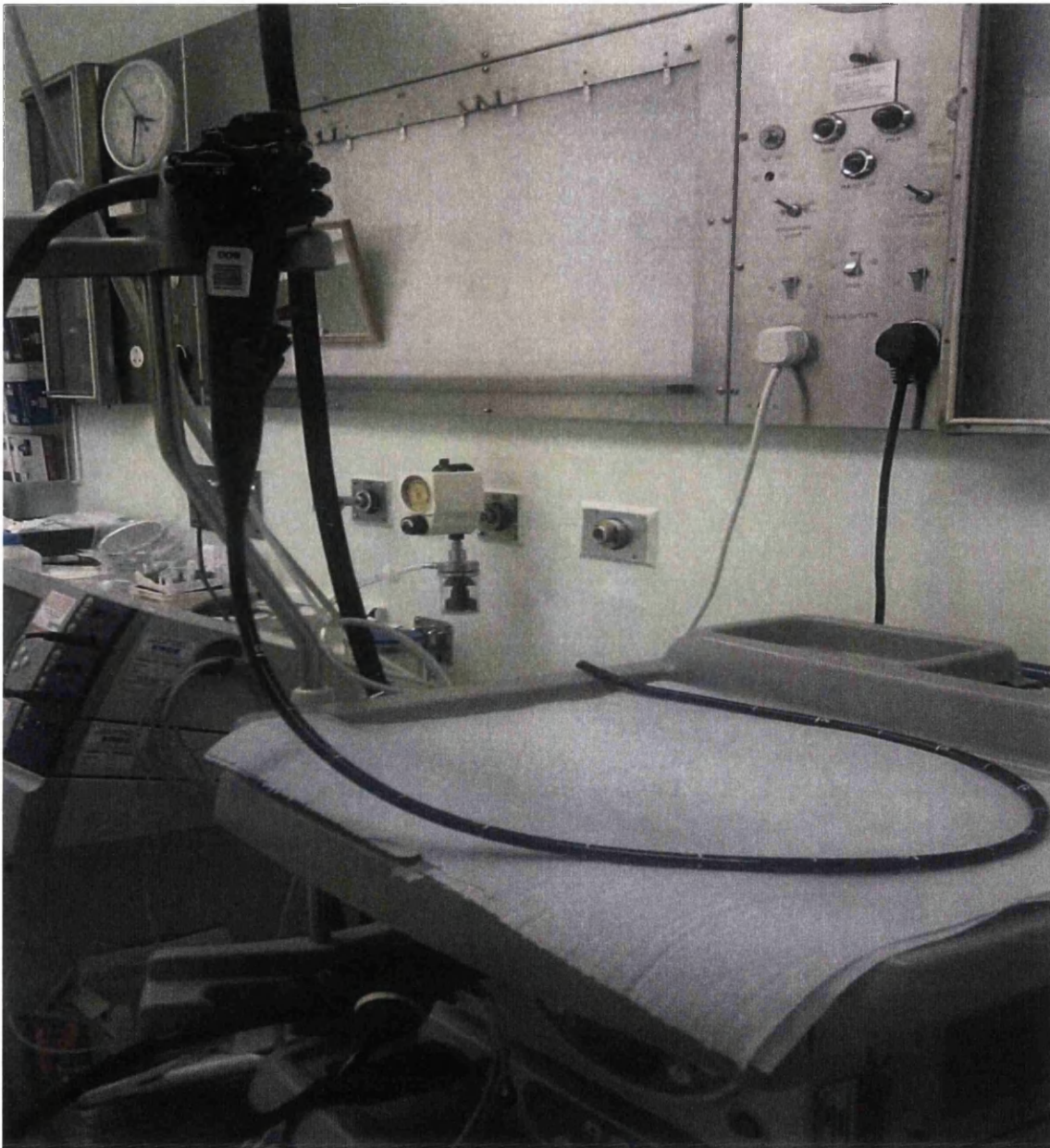


Figure 2.1 shows an upper gastrointestinal endoscope.

Figure 2.2 Cytology brush

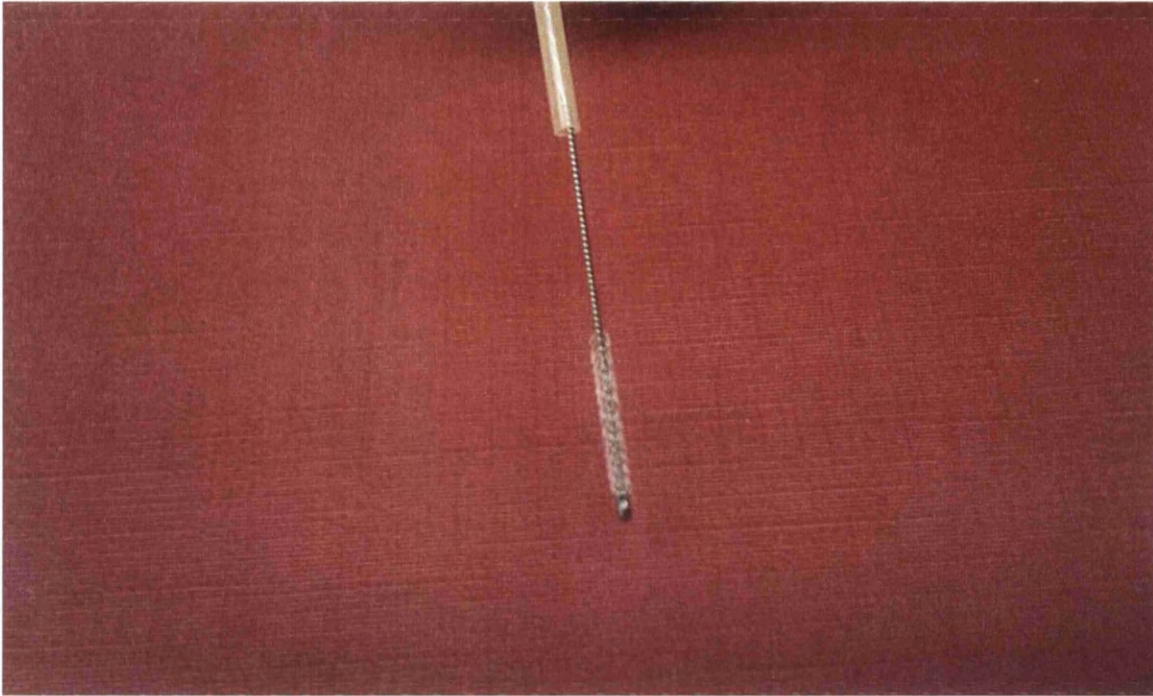
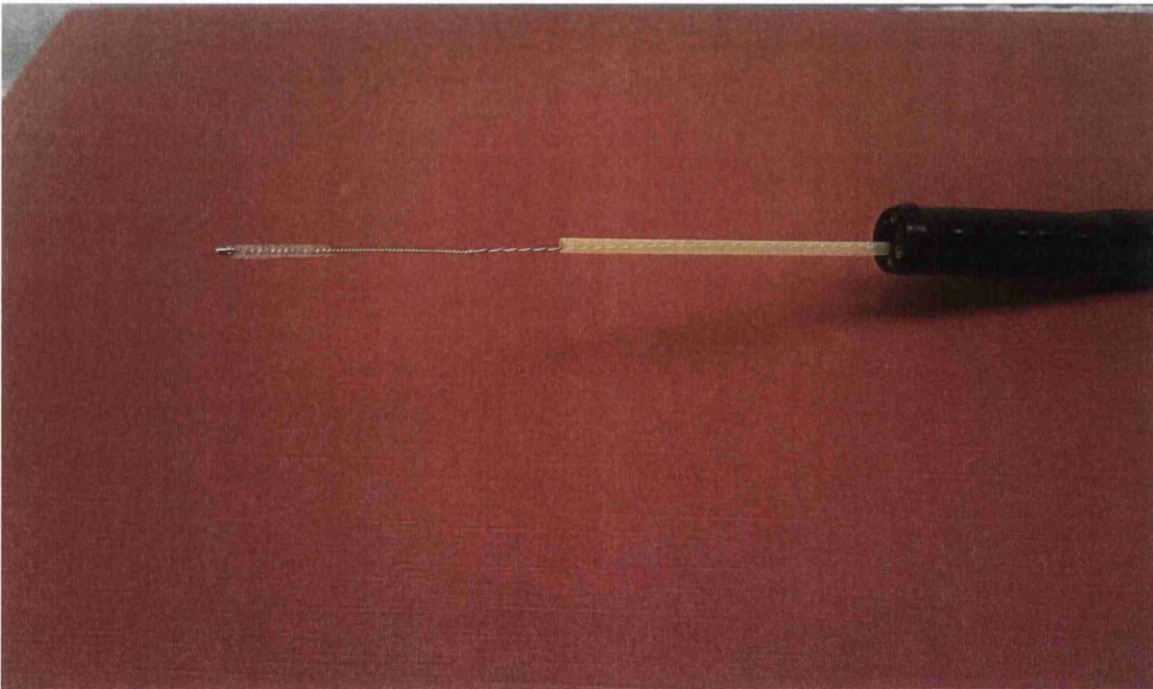


Figure 2.3 Cytology brush through an endoscope



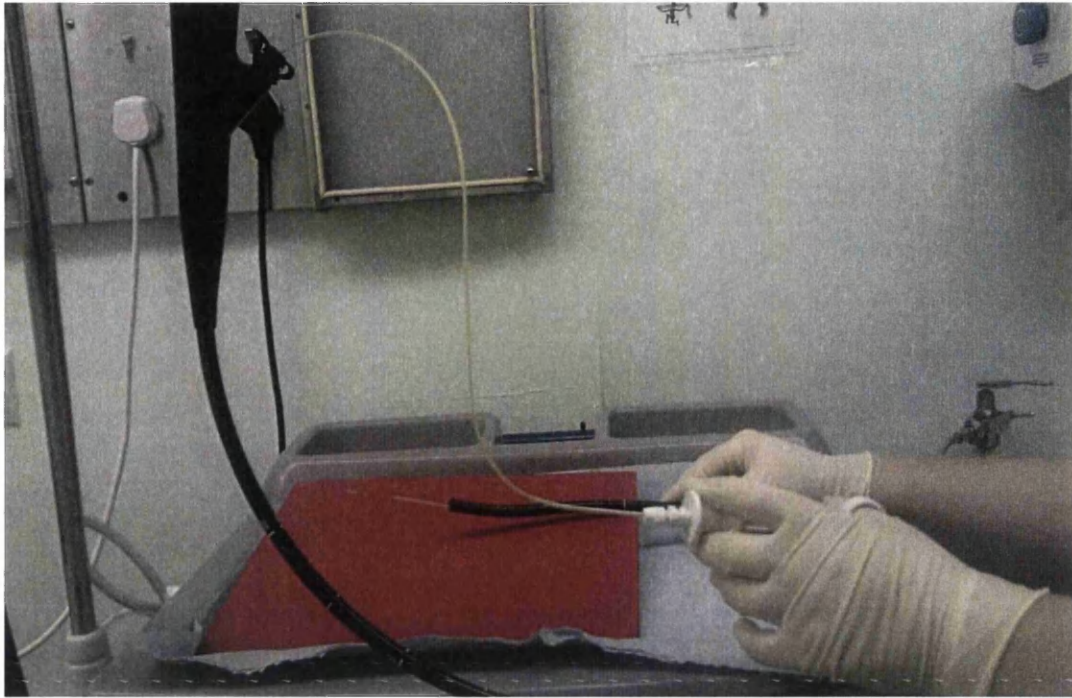


Figure 2.3 shows a close up view of a cytology brush coming out of a biopsy channel and the figure below shows how the cytology brush is used by an endoscopist to obtain gastric cells during an endoscopy.

The yield of gastric cells was poor during the initial stage of the study. This was thought secondary to the difficulty in maintaining good contact between the cytology brush and the gastric mucosa due to the angle between the gastric wall and the endoscope. The yield was also poor if any food residue, bile or blood were present in the stomach. The bile, food and blood were sucked through the endoscope, the area was flushed with 20 – 30 ml of sterile water and the excess water was sucked prior to the use of gastric cytology brush.

The cytology brushes were immediately placed into universal tubes containing 10 ml of ETN buffer (0.1M EDTA, 0.01M Tris-HCl, 0.02M Sodium Chloride, pH 7) as described for oesophageal brushes (Doak et al 2003), on ice, in the endoscopy department for transportation to the University for laboratory analysis. Vigorous shaking of brushes immediately after the brush was placed in the ETN buffer was found to improve the cell yield. The cells were usually harvested within 24 to 48 hours.

Occasionally, the cells were stored up to 4 days. At first, the brushes were transported from the Endoscopy unit in 10ml of 90% methanol, on ice, but in spite of harvesting these cells within 4 hours, the cell yield was unreliable and the medium was changed to ETN buffer.

2.3 Cell Cleansing

Gastric cells were difficult to harvest and were partially digested with no clear cell membrane or cytoplasm. The oesophageal cell yield was adequate most of the time. This improved with the change in the media to ETN buffer.

The cell suspension was centrifuged at 1500 rpm for 10 minutes and the resultant cell pellets were re suspended in ETN buffer. This washing step was repeated twice in order to remove cell debris and bacteria that would affect the scoring of the signals. If the cell pellet produced was small, the third wash was omitted but otherwise this was performed. The resultant cell pellet was re suspended with 0.5 ml of ETN buffer and a cytodot was produced on a glass slide using Cytospin (Thermo Shandon Cytospin 4). Larger pellets were re suspended in 1ml of ETN buffer.

2.4 Slide Generation from Cytology Brushings

Glass slides (pre- cleaned with 100% ethanol to remove the dirt) were assembled in a cytospin clamp with a filtercard and funnel according to the manufacturer's instructions and placed into a cytospin (Thermo Shandon Cytospin 4). One Hundred microlitres of the resultant cell preparation was added to the cytofunnel in the cytospin and spun for 5 minutes at 1200rpm to produce a cytodot. The cytodot was examined by a light microscope, and the respective cell suspensions was diluted or concentrated accordingly to ensure that an adequate number of single layered cells were present on the slides for the interphase FISH. The resultant slides were fixed in 90% methanol for 10 minutes, left to dry and then stored at -20C. An average of 2-5 slides was produced per sample.

2.5 Fluorescence *In situ* Hybridisation

2.5.1 Reagents

1. 20x SSC

3M NaCl plus 0.3 Tris – Sodium Citrate.

87.5grams NaCl/ Tris – Sodium Citrate in 50 ml of deionised water

2. 2xSSC

50 ml of 20x SSC in 450 ml of deionised water adjusted to pH7

3. 2x SSC/ 0.1% NP-40:

500µl of NP- 40 in 500 ml of 2x SSC adjusted to pH 7

4. 4xSSC/0.3% NP-40.

1.5ml of NP-40 plus 100 ml 2xSSC in 400 ml of deionised water adjusted to pH – 7.5.

All the above reagents were stored under ambient conditions for up to 6 months.

5. 70%, 80% and 95% ethanol made up with deionised water.

6. 1x PBS : 0.1M Na₂HPO₄ plus 0.1 M NaH₂PO₄ plus 0.1% w/v NP- 40

17.9gNa₂HPO₄ plus 7.8gNaH₂PO₄ plus 500µl NP 40 in 500 ml of deionised water

This reagent was stored at 4°C and discarded after 6 months.

2.5.2 Pre treatment of interphase cell preparation

Treatment with pepsin was performed to remove the cytoplasmic protein probe improving the penetration of the probes into the cells. Interphase cell preparations and 300µl/ ml of 0.01M of pepsin (pH 2.7-3) were incubated at 37°C for 10 minutes and then treated with pepsin at the same temperature. Several drops of pepsin were applied to the cytodot and they were left at 37°C. The cytodot treatment with pepsin was left for 5 minutes but this was reduced to 2 minutes as it was thought that treatment for 5 minutes could be responsible for over digestion of gastric cells resulting in the loss of nuclei. This suggested that partial gastric cell digestion occurs after the collection phase of the endoscopy and treatment with pepsin for more than 5 minutes could have

accelerated this process resulting in poor interphase FISH. The slides were washed in PBS for 5 minutes and followed by a further 5 minutes in PBS/MgCl₂ at room temperature. This is to arrest the enzymatic action of pepsin. The slides were then dehydrated with increasing concentration (70%, 80%, and 95% for 2 minutes each) of ethanol to prepare them for the denaturation step prior to FISH.

2.5.3 FISH probes

Commercially available centromeric enumeration probes (CEN) for chromosomes 1 and 4 (Abbott/Vysis Cat no Cep1 orange: 06J36-001 CEP4 green: 06J37-004) were used. The probe vials were briefly centrifuged for a few seconds before opening them in order to collect the probe at the bottom of their containers, thus minimising any loss of the probes.

2.5.4 Probe Mixture

A probe master mixture was made in a 0.5ml microfuge tube with the following components for each slide:

3.5µl of hybridisation buffer (supplied by the manufacturer with the probe)

0.5 µl of each probe for hybridisation

Purified water to bring the volume of probe plus water to 1.5µL (For example if using one probe: 0.5µL of probe plus 1µl of water and doubling these when using for two probes)

This mixture was pipette repeatedly to mix the contents thoroughly.

These two probes were used simultaneously and the FISH was performed according to a slightly modified manufactures instructions. In 51 samples both the probes were used at first but the signal from the probe used for chromosome 4 were nonspecific, therefore only chromosome 1 was studied during the latter part of the study involving 10 samples. 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 75degreesC hotplate for 2 minutes (the edge of the cover slip was sealed using a rubber cement) and was incubated in a humidified chamber which has been preheated to 37C for 30 minutes. The cover slip was removed and the slides were washed in 0.4 SSC solutions for 2 minutes for 2 minutes, followed by washing it again with 2 X SSC solutions at room temperature for 30 seconds. This was left to air dry in the dark. To the nuclei was countersigned by adding 10µl of DAPI (Vectorshield with DAPI Cat no. H-1200) to the slide.

2.6 Signal visualisation and scoring

An (Zeiss Imager Z1 Axio) microscope and (Bio Rad) software was used to score each slide. An average of 182 cells was studied per sample. Nuclei that were smeared or overlapping were excluded.

CEN probes highlight the centromere of the chromosome and were used to determine the entire chromosomal changes i.e., aneuploidy. A loss of a CEN signal said to be due to deletion of that chromosome and more than 2 signals denotes amplification. Slides were coded prior to scoring, with no knowledge of the histological details of the tissue samples. Figure 2.4 shows an example of a normal cell and an aneuploid cell.

2.7 Statistical analysis of chromosomal abnormalities

Paired two tailed student's t test was used to compare the chromosomal changes between the various histological diagnoses. Statistical significance was achieved if the p value was less than 0.05. Correlation coefficient was calculated between the age and aneuploidy levels. If the correlation coefficient was between -0.09 to 0.09, there was no correlation between these two variable, if the correlation coefficient is between -0.3 to 0.3, then the correlation between them is small, between -0.5to -0.3 or 0.5 to 0.3, then the correlation between these two variables are medium and the correlation is considered to be strong if the levels are between -.05to-1.0 or 0.5 to 1.0. Standard error of mean is used as it is an estimate of the amount that an obtained mean may be expected to differ by chance from the true mean. The smaller the standard error, the

more representative the sample is to the overall population. The standard error also depends on the sample size. It is inversely proportional to the sample size. For e.g. the larger the sample size, the smaller the standard error because the statistic will approach the actual value.

2.8 Histological Diagnosis of tissue biopsies

The histological diagnosis of the endoscopic biopsies taken at the Neath Port Talbot Hospital were determined by Dr AP Griffiths, Consultant Histopathologist based at the ABM University Hospital and also the histologists based at the Royal Glamorgan Hospital.

2.9 Gastric Biopsies at endoscopy

During the upper GI endoscopic examination, gastric biopsies were obtained from the same area after the cytology brushing was done. Two biopsies were taken from the same area of brushings and a third biopsy was obtained if the patient tolerated the procedure. A typical endoscopy takes approximately 5 minutes and taking the samples prolonged the procedure by an extra 2-3 minutes as they have to go into the relevant transporting medium and labelled before proceeding to the next sample. It was not always possible to get the third sample for various reasons. Of the three biopsy samples, the first was stored in RNA later, the second one was sent to histological analysis and the third one was used to perform CLO test (Bripharm Ltd., UK).

2.10 *H. pylori* testing method

Gastric biopsies were obtained during the endoscopy; one of the biopsies was used for the detection of *H. pylori* infection. CLO test was used for this in the endoscopy unit. The biopsy was placed on a special slide containing urea and an indicator such as phenol red. The urease produced by *H. pylori* hydrolyzes urea to ammonia, which raises the pH of the medium and this leads to a change in colour (yellow to start with, changed to red/dark pink if it is positive). This may sometime take up to 24 hours and the results are read the day after the endoscopy. There are rapid CLO

test available which can be read within 30 minutes of performing the test and is this is useful in letting the patients of their condition and the need to treat it on the day of the procedure.

2.11 Precautionary measures to prevent RNA contamination

To prevent cross contamination between samples and prevent carryover of nucleic acids from one experiment to another the following precautions were taken:

1. The workstations were wiped with commercially available RNase free solutions.
2. The samples were prepared in a laminar flow hood equipped with a UV lamp and pre cleaned with RNase free wipes (Ambion UK, Cambridgeshire).
3. This area was different from the thermal cycler area to avoid cross contamination with plasmids or amplicons.
4. Gloves were changed frequently between each stage of procedure.
5. Only RNase free filter pipette tips were used.
6. A no- template control was used to all PCR assays to verify that no contamination has occurred during the procedure.

2.11.1 RNase free wipes

Ribonucleases (RNase) is a type of nuclease that degrades RNA into smaller components. They play an important role in the nucleic acid metabolism. They are found in both prokaryotes and eukaryotes and are present in almost every cell type. The human body secretes RNase in tears, saliva, mucus and perspiration and this is used in the defence against invading microorganisms. The primary source of RNase is microorganisms like bacteria, fungus, and their spores. RNase contamination can easily occur in the lab as bacteria, fungi and their derivatives (Eg. restriction enzymes, polymerases) are widely used in the experiments. The prevention, detection and elimination of nuclease contamination are a constant challenge to anyone working with RNA in the lab. All intracellular RNAs are protected by various strategies including 5'end capping, 3'end polyadenylation, and folding within an RNA protein complex (ribonucleoprotein particle or RNP). RNases like RNaseA and T1 are secreted in large

amounts by non specific cells. These can render any RNA that is not in a protected environment to degrade rapidly.

For this entire study, various precautionary measures were taken to ensure that the experiments were performed in an RNase free environment. This has been listed earlier in this section. Laboratory surfaces such as bench tops, centrifuges and pipettes should be assumed to be contaminated with RNAases and necessary precautions taken to get rid of them before starting the experiments. Elimination of RNases from these surfaces is easy and can be done by using RNase decontamination solution such as Ambion's RNaseZAP. RNaseZap is a combination of three different chemicals that will completely inactivate RNases immediately on contact. This solution is sprayed directly to the surface and wiped clean with nuclease free water. RNase wipes are towelettes that are pre-soaked with RNAase decontamination solution (Ambion RNaseZap wipes was used in this study). These are particularly convenient for decontaminating pipettes, work tops and other surfaces.

2.12 Extraction of RNA

The gastric tissue sample was cut into small pieces and transferred into a 2.0mL safe- lock micro centrifuge tube containing 500 μ L TRIZOL reagents. The sample was then homogenised using a rotor- stator homogeniser for 30 seconds and was incubated at room temperature for 5 minutes. The homogenised mixture was immediately frozen in -80 $^{\circ}$ C or immediately proceeded to phase separation. Almost all the samples underwent immediate phase separation. One hundred micro litres of chloroform was added to this and the resultant mixture was vortexed for 20 seconds, followed by incubation for 5 minutes at room temperature. The resultant mixture was then centrifuged at 12,000 revolutions per minute in a pre – cooled centrifuge at 4 $^{\circ}$ C. Following centrifugation, the mixture separates into a lower red, phenol – chloroform phase, an opaque interphase, and an upper colourless aqueous phase. The RNA remains exclusively in the aqueous phase. Precaution was taken not to disturb the resultant centrifuged mixture and the red chloroform phase and the opaque interphase were discarded. The aqueous phase was pipette using a RNA free filter pipette tips and transferred to a 2.0 mL safe – lock micro centrifuge tube and 70 % ethanol was added

to bring the total volume to 350 μ l. The resultant mixture is mixed thoroughly by using a pipette with a RNA free filter tip.

Seven hundred microlitres of the sample was transferred to an RNeasy column placed in a 2 ml collection tube and the flow through is discarded. Three hundred and fifty μ L of buffer RW1 was added to the RNeasy spin column and this was centrifuged at 10,000 rpm to wash the spin column membrane. The flow through was discarded. Ten μ L of the DNase stock solution was added to the 70 μ L of the buffer RDD. This was mixed by gently inverting the tube and centrifuging briefly to collect any residual liquid from the sides of the tube (RDD buffer is supplied with the RNase – Free DNase set). The DNase incubation mix (80 μ L) was added directly to the RNeasy spin column membrane, and placed on the bench top at 20-30 $^{\circ}$ C for 15 minutes. Three hundred and fifty μ L of RW1 buffer was added to the RNeasy spin column and this was centrifuged at 10,000 rpm and the flow through was discarded.

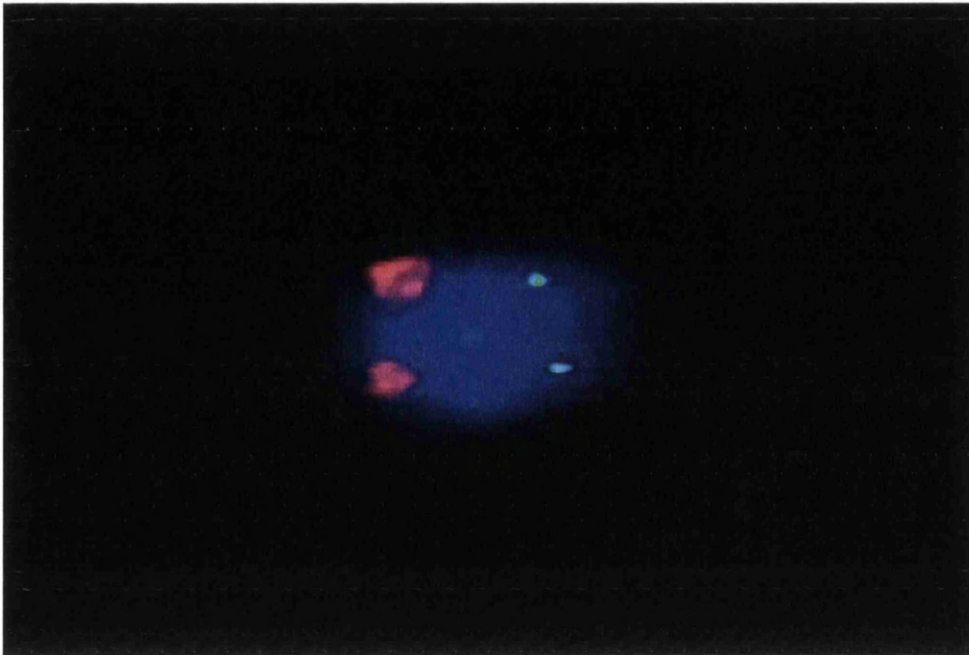
Add 500 μ L of the buffer RPE (buffer supplied by the supplier) to the RNeasy spin column. The lid was closed gently and centrifuged at 15rpm to wash the spin column at 8000rpm. The flow through was then discarded. The collection tube was used later. After centrifugation, the RNeasy spin column was carefully removed from the collection tube so that the column did not contact the flow – through. The RNeasy spin column was placed in a 1.5 μ L collection tube and 30 -50 μ L of RNase free water was added directly to the spin column membrane. This was centrifuged at 8000 rpm for a minute to elute the RNA. If the expected RNA yield was less than 30 μ gm, this step was repeated to obtain more RNA from the sample. Each resultant sample RNA was divided into 10 μ l aliquots, stored at -70 $^{\circ}$ C and discarded after freeze/thawing twice.

2.13 Reverse transcription of RNA

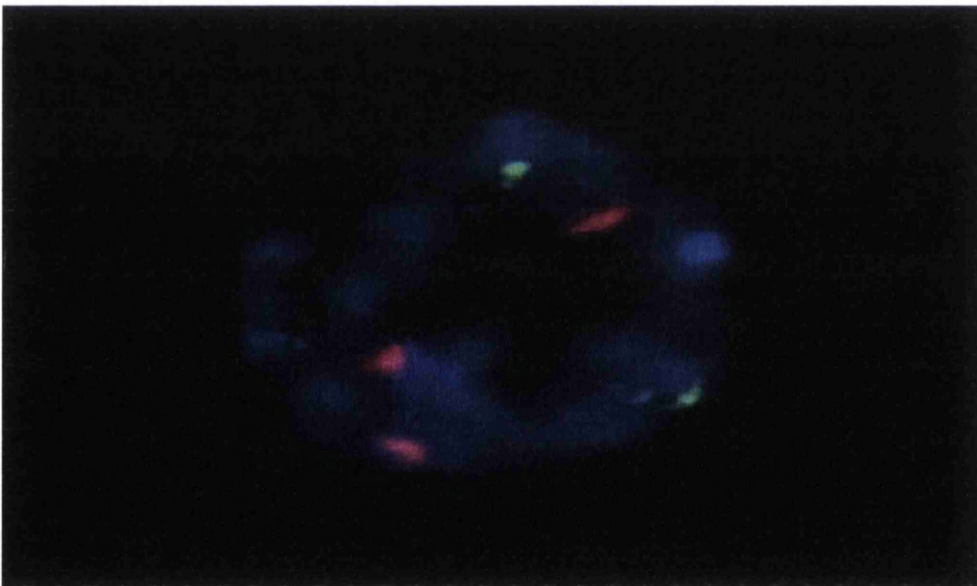
A 10 μ l aliquot of RNA obtained and stored at -80°C and a reference RNA, also stored in -80°C were thawed per PCR plate at 4°C . The gDNA wipe out buffer, Quantiscript reverse transcriptase, Quantiscript RT wipe out buffer, RT primer mix (Qiagen quantiscript reverse transcription kit) and RNase free water were also thawed in a lamina flow hood that has been thoroughly pre cleaned with RNase wipes (Ambion UK, Cambridgeshire) at the room temperature for approximately 15 minutes.

The gDNA reaction was performed in a 0.2ml microfuge tubes... The RNA sample were normalised to $1\mu\text{g}$ RNA in $12\mu\text{L}$ RNase free water. Two micro litres of gDNA wipe out buffer was added to the $12\mu\text{l}$ of RNA sample to make up a total volume of $14\mu\text{L}$. The resultant mixture of RNA and gDNA wipe out buffer was vortexed briefly for few seconds to allow thorough mixing of the RNA sample and gDNA wipe out buffer, followed by centrifugation and incubation for 2 minutes at 42°C using a gDNA protocol on BIO-RAD *i* cycler. Figure 2.4 shows the gDNA reaction conditions.

Figure 2.4 A normal and aneuploid cell.



Normal Cell with diploid nuclei. The two red dots represent chromosome 1 and the two green dots represent chromosome 4.



Aneuploid y of chromosome 1

The RT master mix was prepared by mixing:

- 1 μ L of Quantiscript reverse transcriptase,
- 4 μ L of Quantiscript RT buffer
- 1 μ L of RT primer mix

This was prepared by initially adding 1 μ l of RT primer mix followed by 4 μ l of quantiscript RT buffer and adding the 1 μ l of reverse transcriptase just before the RNA sample is taken out from the BIORAD *icycler* machine. This was vortexed briefly to allow efficient mixing of all the constituents and the resultant solution was centrifuged briefly. The total amount of reverse transcriptase master mix solution required, during that particular experiment was calculated by multiplying the amount required for each sample i.e., 6 micro litres by the number of samples. Six micro litres of this RT master mix was added to each gDNA elimination reaction (14 μ L), resulting in a total volume of 20 micro litre of total volume. This was vortexed briefly to allow them to thoroughly mix and was centrifuged following this

This was then taken back to the BIORAD *icycler* (Figure 2.6 show the Bio Ra RT PCR machine) and the reverse transcription reaction was performed by using RT protocol setting on BIO-RAD *i cycler*. This was a predetermined setting and was not changed thorough the study. The only variable which could be changed was the volume used during a particular experiment, which was again set at 20 μ l thorough out the study. The RT protocol involved incubating the samples for 15minutes at 42 \square C, followed by 3 minutes at 95 \square C. Figure 2.5 shows the conditions used in the reverse transcriptase procedure. The reverse transcription negative controls were not performed. The primers used were intron spanning and were optimised in previous experiments performed in our lab, thereby minimising the carry over of any gDNA. Also, gDNA digestion buffer was used in the RNA extraction process and also gDNA wipeout buffer was used during the reverse transcriptase reaction.

Figure 2.5 Reverse Transcriptase Procedures

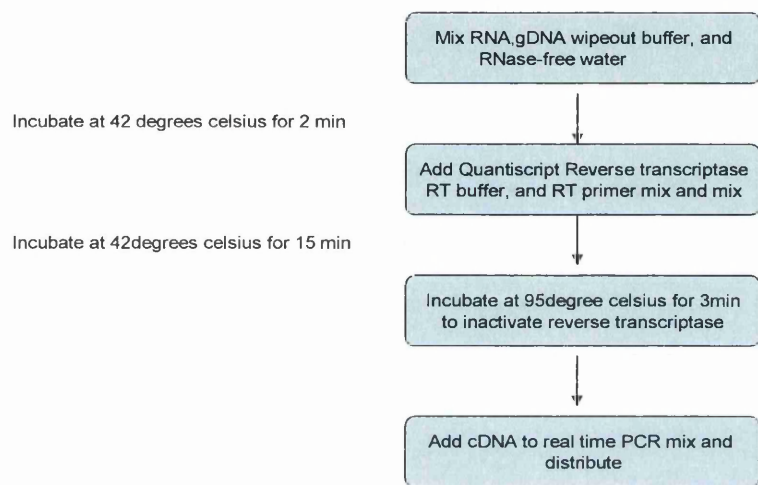


Figure 2.6 Bio Rad RT PCR machine



Figure 2.6 shows the Bio Rad RT PCR machine.

Serial dilution of reference cDNA was made in order to get a 1:10, 1:100 and 1:1000 dilution of the reference RNA. This was achieved by pipetting 18µL of RNase free water in three 0.2mL tubes and they were labelled as 1:10, 1:100 and 1:1000. Two µL of neat reference RNA was added to the first tube labelled as 1: 10 and this was mixed by vortexing briefly and centrifuging for 1- 2 minutes approximately. Two micro litres of this solution (1 in 10 dilutions) was added to the tube marked 1:100 (this has 18 µ l of RNase free water) and this was vortexed and centrifuged. Two micro litres of 1 in 10 dilution solution was added to 18 µl of RNase free water, giving a dilution of 1 in 100. The resultant solution of a 1 in 100 dilution was briefly vortexed and centrifuged for 1 – 2 minutes. Two micro litres of 1 in 100 dilution solution was added to the tube marked 1:1000 (this has 18 µl of RNase free water) and the resultant solution was vortexed briefly and centrifuged for 1 – 2 minutes giving a 1 in 1000 dilution.

2.13 Primers

Primers are designed to amplify a fragment of cDNA 100-150bp in size from the MAD2, BUB1 and β Actin genes. The primers are intron spanning and were designed and optimised in our lab for an earlier study(Doak et al 2003) In order to allow analysis of all the targets simultaneously using the same thermal cycling conditions, the primers were designed to anneal at the same the same temperature, the resultant primer sequences are detailed in the following Table 2.1

Table 2.1 Real time RT-PCR primer sequences

Gene for amplification	Forward Primer Sequence	Reverse Primer Sequence
MAD2	GGTCCTGGAAAGATGGCAG	ATCACTGAACGGATTTTCATCC
BUB1	AGGATCTGCCCGCTTCCC	GTCGTCTGATAGGTTACTGG
B Actin	GATGGCCACGGCTGCTTC	TGCCTCAGGGCAGCGGAA

2.14 Reference gene

The house keeping or reference genes are routinely used in qPCR to normalise experimental data, such as differences in RNA quantity and quality, the overall transcriptional activity and differences in cDNA synthesis (Thellin et al 1999). Theoretically, all reference genes are supposed to exhibit consistent, non – regulated, stable expression among different tissues (Radonic et al 2004, Vandesompele et al 2002). However, cancer development is a very complex stepwise process involving altered cell functions at many steps, through changing almost all genes in gene expression (Brumy et al 2005, Lyng et al 2008). Many experimental evidences indicate that even the so called housekeeping genes (HKSs) are involved in tumorigenesis, including breast, prostate, colorectal, and bladder-cancer (Lyng et al 2008, Hsiao et al 2001, Buttè et al 2001, De Kok 2005, Schmittgen et al 2000, Goiding et al, Ohl et al 2005, Khimani et al 2005). Typical housekeeping genes including glyceraldehydes 3-phosphonate dehydrogenase (GAPDH), beta-actin (ACTB), TATA-binding protein (TBP), 18S ribosomal RNA (18S) and many more have been adopted from the literature without taking into account their specific tissue dependent behaviour or the special design of the respective study (Vandesompele et al 2002, De Kok et al 2005, Khimani et al 2005). The possibility of deregulation of the so called housekeeping genes for qPCR normalisation in cancer research may lead to unreliable results and misinterpretation of the results (Schmittgen et al 2000, Ohl et al 2005, Dheda et al 2005). A comprehensive literature review of expression studies in high impact journals showed GAPDH, ACTB, 18S RNA and 28sRNA were used as a single control gene in more than 90 % of cases (Vandesompele et al 2002).

Reference genes have been described for RT-PCR in various studies in various cancers of other tissues (Jung et al 2007, Huang et al 2003, Gao et al 2008). However, there seems to be no consensus on reference genes for gene studies in stomach cancer (Hyun et al 2010). Hyun et al found out 115 articles published between May 2007 to November 2009 – GAPDH (46%) and ACTB (35.7%) were the most frequently used reference genes in gastric cancer studies. They also showed that ACTB and GAPDH

showed most abundant expression in both 'stomach cancer cell lines' and 'non stomach cancer cell line'. Hyun et al also studied the stability of various reference genes and the best single reference gene for each group as follows ; 'non- stomach cancer cell line'- GAPDH, 'stomach cancer cell lines' – RPL29(ribosomal protein29),'tumour stomach tissue' –RPL29,and 'all stomach cell lines and tissues'- ACTB. Beta actin (ACTB) was used in this study as it is one of the most commonly used housekeeping genes in the gastric cancer study. The choice of reference gene should depend on the cell lines or/tissue under study and there is no single, universal optimal reference gene.

2.15 Limitations of SYBR green

Real time PCR can be performed by using probe based systems –

1. Taqman probes (Heid et al 1996)
2. Molecular beacons (Piatek et al 1998)
3. Fret probes (Chen et al 1999)
4. Scorpions (Solinas et al2001)
5. iFret probes (Howell et al 2002).

An alternative to the probe based system is the use of fluorescent double stranded DNA (dsDNA) – specific intercalating dyes and the example of this are YO-PRO -1(Ishiguro et al 1995) SYBR green1 (Wittwer et al 1997, Ririe et al 1997), BEBO (Bengtsson et al 2003), and LC Green (Witter et al 2003) and they have been tested in real time PCR applications.

SYBR green 1 is used commonly as it is cost effective compared to the probe based system and it also allow for generic detection of amplified DNA, and can be used to differentiate DNA by DNA melting curve analysis (Wittwer et al 1997, Ririe et al 1997).However, the limitations of SYBR is that it needs to be optimised and certain agents needs to be added to improve reaction efficiency, such as bovine serum albumin and Triton- X100 (Bengtsson et al 2003). The SYBR green used in this study was an optimised Super mix produced by BIO RAD systems, which contained 2 reaction

buffers, iTaq DNA polymerase, Magnesium hydrochloride, SYBR green1, fluorescein, and stabilisers.

Another limitation of the SYBR green is that depending on the reaction conditions, the dye also appears to have an inhibitory effect on the PCR in a concentration dependent manner (Wittwer et al 1997, Witter et al 2003). This has been shown to be overcome by increasing the concentration of magnesium chloride in the reaction (Bengtsson et al 2003, Witter et al 2003). The degradation products of the dye have been shown to be inhibitory to the PCR reaction (Witter et al 2003). A further limitation of SYBR green 1 is that it appears to have limited application for the analysis of multiplex PCR and has selective detection of amplicon during DNA melting curve analysis of multiplex PCT. In a study by Giglio (2003) et al multiplex PCRs for *Vibrio Cholerae* and *Legionella pneumophila* analysed by DNA melting curve analysis using SYBR green found only one amplicon could be detected by melting curve analysis but both amplicons were amplified as determined by agarose gel electrophoresis.

2.16 Real time RT PCR

All reactions were set up in a laminar flow fume hood using sterile nuclease free pipette filter tips. The fume hood and all equipment to go into it were cleaned with RNase free wipes prior to use and no tip boxes were opened outside the hood.

The components of each reaction included:

12.5 µl of 1Q SYBR Green Super mix (BioRad, Hertfordshire, UK)

µl of Forward Primer

µl of Reverse Primer

BIO-RAD *iQ* SYBR Green super mix, primers (10µM forward and reverse) and RNAase free water were thawed at room temperature.

A PCR master mix were prepared for each gene i.e., MAD2, BUB1 and β actin in 1.5ml tubes for all reactions. Twelve and half micro litres of SYBR green, 5µL of

forward and reverse primers, 6 μ L of nuclease-free water were mixed for each reaction. A total amount of 450 μ L of SYBR green, 180 μ L of primers, 216 μ L of nuclease-free water was prepared for each 96 well 0.2 ml PCR plate (BioRad, Hertfordshire), which was used for the three genes used in this study (MAD2, BUB1 and β actin). The PCR master mix was vortexed briefly and centrifuged for 2 – 3 minutes to ensure that the exact component quantities were present in well in the PCR plate.

Seventy five micro litres of this PCR master mix is aliquoted into 0.2mL tubes, and 5 μ l of the sample DNA was added to this. Five micro litres of the diluted reference cDNA was added to each aliquot of the 75 μ l of PCR master mix and 5 μ l of RNase free water was added to the no template control tube (this was included to as negative controls). All of these were vortexed briefly and centrifuged for 2-3 minutes. Twenty five microlitre of PCR master mix/cDNA (sample DNA, reference DNA in serial dilution and no template control) was pipetted to a 96 well plate in triplicates, ensuring exact quantities were present in each triplicate. Figure 2.7 illustrates a 96 well plate. Once all 25 μ l reactions were loaded into the wells, the plate was sealed with Optical Sealing Tape (BioRad, Hertfordshire, UK). This was centrifuged briefly to collect all the contents in the bottom of the wells. The sample plate was then slotted into an *iCycler iQ* Thermal cycler (BioRad, Hertfordshire, UK) and real time PCR was performed using a PCR program 'Sher 60'. The cycling conditions for QPCR are illustrated in table 2.2. The programme was performed with the setting of persistent well factors, which was used throughout the run. The data obtained was stored in the computer attached to BioRad *icycler*.

Table 2.2 Cycling conditions of qPCR

	Cycle1		Cycle2		Cycle3	Cycle 4	Cycle5
	Step 1	Step1	Step2	Step3	Step1	Step1	Step1
Repeats	1		40		1	1	40
Dwell time (Mins)	3.00	0:30	0:30	0:30	0:30	0:30	0:30
Set point	95.0	94.0	60.0	72.0	55.0	95.0	55.0
			PCR				Melt curve

End temperature of 94°C.

Table 2.2 shows the cycling conditions used in the *Sher 60* protocols. There are three major steps in PCR, which are repeated for 30-40 cycles. During Cycle 1 denaturation takes place at 95°C, the double stranded DNA melts open to single stranded DNA, all enzymatic reactions stop. During the cycle 2, annealing occurs when ionic bonds are constantly formed and broken between the single stranded primer and single stranded template. The more stable bonds lasts a bit longer and on that little piece of double stranded DNA (template and primer); the polymerase can attach and start copying the template. Once there are few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore. At 72°C, extension occurs and this is the ideal temperature for the polymerase. As both strands are copied during this, there is an exponential increase in the number of copies of the gene.

Figure 2.7 96 Well plate

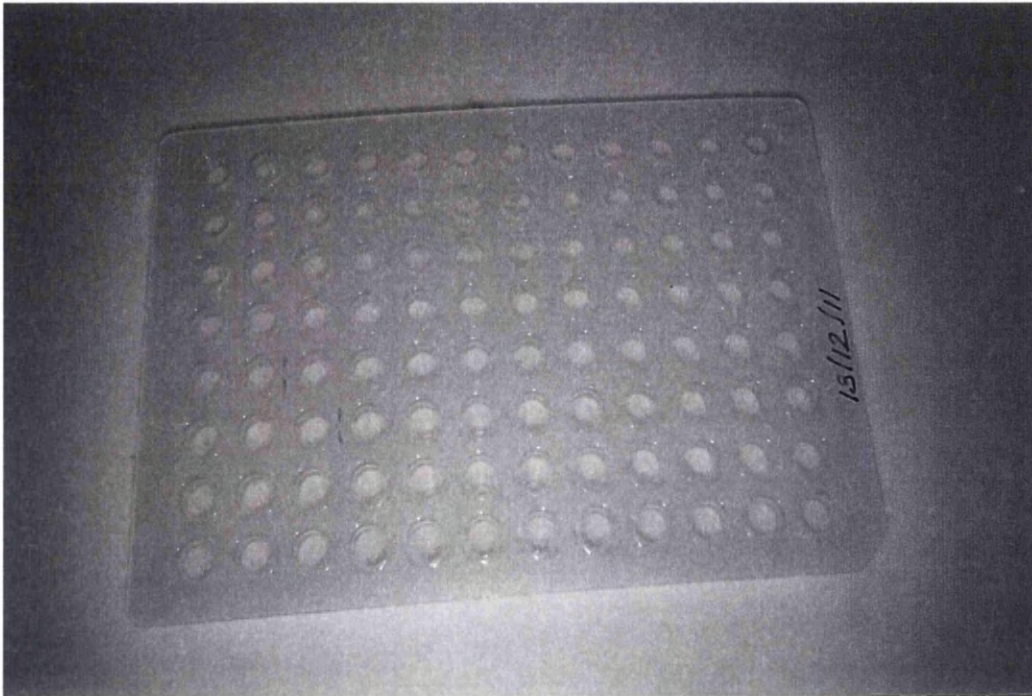


Figure 2.7 showing a 96 well plate that was used for the RT-PCR.

2.17 Data analysis

All data analysis was performed on the iCycler iQ5 software version 2.1 and each sample was individually analysed.

The melting curves generated for all the samples were first analysed to determine the specificity of the PCR products that has been generated. Samples without any correct melting curve were removed from the subsequent analysis. An example of meltcurve is shown in figure 2.8. Samples that did not have correct melting temperatures were excluded from the study.

Figure 2.8 Primer test – Melt curve

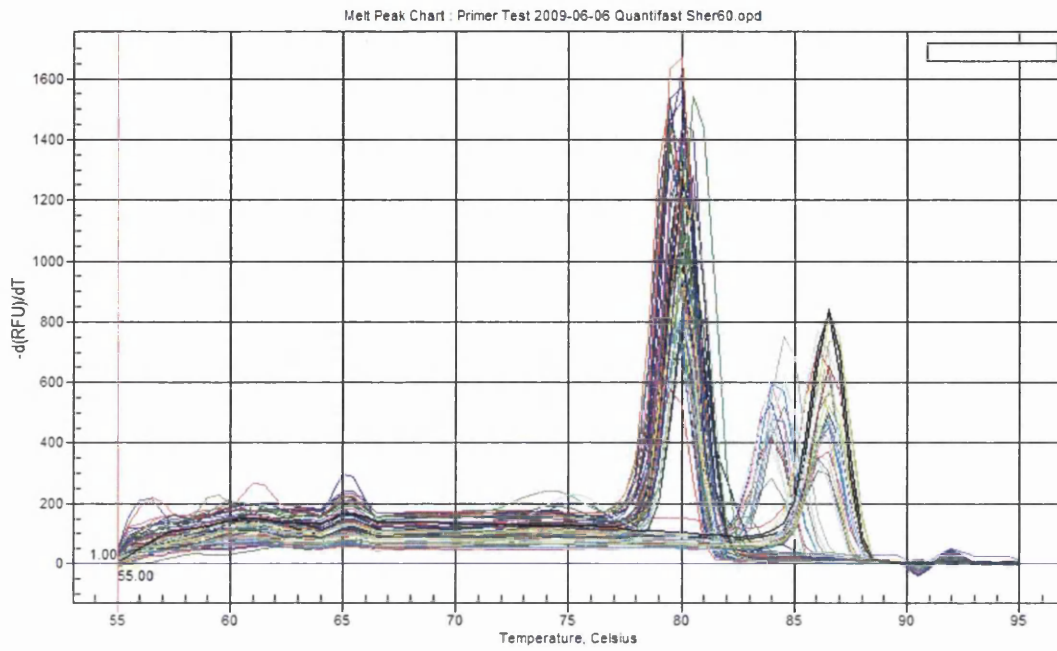


Fig 2.8 shows example of a melt curve for a MAD2, BUB1 and ACTB PCR products.

The software calculated the C_t value (the threshold value – this is calculated by detecting the fluorescent signals over the baseline cycle range and then setting it fixed to fixed threshold level at a statistically significant point above the baseline). Once set, the resultant C_T is recorded for each member of each triplicate is examined for any individual outliers and if there are any outliers, these were removed and regarded as an invalid data as it could have been due to experimental error for example pipetting etc., and as this could potentially skew the results. Figure 2.9 shows an amplification plot for a sample with its C_T (threshold cycle).

The software used then generated a standard curve from the β actin dilution samples and this was used to assess the quality of experiments involving the extracted DNA. The efficiency of each PCR reaction was determined by an equation

$$E = [10^{(-1/a)}] - 1, \text{ where } a = \text{slope of the standard curve.}$$

An optimum efficiency of 1.0 is obtained when the standard curve slope is -3.3.

Figure 2.10 shows the examples of typical real time PCR standard curves.

Figure 2.9 Amplification plot for a sample with its C_T (threshold cycle)

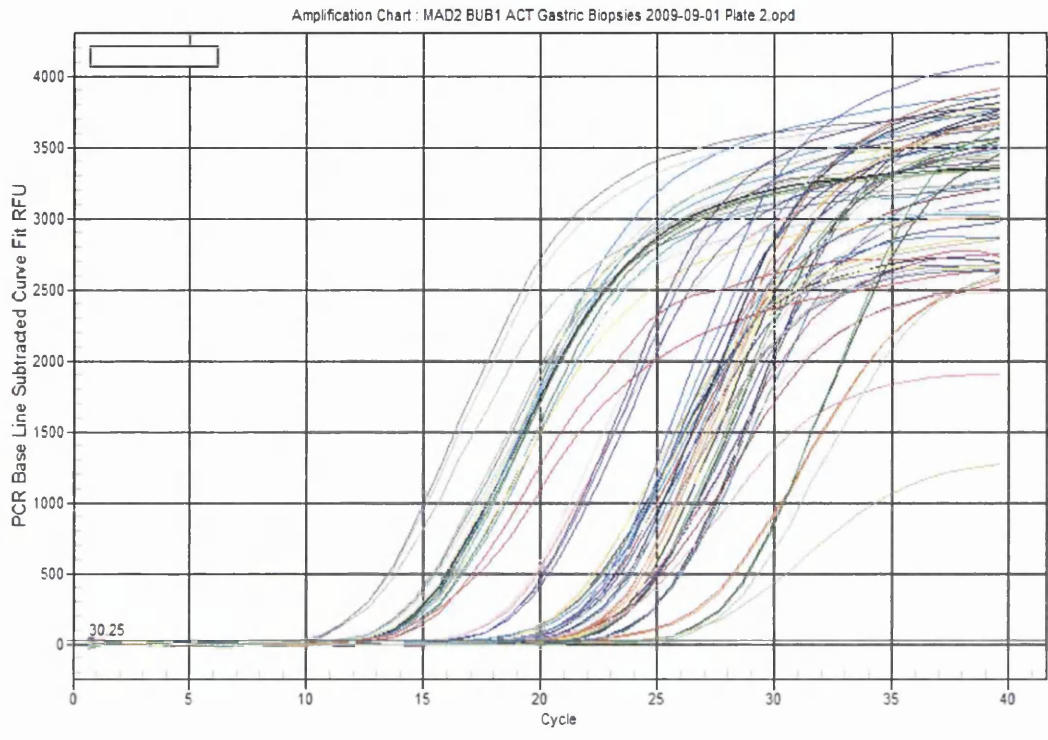


Fig 2.9 shows an amplification plot for a sample in triplicate illustrating its C_T (threshold cycle) and the plots baseline.

Figure 2.10 Example of a typical realtime PCR standard curve

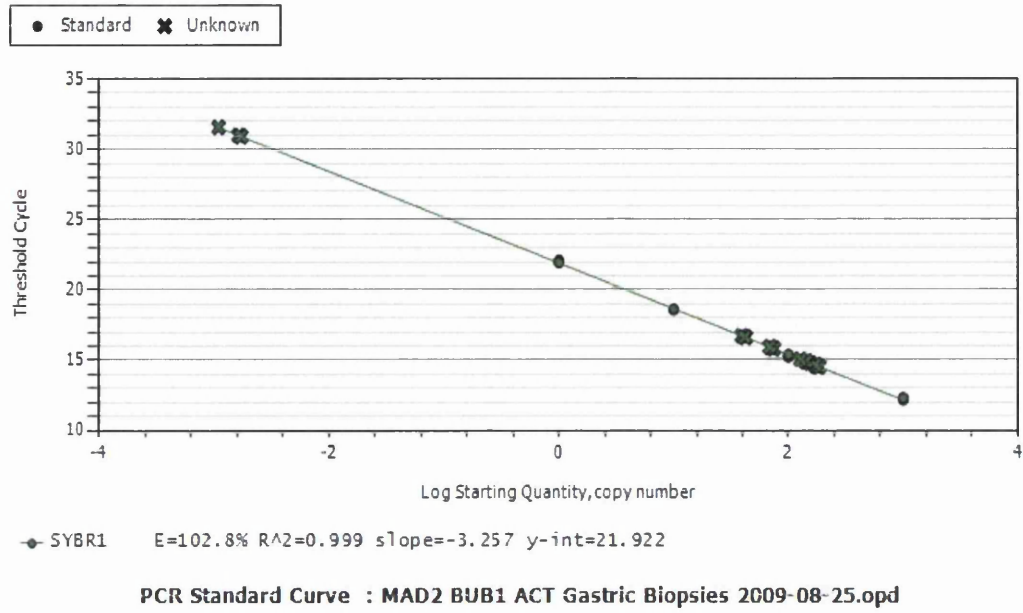


Figure 2.10 shows the examples of typical real time PCR standard curves.

2.18 Statistical analysis of the gene expression levels

Paired two tailed student's t test was used to compare the MAD2, BUB1 expression levels between differing histological diagnoses. Statistical significance was achieved if the calculated p values were less than 0.5, documented as $p < 0.05$. Again, Correlation coefficient was calculated between the age of the patients and the MAD2 and BUB1 expression levels and the strength of correlation coefficient is calculated as explained above.

2.19 Justification of the statistical methods

T-test was used as it is a simple, straightforward, easy to use and adaptable to a broad range of situations, T-test is most commonly used method to evaluate the differences in means between two groups. T –test can be used even in the sample sizes that are very small. Paired two tailed T-test was used to look for variation of statistical significance in both directions of normal distribution in the given sample. Correlation coefficient was used to look for any correlation between two variables like age and aneuploidy as it measures the strength of linear relationship between the two variables.

2.20 Histological analysis of the gastric samples

All the samples used for the real time PCR were obtained at the Neath Port Talbot hospital. The histological staging was performed by Dr. AP Griffiths at the ABM University Hospital.

Chapter 3

The analysis of chromosome abnormalities in gastric premalignant tissue using Interphase Fluorescence In situ Hybridisation (FISH)

3.1 Introduction

Correa's pathway suggests that there is a multistep precancerous process that leads eventually to the development of gastric cancers. Gastric carcinoma is a result of various genomic changes that affects the cellular function and results in cancer development. The studies looking at the cytogenetic abnormalities in gastric cancer are summarised in table 3.1.

Table 3.1 The results of a number of cytogenetic studies looking at chromosomal aberrations in gastric cancer.

Author	Sample size	Cancer type	Chromosome
Panani 1995(Greece)	11	10/11 intestinal (3 metastatic)	3,- gain and loss 6- gain and loss Translocation between chromosome 3 and 5 Trisomy of 8 in one patient.
Kokkola 1998 (Finland)	22	Intestinal	Gain-20q, 17q,loss-18q,4q
Koo 2000 (South Korea)	37	50% intestinal (22/37 metastatic)	Gain-8q, 7pq,13q,7 q Gain-18q,20pq,loss-17p
Fringes 2000 (Germany)	20	Intestinal/diffuse	Gain-1(63%),17
Beuzen 2000 (France)	60	Cardia, antrum and oesophageal	7,8,11,17,18,Y (40-65% all cancers)
Han 1996 (South Korea)	18	Diffuse	Widespread numerical rearrangements
Chun 2000 (South Korea)	6	Advanced	Loss-17p (100%) Gain-7
Igashari 2000 (Japan)	39	Gastric carcinoma	1p lost in advanced gastric carcinoma
Kitiyama 2000 (Japan)	24	Various stages of gastric carcinoma	1,2- early 1,2,4,20- later

Sud 2001 (UK)	26	17 intestinal 9 diffuse	Loss-22q,14q,4q,17q
Wu 2001 (Taiwan)	53	65% advanced 55% intestinal 45% diffuse	Gains- 8q, 6q, 11q, 13q, 7p, 17p, 20p Loss- 16q,19p,5q,3p,4q,1p
Sugai 1999 (Japan)	99	65 intestinal 34 diffuse	Aneuploidy in 73%
Kitayama 2003 (Japan)	51		Aberrations of chromosome 1,8,17,20 and X
Rodriguez 1990	9	Gastric and oesophageal adenocarcinoma	11p 13-15 in 8
Falchetti (2008)	159	All types of Gastric cancer	Microsatellite instability
Ottini(2006)	Review	All types of Gastric cancer	Microsatellite instability
Hamamoto(1997)	15	Gastric cancers with precancerous lesions	Frequent alteration at the locus D1S191(1q)

In this series of *in vivo* experiments patients with premalignant gastric disease were enrolled in the endoscopy, brush cytology, biopsies were taken to determine the histological stage and for *H. pylori* detection. Brush cytology using exfoliated gastric cells obtained during the endoscopy were used to look for aneuploidy in chromosome 1. Fluorescent *insitu* hybridisation technique was used to determine the chromosomal abnormalities. Although, the aim of this study was to look for the aneuploidy levels of chromosome 1 and chromosome 4, only the data from chromosome 1 was used as the signals received from Chromosome 4 were non specific.

3.1.1 Factors affecting the specificity of DNA probe hybridisation

There are various factors which could affect the specificity of a probe. If the exact nucleotide sequence of the DNA in the cell is known, a precise complementary probe can be designed. If even 5% of the base pairs are not complimentary, the probe will only hybridize loosely to the target sequence. In addition to this, the melting behavior of the probe depends on the temperature, the composition of the hybridization

buffer and on the probe length. Any variation in the above mentioned factors may lead to the probes being washed away during the wash steps, probes may not be detected, or only some of them may be detected or may be non-specific and will not be accurately representative. FISH experiments used the same hybridization conditions for both Chromosome 1 and 4. The signals from chromosome 1 were specific and were used in the study, where as the signals from chromosome 4 were non-specific and could not be used. This is most probably due to the nucleotide sequencing in the DNA probe used.

3.2 Results

3.2.1 Patients enrolled in the study

There were 72 patients enrolled at the Endoscopy department of the Neath Port Talbot and Royal Glamorgan hospitals. Samples from 61 patients were suitable to perform FISH. Initially, samples did not yield good amount of cells for the study but this improved as the study progressed as improvements were achieved in collecting, transporting and fixing the samples. Patient details were collected from the patients prior to the endoscopy and the information collected was not confirmed by checking their medical notes as this would have resulted in prolonging the procedure time and increased the waiting time for other patients in the list. This may have resulted in underreporting of the risk factors associated with the development of gastric cancers.

Table 3.2 shows the age, sex, family history, smoking and dietary habits, alcohol intake and histological diagnosis of these patients.

Table 3.2 Patient characteristics

Patient No.	Histology	Sex	Age at Endoscopy	PPI	NSAID	Smoking	Diet	Clo	Alcohol
1	Normal	m	52	No	No	DK	Normal	Not done	within recommended limit
2	Gastritis	m	55	No	No	DK	Normal	Not done	within recommended limit
3	Atrophy	m	34	No	No	DK	Normal	Not done	within recommended limit
4	Gastritis	f	29	Yes	No	DK	Normal	Not done	within recommended limit
5	Normal	f	49	No	No	DK	Normal	Not done	within recommended limit
6	Gastritis	m	88	Yes	Yes	DK	Normal	Not done	within recommended limit
7	Normal	m	57	No	No	DK	Normal	Not done	within recommended limit
8	Gastritis	f	64	No	No	DK	Normal	Not done	within recommended limit
9	Gastritis	m	70	Yes	Yes	DK	Normal	Not done	within recommended limit
10	Gastritis	m	62	Yes	Yes	DD	Normal	Not done	within recommended limit
11	Normal	f	51	Yes	Yes	DK	Normal	Not done	within recommended limit
12	IM	m	51	Yes	No	DK	Normal	Not done	within recommended limit
13	Atrophy	m	69	Yes	Yes	DK	Normal	Pos	within recommended limit
14	Gastritis	f	57	No	No	DK	Normal	Not done	within recommended limit
15	Gastritis	m	83	Yes	No	DK	Normal	Not done	within recommended limit
16	Normal	f	23	Dk	No	DK	Normal	Not done	within recommended limit
17	Gastritis	f	82	No	Yes	DK	Normal	Pos	More than recommended limit
18	Gastritis	m	67	No	Yes	DK	Normal	Not done	within recommended limit
19	Gastritis	f	74	No	No	DK	Normal	Not done	within recommended limit
20	Normal	f	58	No	Yes	DK	Normal	Not	within

								done	recommend ed limit
21	Gastritis	f	49	No	No	DK	Normal	Pos	within recommend ed limit
22	Gastritis	m	50	DK	No	DK	Normal	Not done	within recommend ed limit
23	Gastritis	m	75	DK	No	DK	Normal	Pos	within recommend ed limit
24	Gastritis	f	47	DK	No	DK	Normal	Not done	within recommend ed limit
25	Gastritis	m	43	DK	No	DK	Normal	Not done	within recommend ed limit
26	Gastritis	f	60	DK	No	DK	Normal	Not done	within recommend ed limit
27	Gastritis	f	38	No	No	Dk	Normal	Not done	within recommend ed limit
28	Normal	m	57	DK	No	Dk	Normal	Yes	within recommend ed limit
29	Gastritis	f	41	No	No	No smoking	Normal	Not done	within recommend ed limit
30	Gastritis	f	78	Yes	No	No smoking	Normal	Not done	within recommend ed limit
31	Gastritis	m	64	Yes	No	No smoking	Normal	Not done	within recommend ed limit
32	Normal	f	70	No	Yes	No smoking	Normal	Not done	within recommend ed limit
33	Barretts	m	60	No	No	Ex smoker	Normal	Not done	within recommend ed limit
34	Barretts	m	85	No	No	No smoking	Normal	Not done	within recommend ed limit
35	Normal	m	56	No	No	Smoker	Normal	Not done	within recommend ed limit
36	Barretts	m	56	No	No	No smoking	Normal	Not done	within recommend ed limit
37	Gastritis	f	60	No	Yes	Smoker	Normal	Not done	within recommend ed limit
38	Gastritis	f	55	No	No	No smoking	Normal	not done	within recommend ed limit
39	Barretts	m	68	No	No	No smoking	Normal	not done	within recommend ed limit
40	Normal Oes	f	69	No	No	No smoking	Normal	Pos	within recommend ed limit

41	Oesophagitis	m	45	Yes	No	Smoker	Less than 5 portions of fruits/vegetable	Not done	within recommended limit
42	Gastritis	m	50	No	No	No smoking	Normal	Not done	within recommended limit
43	Gastritis	m	61	No	No	No smoking	Normal	Neg	More than recommended limit
44	Gastritis	f	68	No	No	No smoking	Normal	Pos	within recommended limit
45	normal	m	55	No	No	Ex smoker	Normal	Neg	within recommended limit
46	Normal	m	49	No	No	Smoker	Normal	Neg	within recommended limit
47	Oesophagus	m	55	No	No	Ex smoker	Normal	Neg	within recommended limit
48	Gastritis	f	81	No	No	No smoking	Normal	Not done	within recommended limit
49	IM	m	70	No	No	No smoking	Normal	Not done	within recommended limit
	Oesophagus	f	81	No	No	No smoking	Normal	Not done	within recommended limit
	Gastritis	f	63	Yes	No	No smoking	Normal	Neg	within recommended limit
	Gastritis	m	36	Yes	No	Smoker	Normal	Nd	within recommended limit
53	Gastritis	f	79	Yes	No	No smoking	Normal	Yes	within recommended limit
54	Gastritis	f	54	No	No	No smoking	Normal	Not done	Normal Limits
55	Gastritis	m	73	DK	DK	No smoking	Normal	Not done	Normal Limit
56	Gastritis	m	54	DK	DK	No smoking	Normal	Not done	Excess Alcohol
57	Gastritis	m	49	DK	DK	No smoking	Normal	Not done	Within normal limits
58	Gastritis	f	54	DK	DK	No smoking	Normal	Not done	Within normal limits
59	Intestinal metaplasia	m	60	DK	DK	No smoking	Normal	Not done	Within normal limits
60	Intestinal metaplasia	f	69	DK	DK	No smoking	Normal	Not done	Normal limits
61	Gastritis	m	78	DK	DK	No smoking	Normal	Not done	Normal limits

Smoking was considered significant if they smoked more than 5 cigarettes per day and the alcohol history was considered significant if the alcohol consumption was above the national recommended limits (21 units a week for men and 14 units per week for women) .Family history of all upper GI cancers were noted. A total number of 61 patients were enrolled in the study. Fifty percent of the patients were male.

3.2.2 Histological diagnosis in the study

Figure 3.1 shows the histological distribution of the samples collected. The histology was broadly divided into normal, gastritis, Intestinal metaplasia or atrophy. The gastritis was not further subdivided into active, chronic or reflux gastritis due to the number involved. Again, we have not differentiated between the atrophy of gastric glands and the intestinal metaplasia due to the numbers involved. These two histologies represent the later stages of neoplastic development in the Correa's pathway (Correa 1988) and they all represent various precancerous stages in the development of gastric cancers. It should be remembered that the progress through the stage could be arrested at any point in the pathway and in instances regression of the staging could take place.

Figure 3.1 Illustrating the histology of samples collected.

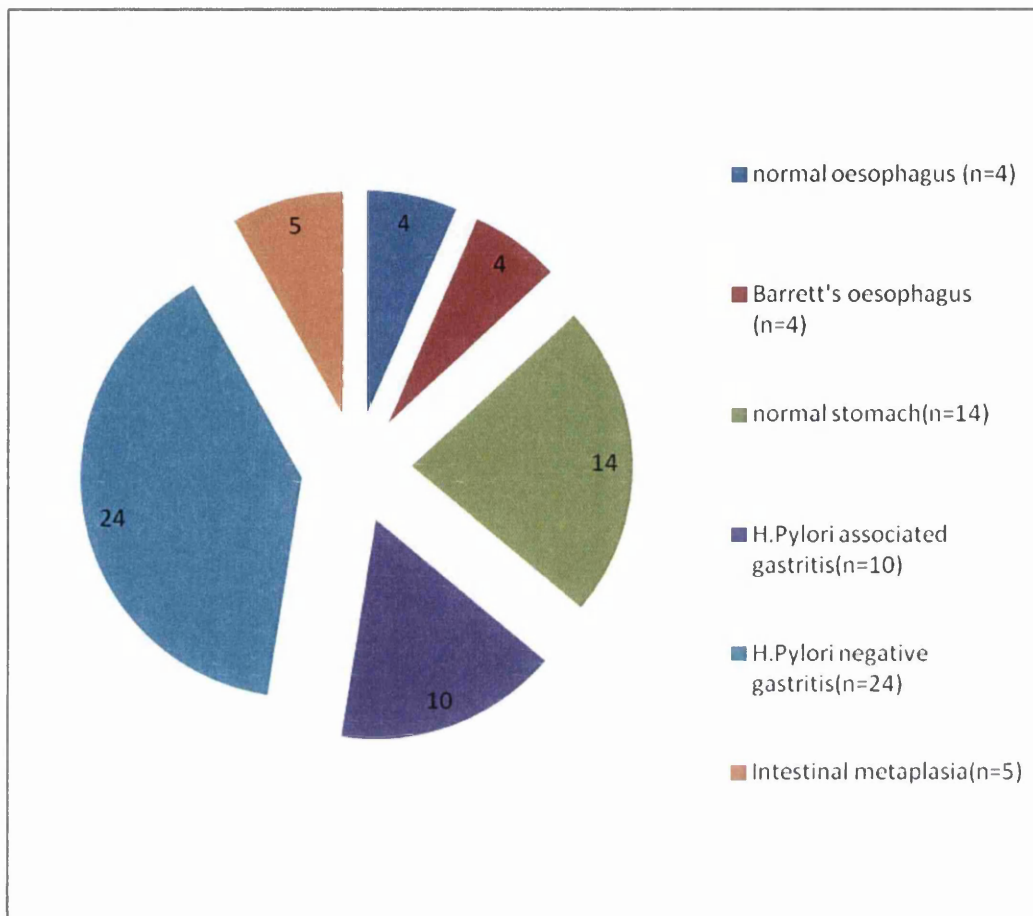


Fig 3.1 Histology of the samples collected during the study. Predominant abnormal histology is *H. pylori* negative gastritis (n=24), followed by *H. pylori* associated gastritis (n=10) and then intestinal metaplasia (n=5).

As explained in the previous chapter, cytology brushes/biopsies were collected from the gastric antrum. Of the sixty one patient samples collected, fourteen patients had normal gastric mucosa, ten patients had gastritis which was associated with *H. pylori* and twenty four had gastritis but were *H. pylori* negative. Five patients had intestinal metaplasia or atrophy. Eight patients of sixty one had their oesophageal brush analysed in this study. Four of them had Barrett's oesophagus and the other four were normal. These samples acted as an internal control for this study.

3.2.3 Risk factors associated with gastric cancer

Risk factors commonly associated with gastric cancers are male sex, increasing age, family history and poor socioeconomic conditions. Smoking, high alcohol intake and poor dietary habits have also been implicated in the development of gastric cancers (Wu et al. 2001). It has been noted that the risk of gastric cancer in smokers is dose dependant and the risk is even higher if this is combined with high alcohol intake (Sjödahl et al. 2007). It has been noted that patients who have first degree relatives with gastric cancer are likely to have increased incidence of gastric cancer when compared to the general population and this seems to vary with the ethnic group (Yaghoobi et al. 2009).

3.2.4 Aneuploidy levels in males and females

There was no significant difference in aneuploidy levels between male and female patients (aneuploidy levels in male 4.56% and females 3.55%, p value 0.45). We had approximately equal number of patients and it is interesting to observe that there was no difference in aneuploidy levels between the genders although gastric cancers are considered to be predominantly a disease of men.

Figure 3.2 shows the difference in the aneuploidy levels between males and females.

Figure 3.2 The difference in the aneuploidy levels between males and females, patients below 60 years and above 60years.

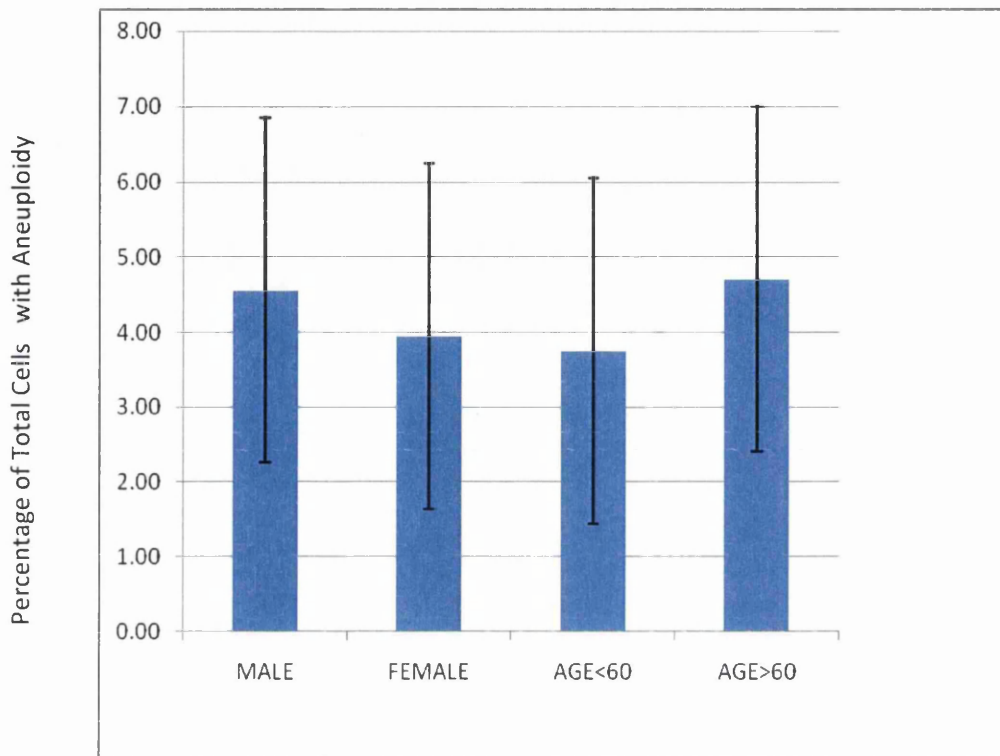


Figure 3.2 Twenty three male patients had 4.56% of aneuploidy cells and the rest of the females had 3.55% of aneuploidy cells. 24 Patients were less than 60 years old with 3.74% of aneuploid cells and the percentage of aneuploidy cells were 4.7% in patients above 60 years.

3.2.5 Aneuploidy levels according to the age

Correlation coefficient was calculated between the aneuploidy level of chromosome 1 and the age of the patients. The correlation coefficient was 0.12, indicating a weak positive correlation.

Figure 3.3 shows the correlation between the age of the patients studied and the level of aneuploidy found in them.

Figure 3.3 The aneuploidy level according to the age.

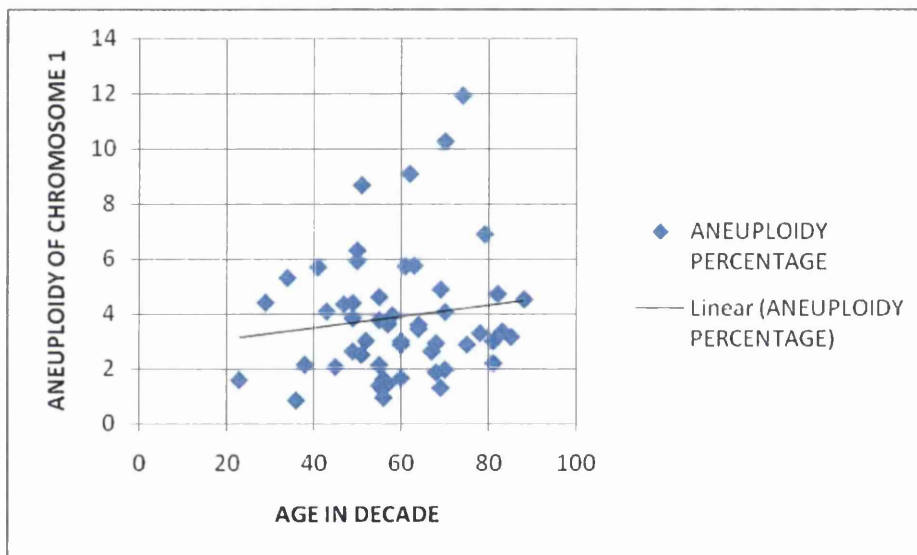


Figure 3.3 shows the correlation between the age of the patients and the aneuploidy level of chromosome 1 (correlation coefficient -0.12).

3.2.6 Aneuploidy levels in high risk older men and low risk younger females

Therefore it was interesting to see if there was any difference in the aneuploidy levels between young female patients (age less than 60 years), whose relative risk of developing cancer is low with older men (age above 60 years), who are considered at higher risk to develop gastric cancer. However, this also failed to show significant differences (aneuploidy in males over 60years is 4.80% and females under 60 years is 3.15%, p value 0.07)

Figure 3.4 shows the aneuploidy levels between the males above 60 years and females below 60 years and vice versa.

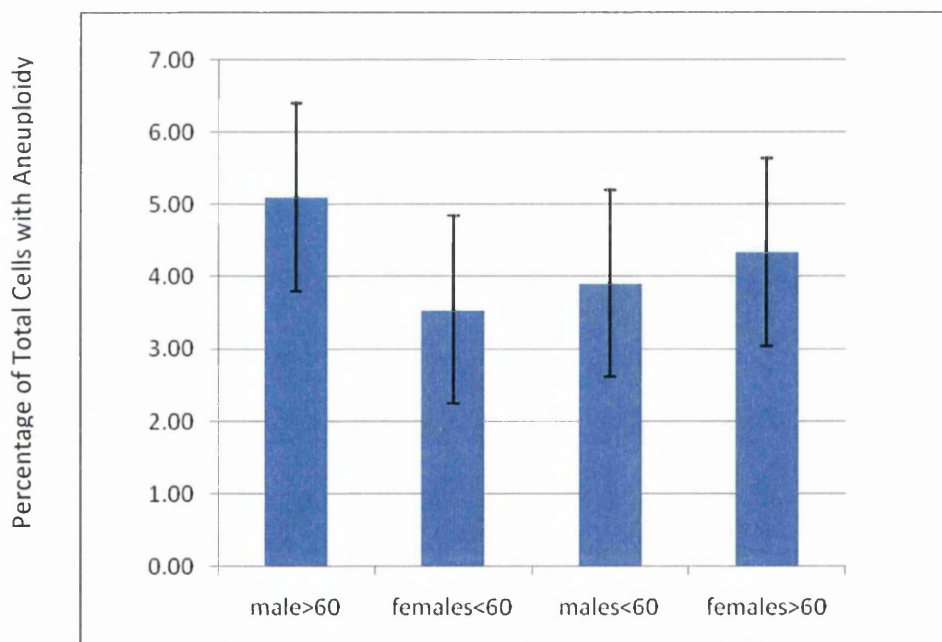


Figure 3.4 There is no significant difference between the aneuploidy levels between the high risk elderly males and low risk females in this study. 10 male patients above 60 years had aneuploidy levels of 4.80% and 10 female patients below 60 years had aneuploidy levels of 3.15% (p value – 0.07).

3.2.7 Aneuploidy levels in *H. pylori* infection

Helicobacter pylori infection has been strongly implicated in the development of gastric cancer. The determination of *H. pylori* in the gastric mucosa of the samples collected was performed using a standard haematoxylin and eosin staining of the gastric mucosa and also included searching for the organism with an oil immersion lens when gastritis was present by a consultant histopathologist with an interest in gastroenterology. An additional biopsy was also taken when possible to look for *H. pylori* infection using a CLO test and this involves change in colour of a special medium by an enzyme called urease produced by *H. pylori* in the gastric mucosa. The change in colour could take up to 24 hours and the test is usually considered negative after observing the media for at least twenty four hours to forty eight hours. This is usually performed by specialist nurses who work at the endoscopy department of the hospital.

A clinical questionnaire was obtained from the patients prior to their upper GI endoscopy to ascertain their drug history and they were specifically asked regarding the use of proton pump inhibitors and also use of non steroidal anti-inflammatory drugs. The use of proton pump inhibitors could mask the infection of *H. pylori* as it can make them migrate to the upper part of the stomach and this could lead to false negative results in the CLO test as the biopsies were commonly taken from the gastric antrum in patients with normal gastric mucosa. The aneuploidy for chromosome 1 was 3.78 in patients with gastritis and 5.055 in patients with *H. pylori* associated gastritis and this was significant ($p = 0.03$).

Figure 3.5 shows the difference in aneuploidy levels depending on the *H. pylori* infection status.

Figure 3.5 shows the aneuploidy levels depending on the *H. pylori* status

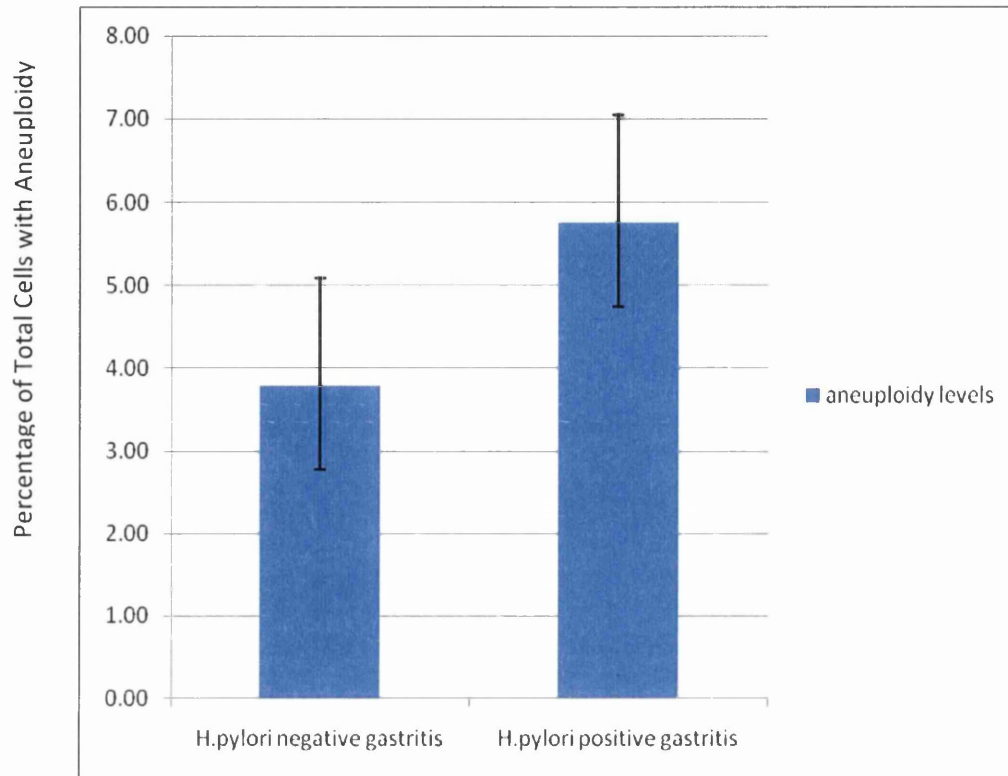


Fig 3.5 the aneuploidy levels (5.74%) in 8 patients with *H. pylori* associated gastritis was significantly higher ($p=0.03$) compared to the aneuploidy levels (3.78%) in the 24 *H. pylori* negative patients.

3.2.8 Aneuploidy levels in patients taking NSAID's and PPI

Information regarding the use of non steroidal anti inflammatory medication was obtained as there are studies which have shown that their use could lead to lower incidence of gastric cancer (Lindblad et al 2005). Hence, it would be interesting to observe if their use led to any difference in the aneuploidy levels. These questionnaires were filled in by the patients and sometime by the nursing staff due to the constraints of the time placed by a busy endoscopy list. We had to rely on patient's memory and corroborative evidence for this was not sought by looking through their clinical notes or ringing their primary physician responsible for their care.

There was no significant difference in the aneuploidy levels between patients who were taking a proton pump inhibitor and there was no significant difference in the aneuploidy levels depending on the use of non steroidal anti inflammatory medications. Figure 3.6 shows the difference in the aneuploidy level with the PPI use. There was no difference in the aneuploidy levels between taking a proton pump inhibitor or not if the histology was not taken into effect. Even if the histology of the patient was taken into effect, there is no difference in the aneuploidy levels in the histological subgroups of normal or gastritis patients.

The data for non steroidal anti inflammatory drug consumption was available only for 16 patients. The aneuploidy levels are higher in patients who took the non steroidal anti-inflammatory medications when compared with the patients who did not take them (aneuploidy level of 4.13 in patients who took NSAIDS and aneuploidy level of 3.42 in patients who did not take the NSAIDS, p value 0.376). This is contrary to the belief that the anti inflammatory medications may play a protective role in the pathogenesis of gastric cancers. But the numbers here are relatively small and it was not possible to analyse the effect of the anti inflammatory medications in comparison with the histological stages. Figure 3.9 shows the difference in the aneuploidy levels with NSAID use.

Figure 3.6 shows the aneuploidy levels and the use of proton pump inhibitor

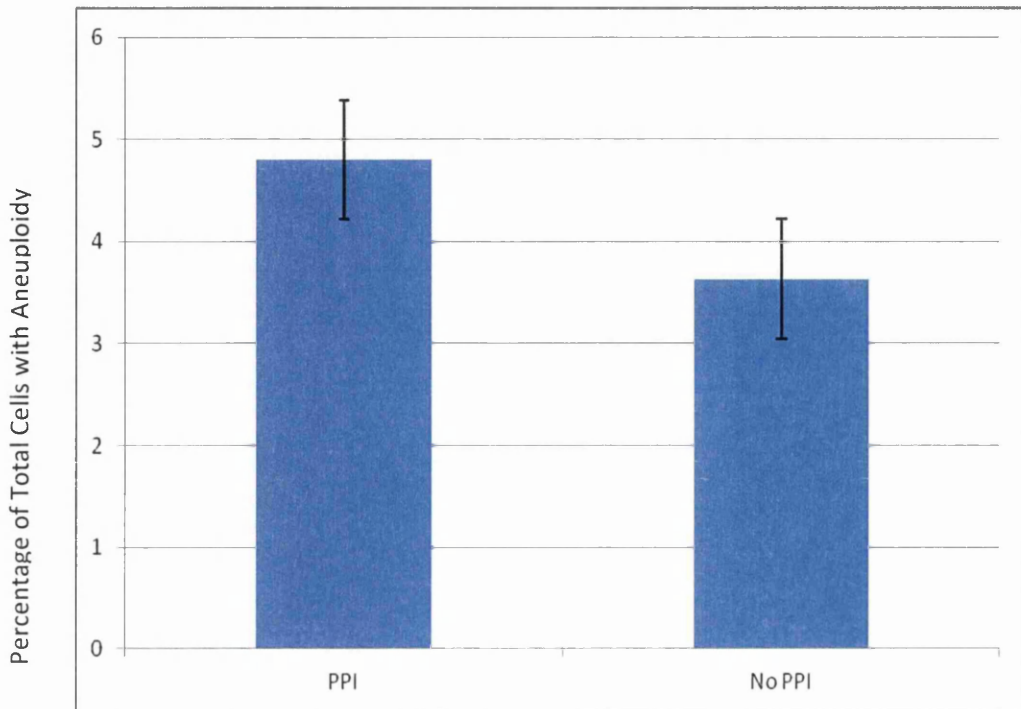


Fig 3.6 Fourteen patients were on a proton pump inhibitor and the aneuploidy levels in those patients were 4.80% whereas 8 patients were definitely not on a PPI and the aneuploidy levels in them were 3.65%. There was no significant difference in the aneuploidy levels between them (p=0.37)

Figure 3.7 shows aneuploidy levels with NSAIDS use

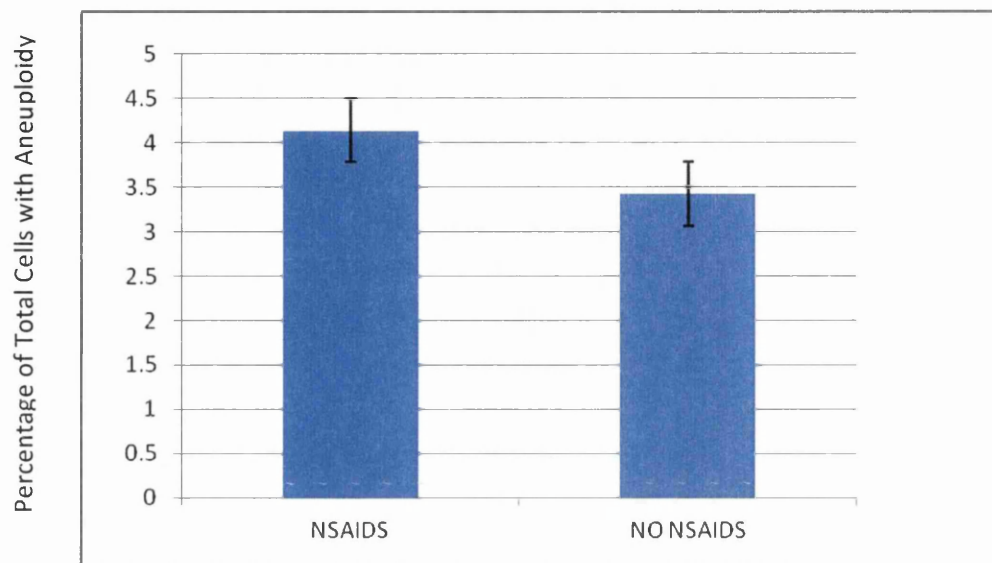


Fig 3.7 Ten patients were on a non steroidal anti inflammatory medication (NSAID) and the aneuploidy levels in them were 4.13 and there were eight patients who were not on a NSAID and the aneuploidy levels in them were 3.42. There was no significant difference in their aneuploidy levels between them p-0.20).

3.2.9 Chromosomal abnormalities in gastric tissue at different stages of disease severity

Cells from gastric tissue with different histological stages were examined for abnormalities for chromosome 1 using interphase FISH. Centromere probes were used for this purpose. Cells were also obtained from the oesophagus of some patients as an internal control. Loss or gain of chromosome 1 was noted and figure 3.9 shows the breakdown of the losses or gains of chromosome 1 in this study. The percentage of aneuploidy was higher in abnormal gastric tissue i.e., gastritis and intestinal metaplasia when compared with normal gastric tissue. This was observed in spite of the fact the normal gastric tissue was found to be more unstable than the oesophageal tissue. There was an obvious trend for the instability to increase with histological progression, from normal gastric through to gastritis and then to intestinal metaplasia and atrophy. In the gastritis group, it was observed, in patients with *H. pylori*, the instability was higher when compared with patients who were found to be not infected with *H. pylori* using the above mentioned methods to detect it. (aneuploidy levels in *H. pylori* negative samples is 3.74 and in *H. pylori* positive samples is 5.055, p value 0.03)

A total of over eleven thousand cells were studied using CEN probe 1 looking at the abnormalities in chromosome 1. Seventy percent of the abnormality was loss of a chromosome and 15 percent of the abnormality was gain of chromosome 1. The rest of the abnormality was gain of more than one chromosome. Three or four chromosome 1 were found in certain samples.

Figure 3.8 shows the common abnormalities in chromosome 1 found during the FISH analysis. Figure 3.9 represents the variation in the level of aneuploidy across the patient group involved and shows that there are group of patients with high aneuploidy levels (highest 12%) and also patients with low aneuploidy levels (lowest 0.4%). Figure 3.10 demonstrates the variation in the aneuploidy levels in comparison with the histological progression. Error bars denote the standard deviation, showing the variation in the data.

Figure 3.8 shows the abnormalities in chromosome 1 found during the FISH analysis

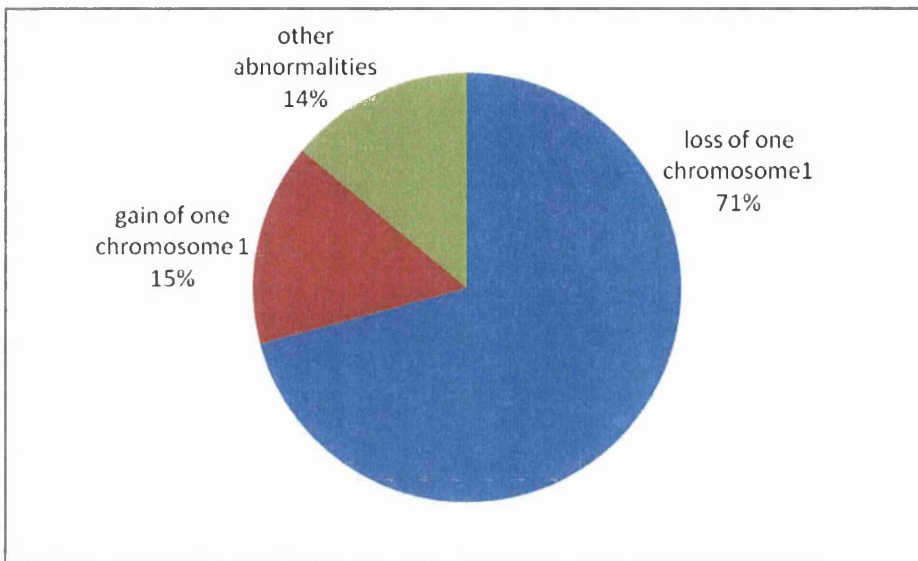


Fig 3.8 The predominant abnormality seen in the chromosome 1 was loss of one chromosome 1, which accounted for more than 70% of the abnormality, followed by gain of chromosome 1 in 15 % of the abnormality. Various other abnormalities including 3 or more chromosome 1 were seen in the rest 15%.

Figure 3.9 shows the variation in the aneuploidy levels in the patients enrolled in this study

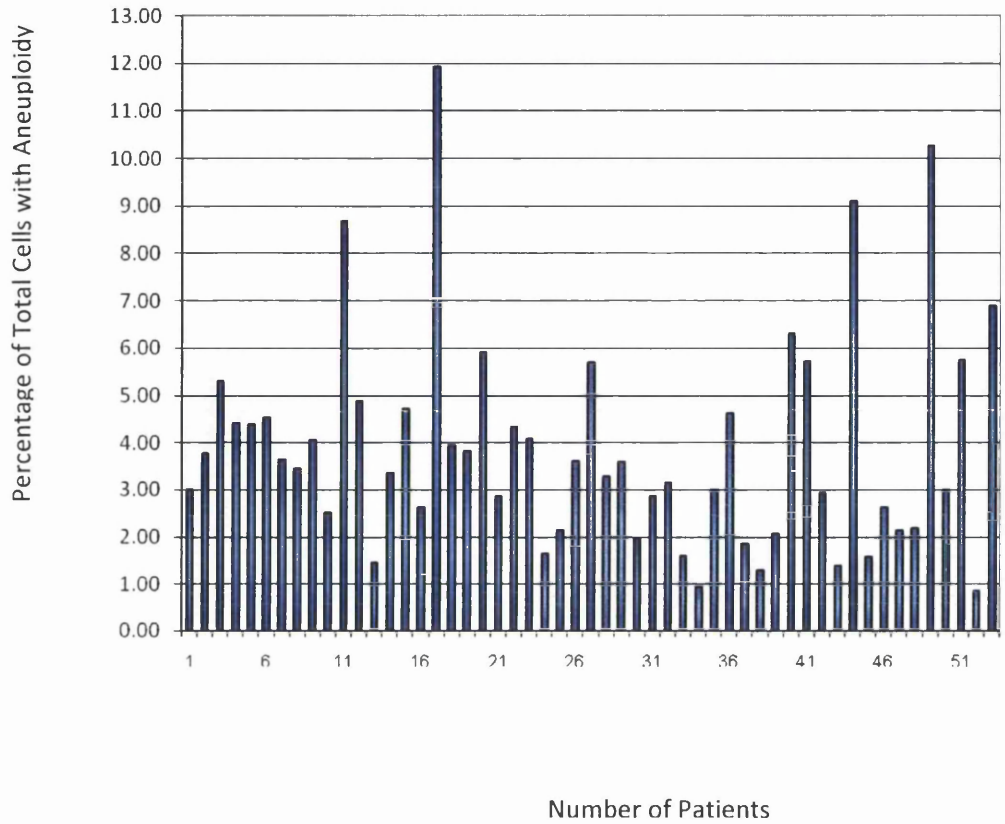


Fig 3.9 represents the variation in the degree of aneuploidy of chromosome I seen in the sample studied. This varied from aneuploidy of 0.4% to very high aneuploidy of more than 10% of the cells studied in some patients.

Figure 3.10 shows the aneuploidy levels and the various stages of histology

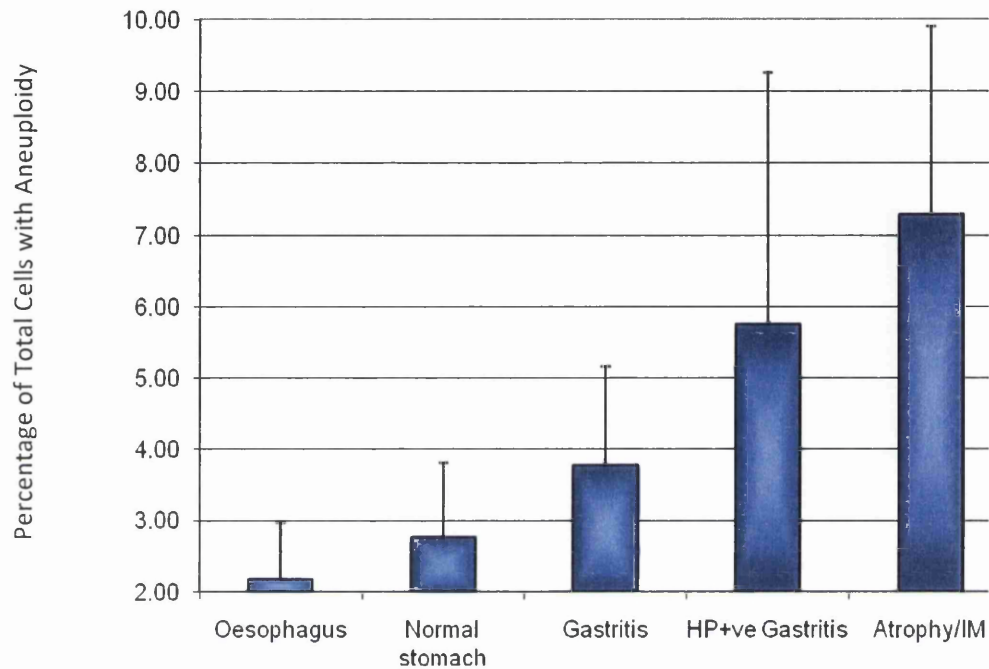


Figure 3.10 the percentage of aneuploidy cells increases with the progression of histology. The aneuploidy of chromosome 1 is greater in the normal gastric mucosa(aneuploidy -2.75%) compared with the aneuploidy levels in the normal oesophageal mucosa(aneuploidy -2.16%). 24 patients had *H. pylori* negative gastritis and the aneuploidy levels in them were 3.78% compared with aneuploidy levels of 5.74% in the 8 *H. pylori* positive patients and this was significant($p=0.03$).The aneuploidy levels in the patients with intestinal metaplasia($n=4$) was 7.28 and this was significant compared to the patients with gastritis($p=0.01$).

Aneuploidy in chromosome 1 was non-significantly higher in normal gastric tissue compared with the normal oesophageal tissue ($p < 0.200$). Chromosome 1 abnormality was higher in patients with gastritis ($p < 0.0381$) and this was significantly abnormal in *H. pylori* associated gastritis ($p < 0.01$) compare to normal gastric tissue. Aneuploidy levels of chromosome 1 were significantly higher in intestinal metaplasia group compared to normal gastric mucosa ($p < 0.001$). The difference in chromosomal abnormality level increased with each successive step of Correa's pathway, with highest percentage of abnormality was seen in the intestinal metaplasia stage

3.3 Discussion

The experiments described in this chapter were aimed at studying chromosomal abnormalities present in gastric tissue in vivo, using inter phase FISH, to investigate the hypothesis that as the histological progression occurs in the development of gastric cancers, there is also an increase in the genetic abnormalities leading to an increase in the aneuploidy levels. Inter phase FISH was successfully used to determine this. The level of aneuploidy of chromosome 1 increased as the histology progressed through the well established Correa's pathway.

As explained in chapter 2, the patients were enrolled during their routine appointments for upper GI endoscopy at the Neath Port Talbot hospital and Royal Glamorgan Hospital, and therefore no selection policy was adopted in recruiting them into the study. Same procedures were adopted in both the hospitals to recruit the patient. They were patients referred by their medical practitioners for varying reasons. An information leaflet regarding the study and consent forms were posted to them in advance and any questions were answered by the well trained endoscopy nurses or by me. A maximum of 2-3 patients were enrolled in the study in a list as there were time constraints on a busy open access endoscopy lists. We did not screen the patients prior to the endoscopy as this would have involve the patient making an additional prior visit to the hospital. The suitability of the patients was assessed during their admitting process in to the unit and a brief clinical questionnaire was given to them to be filled in. Information regarding their risk factors were obtained to ascertain if any of the risk

factors were significant and corresponded to the aneuploidy levels. This information was collected from the patients and we did not seek corroborative evidence for this by either looking through their medical notes or liaising with the referring doctor. A note of all medications was obtained if the patients had their list of medications with them. The accuracy of the information has to be questioned as we did not seek to confirm the information provided by the patients and we are aware that this could have resulted in underreporting of risk factors. Interviewing the patients prior to their endoscopy date and allowing more time for the questions would have resulted in improved accuracy but again this could have not been possible due to the constraints on the time and resources.

Solid tissue has often proven to be difficult to manipulate into a form required to perform FISH techniques. A single layer of cells need to be produced so that FISH probes can be added, and then detected in the nuclei of these cells using microscopy. Therefore, in this study the most important step was to make sure that the cells collected were delivered so that they were suitable for FISH. Modification of techniques described by Doak et al (2003) has shown that cytology brushings from endoscopy is a reliable and safe method of cell collection for this purpose. It also allows adequate cells to be spun onto a microscope slide to form a cytodot to perform FISH in these samples. Inter phase FISH is an observer dependent technique and the technique improved considerably with my experience. Gastric biopsies were taken from the same site and they were used to determine the histological stage, CLO test to determine the *H. pylori* status and also to extract the RNA to perform gene expression levels of MAD2 and BUB1 genes as explained in chapter 2. In order not to prolong the procedure, gastric biopsies were taken only from one site as the risk of complications associated with the procedure increases with increased endoscopy time. As previously explained in chapter 2, brushing of the gastric mucosa precede the biopsies to avoid red blood cells in the cytology samples. The gastric biopsies were taken just adjacent to the site of the gastric brushings. The samples were obtained from the gastric antrum if the gastric mucosa appeared normal during the endoscopy but otherwise the samples were collected from the abnormal site.

Using these cell preparations, our aim was to determine the level of aneuploidy present in the pre-malignant gastric tissue samples from the enrolled patients. The chromosome targeted has been implicated in the development of gastric and

oesophageal adenocarcinomas. A variety of histological stages (i.e., normal, gastritis, intestinal metaplasia) were obtained for the purpose of the study and all of them fitted into the early stages of multi-step model proposed by Correa in the development of gastric cancers.

Initially, a comparison was made of normal oesophageal and gastric tissue from the same patients. The normal squamous oesophageal mucosa had very small amount of aneuploidy in chromosome 1 when compared with normal gastric mucosa. The level of aneuploidy in the normal gastric mucosa was non-significantly higher ($P < 0.2$) when compared with the normal oesophageal mucosa. This is comparable with the results shown by Williams et al (2005).

Histologically, normal gastric tissue showed increased aneuploidy and this may be explained by the different environments in which these two tissues exist in vivo. Gastric cells are bathed in gastric acid, an acidic medium and often contain bile regurgitated from the small intestine, food, mucus, enzymes and bacteria (*H. pylori*). The oesophageal mucosa comes into contact with these only during brief reflux episode. Bile and acid have been implicated in the pathogenesis of upper GI tract cancer (Triadafilopoulos 2001). Bile acids have also been shown to cause DNA damage (Scates et al. 1996,, D K Scates et al. 1996) and are also implicated in the development of chromosomal abnormalities (Jenkins GJ et al 2007).

Against the unstable background found in the gastric tissue, specific chromosomal abnormality was sought. Chromosomal abnormalities occurring at varying stages of gastric cancer pathogenesis have already been discussed. Abnormalities in chromosome 1 occur early in the pathogenesis and the chromosomal aberration expands in a stepwise manner with cancer progression (Kitayama et al. 2000). Chromosomal abnormalities of 1 and 17 were shown to be high in the tumours of the gastric cardia, suggesting high instability at this particular site (Fringes et al. 2000). In addition to the above mentioned chromosomes, various others are implicated in the pathogenesis of gastric tumour. Beuzen et al (2000) have shown that there is high frequency of chromosomal numerical aberrations in oesophageal and gastric

adenocarcinomas, without differences between adenocarcinomas of the gastric cardia and the gastric antrum.

Abnormalities of chromosome 20 have been showed to be associated with the development of adenomas as well as carcinoma (Panani, Roussos 2005), a stage further along than gastritis and intestinal metaplasia. Chromosome 4 aneuploidy has also been shown to progressively increase as the histology progressed. Chromosome 4 amplification has been shown to be significant in the progression of disease from Barrett's oesophagus and to adenocarcinomas (Doak et al 2003, Croft et al 2002). Amplification of chromosome 4 has been shown to be significantly increased in patients with *H. pylori* induced gastritis and has been thought to play an important role in the development of *H. pylori* induced gastric cancer (Williams et al. 2005). Chromosome 4 was studied in 52 of the 61 samples in the study but the data could not be used as the binding of the CEN probe for chromosome 4 was non -specific.

This study has reinforced the concept that interphase FISH can be easily employed to monitor aneuploidy when used in conjunction with brush cytology. It has also been shown that the cells in the brushes can be retrieved even after a day from the day of the procedure. Thirty four of the sixty four samples from Neath Port Talbot Hospital yielded good quality cells for this study. The degree of aneuploidy may have been underestimated in this study as only one chromosome was studied. Chromosome 1 was chosen due to the reports that it is particularly implicated in the gastric cancer (Kitayama et al. 2000, Fringes et al. 2000). It would have been apparent that aneuploidy levels are even higher if we have studied more chromosomes in the premalignant tissues. Our group have previously studied chromosome 4, 8, and 20 and these have been found to have similar overall levels of aneuploidy i.e., 2-3% of normal gastric cells and 7-10% of intestinal metaplasia. Nevertheless, some underestimation is expected if only one chromosome is studied.

Given that chromosome 1 aneuploidy levels correlate well with the histological progression, it is possible that this genetic event is causatively linked to the carcinogenesis, or alternatively that it is the marker for the aneuploidy per se, and that

aneuploidy is co- incidentally linked to carcinogenesis. It is difficult to envisage that the 12% aneuploidy levels encountered in the advanced stage of the Correa's pathway is the only cause for the neoplastic progression. It is more likely that they represent a state of chromosomal imbalance which occurs in the premalignant tissue. This is supported by the previous work performed by our group involving chromosome 4, 8 and 20. It is becoming clear that chromosome instability occurs early in carcinogenesis and this provides a heterogeneous pool of clones for tumour evolution to select from.

Interestingly, the gastric tissue appeared to be genetically unstable. Aneuploidy levels were not only higher in the inflamed gastric tissue but also in the normal gastric tissue than in normal oesophageal tissue analysed alongside. The aneuploid levels in normal gastric tissue (2.37%) was higher when compared to the normal oesophageal tissue (2.2%), which were seen in 4/8 patients. When comparing the aneuploidy levels in the gastric IM tissue to the Barrett's oesophageal tissue (this represents the similar histological stage), they were 4 fold greater in the stomach (6.32% Vs 2.31%). This was also seen in a previous study performed by our group (Williams et al 2005) and probably reflects the harsh environment in the stomach (acidity, bile acids, certain food, nitrosamines).

There was an apparent trend for males having advanced histology (29 males patients had gastritis or intestinal metaplasia or atrophy compared with 21 female patients, 4 out of 5 patients with intestinal metaplasia or atrophy are males). This was a confounding factor when the correlation between the aneuploid level and age and sex was sought. The higher aneuploid levels in men was probably complicated by the fact that the males tended to dominate the later histological subgroup and hence tended to have higher aneuploid levels, as these were correlated with the histology. This was also true in the case of the age as the number of older patients were also higher in later histological group. The mechanism of the sex bias in gastric cancer is unknown, but may be due to the effects of differing sex hormones. A recent study has shown that hormone replacement therapy (HRT) led to a 50% reduced risk of gastric cancer, suggesting that the female sex hormones may protect against this form of cancer (Lindblad et al 2005, Chandanos and Lagergren 2008). Conversely, it has been shown that tamoxifen which blocks the oestrogen receptor might be implicated in the

development of gastric non cardiac tumours (Chandan et al 2006). More work is obviously needed to further unravel the exact mechanisms involved and to establish if oestrogens can ameliorate the molecular causes of gastric cancer, like aneuploidy.

Interestingly, there was a 40 year old female patient in the study with significant family history of gastric cancer and with significant aneuploid levels for chromosome 1(5.7%) and had gastritis, who would probably benefit from regular surveillance. *H. pylori* are associated with gastric cancer pathogenesis (Hansson et al. 1993) and her *H. pylori* status is unknown and would possibly benefit from eradicating it. There is no national surveillance guideline for these patients in this country. Identifying these patients and using aneuploidy levels as a biomarker in assessing their progression is a possibility but larger prospective studies which can follow these patients for a longer length of time would be required. As there is no current evidence to follow these patients, she will be treated as per the current practice of treating her symptoms with a proton pump inhibitor and a standard triple therapy of a proton pump inhibitor and two antibiotics. These patients are not regularly checked for the eradication of *H. pylori* by the treatment. This can be performed by using a urea breath test. Urea breath test to confirm the eradication of *H. pylori* is only offered for patients with persistent symptoms in spite of the treatment.

Long term non steroidal anti inflammatory use is associated with reduced risk of gastric cancers (Lindblad et al. 2005). It was interesting that we did not notice an increase in the aneuploid levels in patients taking non steroidal anti inflammatory medications. As mentioned above, the number of patients taking the medications was low, the information was collected from the patient prior to the endoscopy and the information was not cross checked, the duration of NSAID use was not known.

The aneuploidy level in the gastric epithelium might represent a useful biomarker for neoplastic progression in the stomach and may be indicative of the risk of cancer development. Therefore monitoring this biomarker might be clinically useful. Obviously, close follow up of this particular cohort of patients and the correlation of cancer incidence with aneuploidy is needed to strengthen this link. Using an accepted



method of identifying “aneuploid” patients as those with aneuploidy that was two standard deviations higher than the normal patients, 16 out of 61 patients in this study are classified as “aneuploid”. This approach may help in selecting patients for closer follow up. New evolving methods such as automated scoring of chromosome copy number by image analysis are promising in making this possible. This kind of automated approach will help us in monitoring large number of patients and may represent an effective patient monitoring strategy.

Chapter 4 will look at the gene expression levels of MAD2 and BUB1 genes and will correlate this with the histological progression along the Correa’s pathway. A correlation between the gene expression levels of MAD2 and BUB1 will be sought against the aneuploid levels as well. These spindle check point genes are implicated in the development of aneuploidy.

CHAPTER 4

The analysis of the gene expression levels of MAD2 and BUB1 levels in the gastric premalignant tissues

4.1. Introduction

Mitotic check points have various components which are involved in evoking a cascade response to any defect in spindle cell assembly, preventing the progression of the cell cycle to anaphase. It is a highly regulated feedback mechanism that plays an important role in maintaining the genetic stability and integrity. MAD2 and BUB1 are the most commonly studied genes as they play an important role in the mitotic check point. Mutation of these genes and altered expression levels are reported in breast, lung, colon and gastric cancers (Ouyang et al 2002, Myrie et al 2000, Shigeshi et al 2001, and Tanaka et al 2001).

The experiments described in this chapter looked for the gene expression levels of MAD2 and BUB1 levels in the gastric biopsy tissue samples obtained from the patients at the Neath Port Talbot Hospital using the methodology described in chapter 2. These were the same patients for which aneuploidy level was also assessed in Chapter 3. MAD2 and BUB1 expression levels were sought as they are the most commonly altered genes (within the spindle check point pathway) in many aneuploid cancers as described earlier. The steady state levels of mRNA of both MAD2 and BUB1 were quantified in all the premalignant gastric cancer stages, using real time RT-PCR. These gene expression levels were then correlated with clinical parameters for the patients and with the level of aneuploidy, which was assessed using FISH, as described in chapter 3, in order to determine if alterations in the expression levels of MAD2 and BUB1 mitotic spindle checkpoint genes were involved in inducing chromosomal instability in the samples studied.

4.2 Data Analysis

Real time RT PCR reaction is explained in detail in chapter 2. Real time PCR was performed with each primer set required for the study on a serial dilution (neat, 1 in 10, 1 in 100, 1 in 1000) from each of the cDNA samples. This was repeated at least twice and the transcription levels of MAD2, BUB1 and β Actin were calculated. The mean expression levels of MAD2, BUB1, and β Actin, from these two RT PCR reactions were calculated. This value was used in the analysis to look for correlation between age, smoking habits, aneuploidy levels. This value is represented on the Y axis in this chapter.

4.3 Results

4.3.1 Differences in MAD2 and BUB1 level between men and women

Forty four patients were enrolled in the study and of them, 25 were female and 19 were male. There was no significant difference (MAD2 p value - 0.26/BUB1 value 0.71) in the expression levels of MAD2 and BUB1 between them.

Figure 4.1 shows the difference in the expression levels of MAD2 and BUB1 in different sex.

Figure 4.1 MAD2 and BUB1 expression levels in males and females

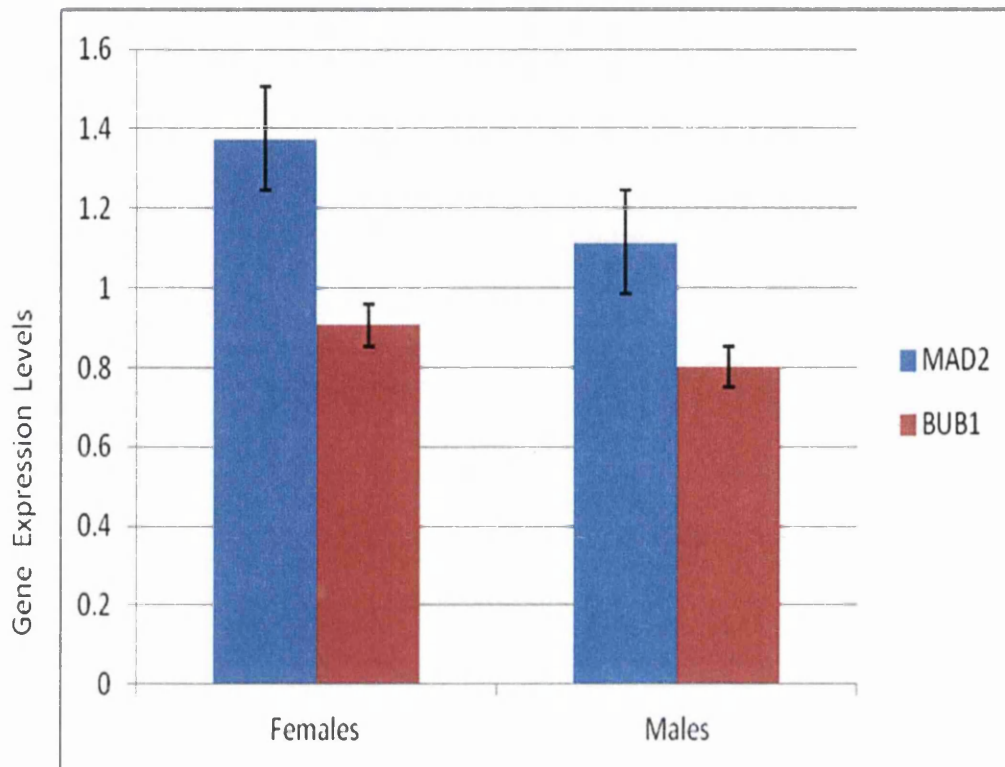


Fig 4.1 Twenty five female patients had an average MAD2 expression level of 1.37 and BUB1 expression level of 0.90. 19 male patients had an average MAD2 expression levels of 1.11 and BUB1 expression levels of 0.80. There was no significant difference between the MAD2 levels ($p=0.26$) or BUB1 levels ($p=0.71$) between males and females.

4.3.2 Differences in MAD2 and BUB1 level depending on the H. pylori status

Infection with *H. pylori* was confirmed either by performing a CLO tests during the endoscopy or by histology. All stages of histology described by Correa were represented in these samples used to analyse the MAD2 and BUB1 expression levels.

Eighteen patients were negative for *H. pylori* and six patients were positive for it. The *H. pylori* status was unknown for the rest of the patients. There was no significant difference in the expression levels of MAD2 and BUB1 depending on their *H. pylori* status (MAD2 p value – 0.09/BUB1 p value – 0.71).

Figure 4.2 explains the differences in the MAD2 and BUB1 levels in patients with *H. pylori* infection.

Figure 4.2 MAD2 and BUB1 expression levels based on CLO results

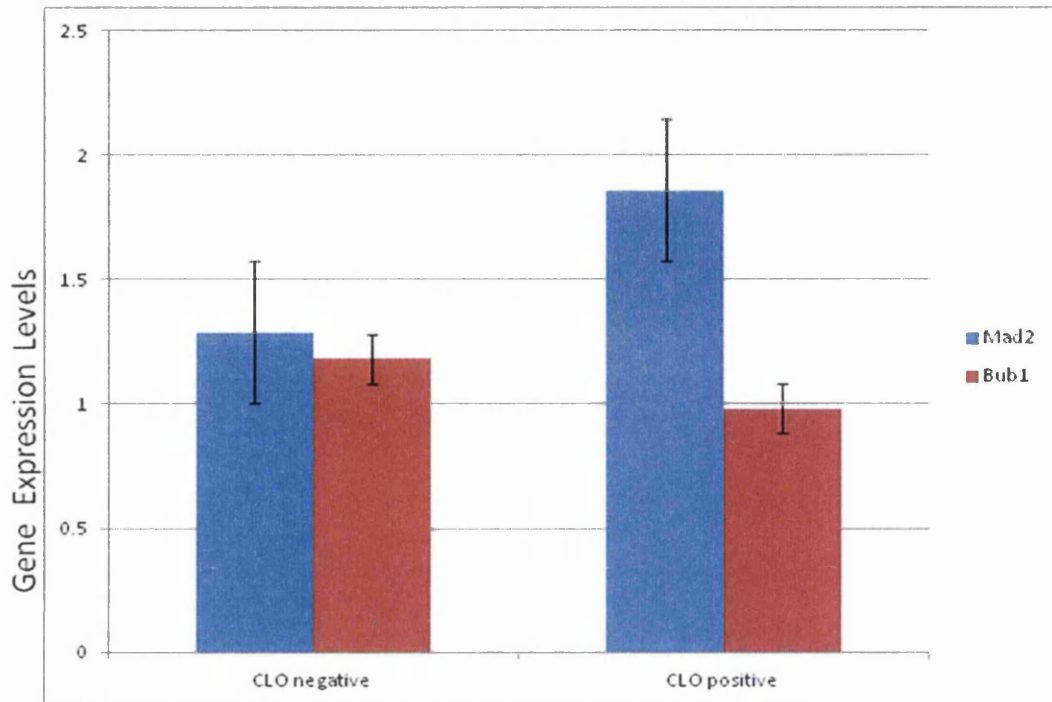


Fig 4.2 Eighteen patients were CLO negative and six patients were CLO positive. MAD2 expression levels were 1.29 and 1.86 between CLO negative and CLO positive patients respectively and there was no statistical difference between them ($p=0.09$). BUB1 expression levels were 1.18 and 0.98 between the CLO negative and CLO positive patients respectively. There was no significant difference in their expression levels ($p=0.71$)

Although the MAD2 expression levels did not reach the significant levels between the patients with or without *H. pylori* infection, the p value of 0.09 suggests that the infection could cause some alteration of MAD2 levels.

4.3.3 Differences of MAD2 and BUB1 level and significant aneuploid levels

MAD2 and BUB1 expression levels were correlated with significant aneuploidy levels. The value of aneuploid level, which is 2 standard deviation above the aneuploid levels seen in patients with normal histology was taken as 'significant aneuploid' levels. The value used was 4.86 % and the method which was used to determine this is explained in chapter 3. There was no difference in the MAD2 or BUB1 expression levels in patients with significant aneuploidy. The difference in MAD2 and BUB1 levels did not reach the level of significance between patients with significant aneuploidy(4.86%) when compared with patients who did not have significant aneuploidy(MAD2 P value 0.30 and BUB1 p value 0.07). The patients with aneuploidy but whose aneuploidy levels did not reach the significant levels was combined with the patients in whom the aneuploidy levels did not reach the 'cut off' of 4.86%.

Figure 4.3 shows the expression level of MAD2 and BUB1 in patients with significant aneuploidy.

Figure 4.3 MAD2 and BUB1 levels in patients with significant aneuploidy

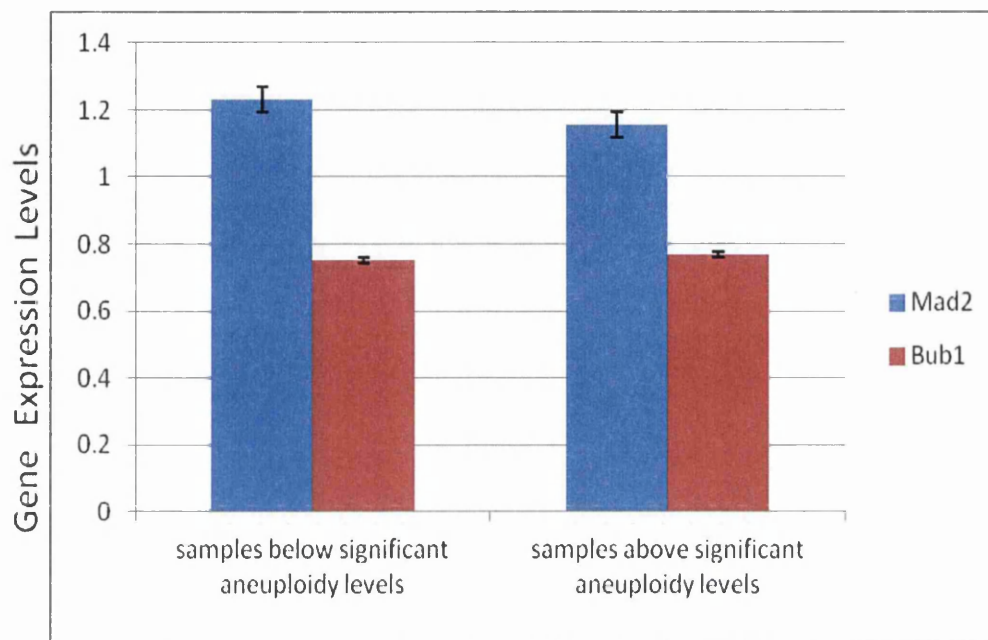


Fig 4.3 Six patients had aneuploidy levels above the significant aneuploidy level cut off of 4.86%. MAD2 expression level were 1.23 and 1.156 in patients below and above the significant aneuploidy levels ($p=0.93$) and BUB1 levels were 0.75 and 0.76 in patients below and above the significant aneuploidy levels ($p=0.98$).

4.3.4 MAD2 and BUB1 expression levels in patients with family history of upper gastrointestinal cancers

Six patients had family history of upper GI cancers. In both the sub sets there was a mixture of histological stages ranging from normal histology to intestinal metaplasia. There was no statistically significant difference in the expression levels of MAD2 and BUB1 gene in patients with family history of upper gastrointestinal cancers when compared with patients with no family history (MAD2 p value -0.45/BUB1 p value -0.55). Although the results did not reach statistical significance, there was a clear decreasing trend in the expression of MAD2 and BUB1 levels in patients with the family history of upper gastrointestinal cancers.

Figure 4.4 shows the expression level of MAD2 and BUB1 in patients with family history of upper GI cancers.

Figure 4.4 MAD2 and BUB1 levels in patients with family history of upper GI cancers

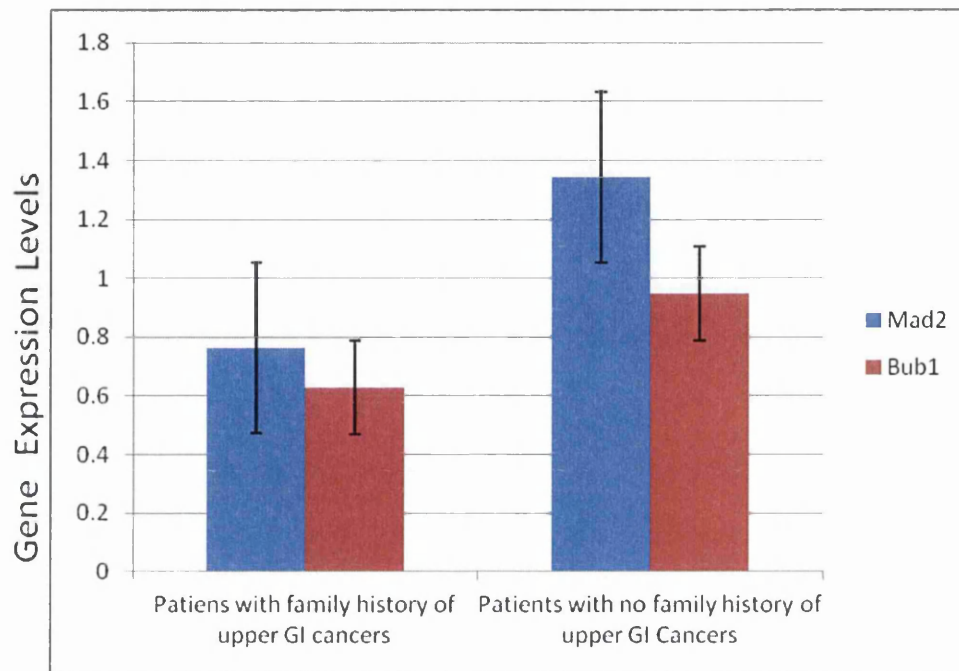


Fig 4.4 Six patients had significant family history. The MAD2 levels were 0.76 and 1.34 in patients without any history of upper gastrointestinal cancers and with history of upper gastrointestinal cancers ($p=0.45$). The BUB1 levels were 0.62 and 0.94 in patients without any history of upper gastrointestinal cancers and with history of upper gastrointestinal cancers ($p=0.55$).

4.3.5 MAD2 and BUB1 expression levels and smoking habits

Of the forty four patients, nine were smokers and there was no significant difference in the MAD2 expression levels between the two subgroups (p value MAD2 - 0.53). BUB1 expression levels were 0.46 and 0.41 in smokers and non smokers respectively (p value – 0.78).

Figure 4.5 represents the difference in the levels of MAD2 and BUB1 between the smokers and non smokers.

Figure 4.5 MAD2 and BUB1 levels in smokers and non smokers

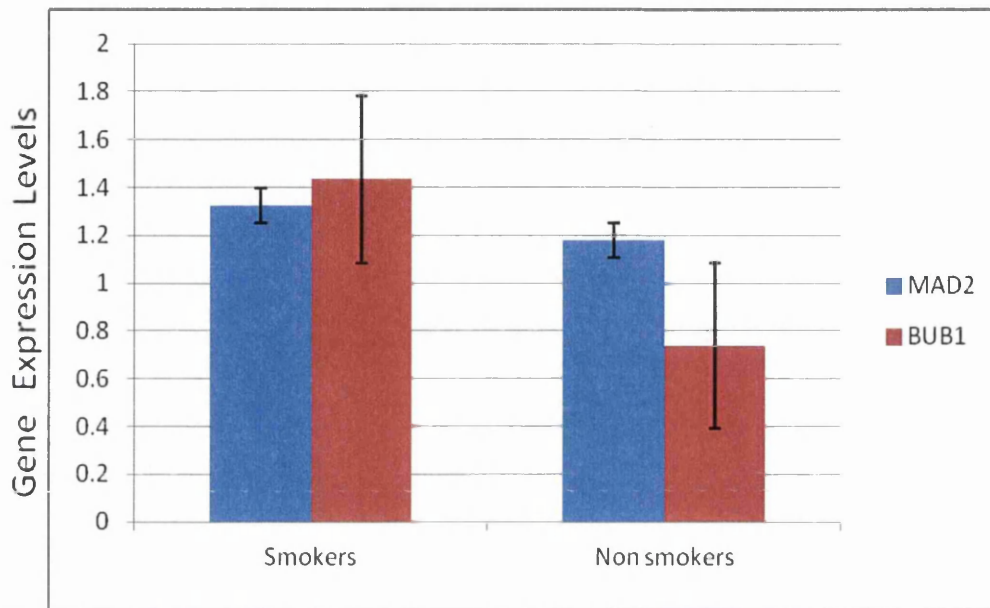


Fig 4.5 Nine smokers and thirty three non smokers were identified in the study. The MAD2 levels were 1.32 and 1.17 in smokers and non smokers respectively ($p=0.55$). The BUB1 levels were 1.43 and 0.89 in smokers and non smokers respectively ($p=0.05$).

4.3.6. MAD2 and BUB1 expression levels according to the age

Correlation coefficient was calculated for MAD2 and BUB1 expression levels and age of the patient. The correlation efficient for MAD2 level was -0.11 and for BUB1 was 0.08. There was no significant positive or negative correlation between the gene expression levels and the age.

Figure 4.6a represents the differences between MAD2 levels in the different age group

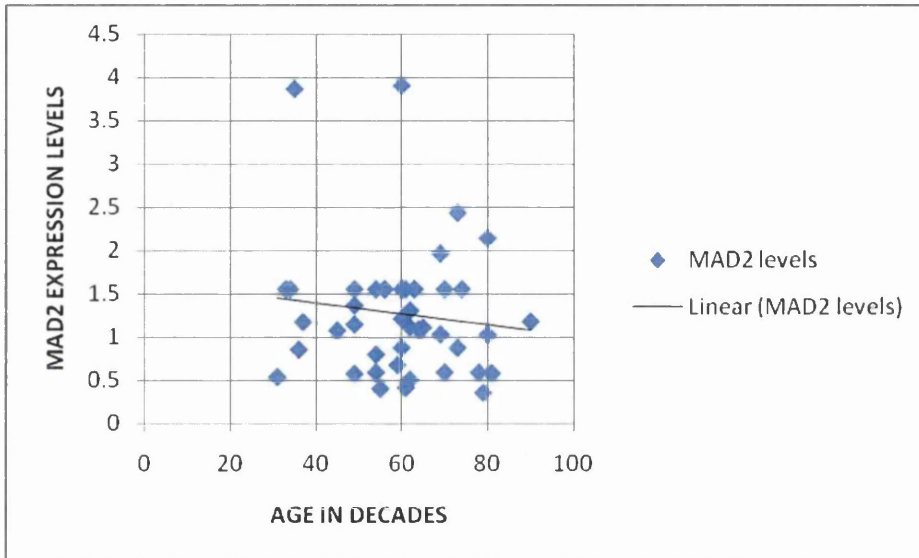
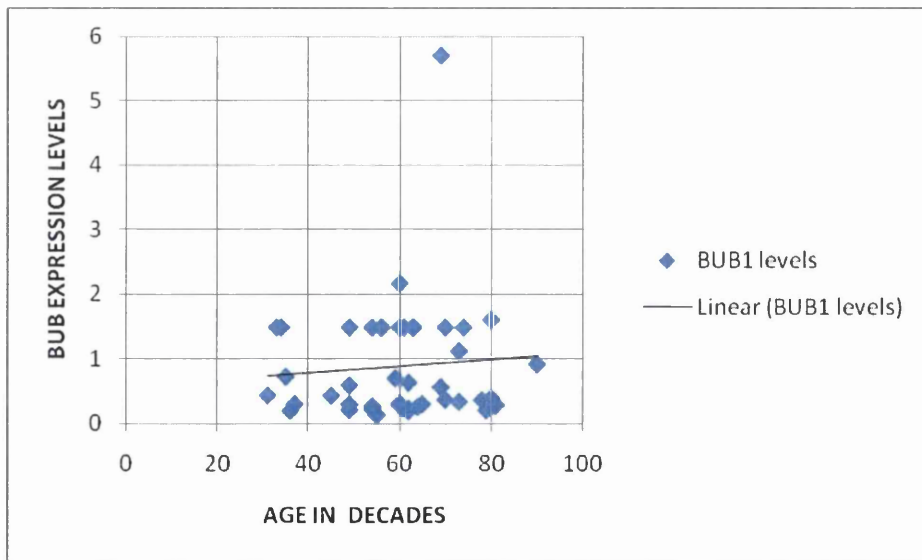


Figure 4.6a and b Shows the MAD2/BUB1 expression levels on the x axis and the age of patients at endoscopy on the y axis. There was no significant correlation between the age of the patient and the MAD2/BUB1 expression levels

Figure 4.6b represents the differences between BUB1 levels in the different age group



4.3.7 MAD2 and BUB1 expression levels and histology

Eleven patients had normal gastric histology and twenty eight patients had chronic gastritis, of which four was *H. pylori* associated gastritis and four patients had intestinal metaplasia. There was no significant difference in the MAD2 expression level between patients with normal histology and gastritis (p value – 0.13) and between patients with normal histology and intestinal metaplasia (p value -0.45). There was also no significant difference between the levels of BUB1 expressed by patients with normal histology and gastritis (p value -0.12) and between patients with normal histology and intestinal metaplasia (p value – 0.47)

Fig 4.7 describes the MAD2 and BUB1 level in the normal, Gastritis; *H. pylori* associated gastritis and intestinal metaplasia.

Figure 4.7 MAD2 and BUB1 levels depending on the histology

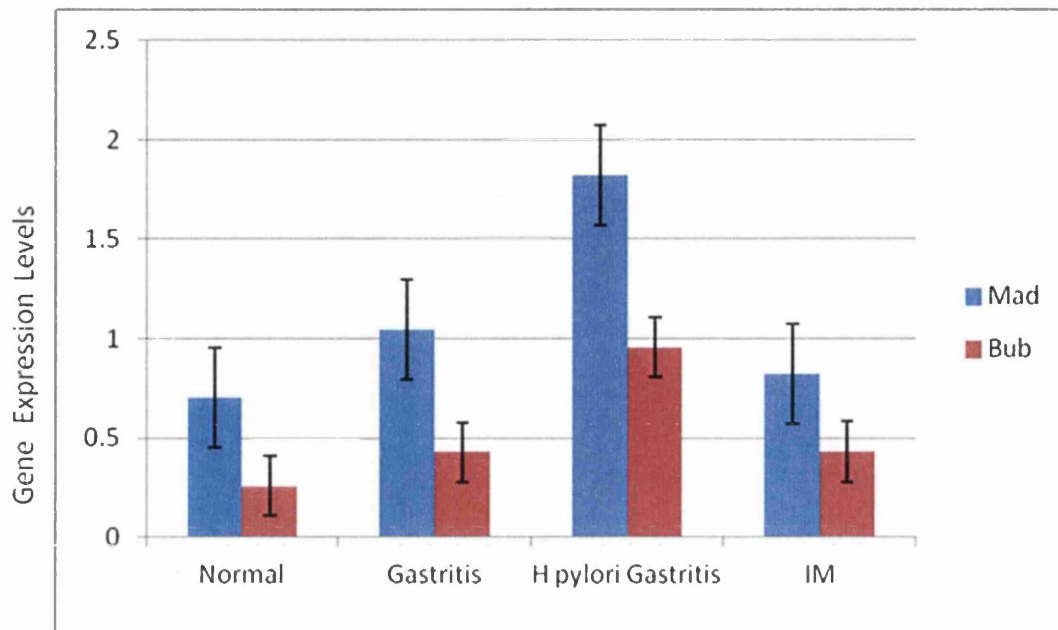


Fig 4.7 Twenty two patients had *H. pylori* negative gastritis, four patients had *H. pylori* associated gastritis and four patients had intestinal metaplasia and the rest were of normal histology . The MAD2 levels were 1.55 and 1.11 in patients with normal histology and *H. pylori* negative gastritis ($p=0.49$). The BUB1 levels were 1.49 and 0.62 in patients with normal and *H. pylori* negative gastritis ($p=0.73$). MAD2 and BUB1 levels were decreased in *H. pylori* associated gastritis (MAD2 -1.81 and BUB1 level-1.07) and this did not reach any significance ($p=0.85$).MAD2 and BUB1 levels were decreased again in the intestinal metaplasia stage (MAD2-0.82 and BUB1-0.43), this also did not reach any statistical significance ($p=0.63$).

But there was significant increase in the levels of MAD2 and BUB1 between patients with normal histology and patients with *H. pylori* associated gastritis (MAD2 p value - 0.01 and BUB1 p value – 0.01)

4.4 Discussion

The experiments described in this chapter have attempted to study the gene expression levels of MAD2 and BUB1 in the gastric tissue, using real time RT –PCR and to investigate the hypothesis that as gastric carcinogenesis progresses, so does the genomic instability. The genomic instability is probably driven by the defects in the spindle check points. We studied MAD2 and BUB1 genes as they play a very important role in the pathogenesis of gastric cancer. There are studies, which has demonstrated that over or under expression can occur, leading to the premature entry into anaphase (Grabsh et al 2003, Tanaka et al 2001).

As explained in the chapter 3, the patients were enrolled from my routine open access endoscopy at Neath Port Talbot Hospital, and as a result no selection process was applied in recruiting them in the study. The cohort of patients enrolled in the study reflects the population of patients with upper GI symptoms, who were referred by the general practitioners in that region. A maximum of 2-3 patients were enrolled in the study per list. This was due to the time constraint on a busy endoscopy list. The patient selection could have been performed, but this would have required patients to have a prior additional visit and this was again restricted by the time and resources. The patients were interviewed on arrival by an experienced endoscopy nurse and a clinical questionnaire regarding their family history, smoking and dietary habits, alcohol intake and drug intake was obtained. This information was not corroborated by checking them in their clinical notes or with their general practitioners as this would be time consuming.

MAD2 and BUB1 expression levels were studied in a large group of patients (n=44). They represented the patient cohort most likely to be seen in the community as they were referred by their general practitioners. The number of patients with intestinal metaplasia was small (n=4) but again there was no way of improving this as it is difficult to predict the histology of the patients depending on their symptoms or by the endoscopy appearances.

Recent studies have looked at the BUB1 levels in the gastric cancers and also the looked at the DNA ploidy levels in the gastric tissue (Grabsch et al. 2003, Grabsch et al 2004). It has been demonstrated that over expression of BUB1, BUBR1 and BUB3 occurs in diffuse type gastric cancer when compared with the intestinal type gastric cancer. Kim H et al (2005) study of gastric cancers and gastric cancer cell lines showed that frequent mutation of MAD2 genes but not BUB1 caused functional defects in spindle checkpoints, which could lead to the development and progression of gastric cancers.

MAD2 expression levels were also studied using the same samples. A steady state level of MAD2 is thought to be important in maintaining the integrity of the spindle cell checkpoint and is recruited as a part of the complex in preventing the cell from progressing to anaphase. This study looked at the MAD2 and BUB1 expression levels in all the stages preceding the development of gastric cancers and also looked at their correlation with the aneuploidy levels in the premalignant gastric tissue.

In this study, there was no correlation with the level of significant aneuploidy and the level of the MAD2 and BUB1 expression levels. Although, the intention was to look for the aneuploidy levels in chromosome 1 and 4, only the results from chromosome 1 could be analysed as the signals from chromosome 4 was non-specific. This decreases the sensitivity of the study. Aberrations of chromosome 1 were seen in gastric cancer especially in the early stages of gastric cancer. MAD2 gene is in chromosome 4 and therefore it would have been interesting to have seen the aneuploidy levels of chromosome 4 as well as chromosome 1. It could be postulated that the aneuploidy occurs early in the pathogenesis and this leads to cumulative loss of particular genes, which encodes the mitotic checkpoint components, which result in over or under expression of MAD2 and BUB1 levels. Again the number of patients with significant aneuploidy (more than two standard deviation of the aneuploidy seen in normal patients) was low. A larger study looking at other chromosomes in addition to the chromosome 1 would provide us with better answer to this question in the future.

The expression levels of MAD2 and BUB1 was not affected by increasing age, or differences in the gender, family history of upper GI cancers or the presence of significant aneuploidy.

Smoking has been directly implicated in the development of various cancers E.g., lung, and bladder cancers. The studies looking at the genetic alterations caused by smoking are rare. Lin et al (2010) have shown that smoking caused abnormalities of *Kras* and P53 genes in small and non small cell lung cancer. Although there was no statistical difference in the levels of BUB1 and MAD2 levels between the smokers and the non smokers, in this study, It would be interesting to look at various other potential genetic alterations that could be caused by smoking and this could contribute to our better understanding of the mechanism involved in the tumour initiation and progression by smoking.

This study has shown that the MAD2 and BUB1 levels were significantly raised in patients with *H. pylori* associated gastritis. This demonstrated that the most important changes may occur due to the *H. pylori* infection and *H. pylori* infection may act trigger a cascade of events responsible for the progression to precancerous intestinal metaplasia stage.

4.4.1 Epigenetics and Cancer

Classic genetics does not explain the variation in phenotypes within a population. It does not explain why there are different phenotypes and different susceptibilities to a disease despite their identical DNA sequences. Epigenetics provide a partial explanation to this. C.H Waddington (1939) introduced this concept and epigenetics was defined later as heritable changes in gene expression that are not due to any alteration in the DNA sequence (Holliday 1987).

Epigenetic changes are generally categorised into four areas: DNA methylation, histone modification, chromatin remodelling and miRNAs (Estellar 2006).DNA methylation play an important role in the control of gene activity and the architecture of

the nucleus of the cell. In humans, DNA methylation occurs in cytosines that precede guanines; these are called dinucleotide CpGs. (Herman 2003, Weber 2007). CpG sites are not randomly distributed in the genome; but there are CpG-rich regions known as CpG islands, which span 5' end of the regulatory region of many genes. These islands are not methylated in normal cells (Herman 2003, Weber2007).The methylation of particular subgroups of promoter CpG islands can be detected in normal tissues. Significant nuclear abnormalities are detected as a result of spontaneous defects in DNMTs (DNA methyl transferase) (Xu1999) or experimentally induced DNMTs (Espada2007).

One of the first epigenetic alterations found is the low level of DNA methylation found in tumours as compared with the level of DNA methylation in the normal tissue (Feinberg 1983). The low level of methylation is mainly due to the hypomethylation of repetitive DNA sequences and demethylation of coding regions and introns – regions of DNA that allow alternative versions of the messenger RNA that is transcribed from a gene(Feinberg 2004). During the carcinogenesis, the degree of hypomethylation of genomic DNA increases as the lesion progresses from a benign proliferation of cells to an invasive cancer (Fraga 2004). There are three ways that are proposed to explain the contribution of DNA hypomethylation in the development of cancers: generation of chromosomal instability, reactivation of transposable elements, and loss of imprinting. Low levels of DNA methylation can favour mitotic recombination, leading to deletions and translocations and can also promote chromosomal rearrangements.(Eden 2003) .It has been shown in experiments that disruption of DNMTs causing low levels of DNA methylation can cause aneupoidy (Karpf2005).

Hypermethylation of CpG – island promoter can affect the genes that are involved in the cell cycle, DNA repair, the metabolism of carcinogenesis, cell-to-cell interaction, apoptosis, and angiogenesis, all of which are involved in the development of cancers (Baylin 2005, Correa 1994). The profiles of hypermethylation of the CpG islands in tumour suppressor genes are specific to the cancer type (Costello 200, Estellar 2001). Each tumour type can be assigned a specific, defining DNA “hypermethylation”

and this can not only be seen in the sporadic cancers but also in inherited cancer syndromes(Esteller2001).

Histone modification can occur in different histone proteins, histone variants, and histone residues such as lysine, arginine, and serine. This can involve various chemical groups e.g., methyl, acetyl, and phosphate groups. The degree of modifications can also be e.g., mono, di, or tri methylation. There are different permutations and combinations that result in a complex array of histone modifications. These modifications affect a variety of nuclear processes like gene transcription, DNA repair, DNA replication, and the organisation of chromosomes. Hypoacetylation of histone is usually associated with transcriptional activation (Mack2006, Berenstein2007). The effect of histone methylation depends on the degree and also on the type of amino acid involved (Mack2006, Berenstein2007). These changes appear early and accumulate during the development of cancer (Mack2006).

The miRNAs are non coding RNAs that regulate gene expression by sequence-specific base pairing in the 3' untranslated regions of the target RNA. The miRNAs are tightly controlled and they play an important role in the cell proliferation, apoptosis, and differentiation (He 2004). The knowledge of the human genes that lose activity due to the attachment of a miRNA to the translated regions is increasing (He 2004, Chen2005). The profiles of miRNA expression differ between normal tissues and tumour tissues and among tumour types (Chen 2005, Cailin2006, Lu 2005).

The DNA methylation and histone modification can be used in the cancer management. DNA hypermethylation markers have a potential to be used as a complementary diagnostic tool, prognostic factors and in the estimation of cancer response to the treatment for e.g., 80-90% of patients with prostate cancers have hypermethylation of glutathione S transferase gene (GPSTP1)(Lee1994, Estellar1998, Cairns2001), hypermethylation of the death-associated protein kinase(DAPK), p16ink4 α epithelial membrane protein 2(EMP3) has been linked to poor outcomes in lung, colorectal, and brain cancer respectively (Estellar 2007). DNA methylation and histone modifications are reversible. It is possible to re-express DNA

methylated genes in cancer cell lines by using demethylating agents (Estellar 2008)
Low dose DNA demethylating drugs like 5-azacytidine (Vidadza) and 5 –aza-2' –
deoxycytidine(decitabine) have been approved in the treatment of leukemia and
myelodyplastic cancers(Esteller 2008).

CHAPTER 5

General discussion

Gastric carcinoma is one of the major health burdens worldwide and a common cause of cancer related death in the world (Jeon 2010). Although the incidence of gastric cancer is gradually decreasing in the developed world, its incidence is still high in certain parts of the world. It is thought to be due to the difference in the socioeconomic conditions and is also related to the prevalence of *H. pylori* infection. The incidence of gastric cancer varies even among different regions in the same Country. The prevalence of *H. pylori* infection is gradually decreasing in certain countries like United Kingdom and Japan. Gastric cancers are diagnosed late and as a result of this has an increased mortality. In Japan, where the incidence of gastric cancer is high, there is an endoscopic surveillance programme, which helps in detecting the cancers at an early stage. This has lead to improved survival rate in this country. Correa described a multistep pathway in the pathogenesis of gastric cancer. As there are several histologically identifiable premalignant stages and the lesion is easily accessible, changes in gastric mucosa is an ideal human research model for investigations into the underlying genetic basis for the development of gastric cancer. The research interest has grown in this particular aspect as prognostic markers are needed to determine those patients at greater risk for developing the malignancy. The development of these markers would help us to stratify the patients into a high risk/ low risk groups for cancer and would help us in streamlining the use of health resource for example- high risk patients could undergo intensive surveillance programme. Currently, the pathogenic events associated with the development of gastric cancer are complex with wide range of chromosomal abnormalities implicated (Uchida M 2010,BuffartTE et al 2009,Sanchez- Perez et al 2009).

Understanding this complex genetic process would help us in predicting the subgroup of patients who has progressive disease and also in designing treatment targeting a particular stage of the pathway to arrest its progression or even reverse the changes concurred by these tissue. The general aim of this thesis was therefore to contribute to the molecular data to the gastric cancer pathogenesis model and hopefully

to define when specific abnormalities arise and become prominent. Hence, the chromosomal aberration was studied in all the premalignant histological stages of gastric cancer development

The initial investigation was intended to characterise when, during the histological progression of the gastric cancer pathway, certain chromosomal alterations first appeared. FISH was the technique of choice, as it involves study of single layer cell analysis and therefore has a sensitivity to detect low frequency abnormalities, which is present in the premalignant lesions. There are number of ways the cytogenetic abnormalities could be studied and some of them have been tested in our lab with not very good yield of cells. Although there are several methods available to study the chromosome aberrations, the use of endoscopic cytology brushes to exfoliate gastric epithelial cells during the upper GI endoscopy was adopted. This was mainly due to the fact that this method has been used successfully in our unit by Doak and Williams in the past and was found to be reliable and sensitive. Following several washing steps, cells were deposited onto slides using cytopsin and their cytoplasm was partially digested if necessary by the treatment with pepsin to allow the FISH probe penetration. This procedure allowed consistent generation of suitable interphase preparation for subsequent FISH analysis. Brushings from the gastric mucosa posed various challenges as the angle of the gastric mucosa was difficult to do the gastric brushings and also the fact that the environment in the stomach is hostile with bile, gastric acid and food residue present in the stomach, which could lead to the digestion of cells. This was improved by washing the gastric mucosa with 5-10ml of sterile water if necessary before using the brush during the endoscopy. The yield of cells improved as the study progressed and there are several factors which may have contributed to this, for example increase in the experience of the endoscopy nurses helping during the procedure, change in the transport medium from ethanol to ETN buffer, decreasing the time of pepsin exposure during the pepsin treatment. As a result of the above mentioned factors, the yield of cells was good even after twenty four hours. The cell preparation, fixation and the actual method of FISH is explained in detail in chapter 2. Following this, centromeric probes for chromosomes 1 and 4 were used to determine the chromosomal aberrations of these chromosomes in the premalignant stages.

In the second part of this study, MAD2 and BUB1 expression levels in all the premalignant stages implicated in the pathogenesis of gastric cancer were studied. MAD2 and BUB1 are important genes in the control of mitotic spindle cell check point. The spindle cell check point is a very well regulated feedback mechanism which prevents the cells from progressing from the metaphase to anaphase if a chromosome lacks bipolar attachment of a spindle. The unattached kinetochore generate signal which triggers a cascade of events, which help in preventing the progress to anaphase. Alterations in the gene expression levels of BUB1 gene has reported in various tumours like breast cancers (Myrie et al. 2000) and colorectal cancers (Shichiri et al. 2002). Recent studies have looked at BUB1 genes in the gastric cancers and have found out that the BUB1 levels are significantly higher in patients with diffuse type gastric cancer when compared with the intestinal type gastric cancer (Grabsch et al. 2004). Over expression of BUB1 has been shown to be associated with increased proliferation of gastric cancer (Grabsch et al. 2003). Over expression of MAD2 has been observed in gastric cancer (Tanaka et al. 2001) but this did not correlate with aneuploidy, clinical or pathological features (Wu et al. 2004). Mutation of MAD2 has also been noted in the gastric cancer which could lead to the development and progression of gastric cancer (H Kim et al. 2005).

Several studies have looked at various gene expression levels and the development of aneuploidy in different cancers. The level of Aurora Kinase A, Aurora Kinase B, MAD2 and BUB1 and development of aneuploidy in colorectal cancer was studied. This study showed that the level of BUB1 was significantly reduced in aneuploidy colorectal cancers when compared with diploid cancers (Burum –Auensen E et al 2008). In an *invivo* study, BUB1 function has been shown to be tension – dependent check point function and leading to aneuploidy and tumorigenesis (Schliekelman et al 2009).

MAD2 and BUB1 genes have been studied by our group in the varying histological stages of Barrett's oesophagus ranging from metaplasia to adenocarcinoma. Both over and under expression of MAD2 and BUB1 levels were noticed in all the stages of the neoplastic progression and there was no particular trend in the levels of MAD2 and BUB1 gene expression levels. There was no correlation with aneuploidy

implicating that other mechanism play an important role in the development of oesophageal carcinoma (Doak S et al 2004).

This study looked at the MAD2 and BUB1 levels in all the premalignant stages of gastric cancer to look for any particular trend in their levels as the disease progresses and also to look at the level of aneuploidy during these premalignant stages. According to our hypothesis, the change in the levels of MAD2 and BUB1 levels should follow a particular pattern with the advancing histological stage. A steady state of these genes are thought to be essential in maintain normal cell division and ensuring that the daughter cells after mitosis is a diploid cell. Over or under expression level of these genes might result in aneuploidy.

The gastric biopsies obtained from the same site after the gastric cytology brushings were used for this. Real time RT PCR was used to determine the gene expression levels of MAD2 and BUB1 genes. This is a sensitive method and analyses all fluorescent signal generated during the PCR process. SYBR green was used and this is an intercalated dye that binds to the minor groove of the double stranded DNA. The method used for analysis is detailed in chapter 4. Any defect in the mitotic check point leads to aneuploidy and there has been great interest in determining the exact defect in the spindle check point that leads to aneuploidy in cancers, which exhibit chromosomal instability. Gastric cancer provides a good model to study both the aneuploidy and the mitotic check point defect as it involves a well defined pathway in the progression to gastric cancer. The correlation of aneuploidy with the expression levels of MAD2 and BUB1 levels was performed to look at the influence the change in the expression level can play in the development of aneuploidy.

5.1. Conclusion

5.1.1 Findings for chromosomal aberrations

Chromosome 1 and 4 were chosen as there has been two recent studies which looked at the aneuploidy levels of chromosome 1 in gastric cancer and has shown that the abnormality of chromosome 1 occurs independent of the gastric cancer subtype (Kitayama 2000) and also abnormalities of chromosome 1 was common in gastric cardia tumours (Fringes 2000). Chromosome 4 was studied as it has been shown that the amplification of chromosome 4 was shown in the development of oesophageal adenocarcinoma (Doak et al 2003, Williams L 2004). The findings from this study are as follows:

1. Aneuploidy level of chromosome 1 is higher in normal gastric tissue (2.37%) when compared with the normal oesophageal tissue (2.2%) and this is in this study thought to be secondary to the harsh environmental condition to which the gastric tissue is exposed.
2. The increase in the aneuploidy level of chromosome 1 progressively increased significantly throughout the histological progression. The aneuploidy level for chromosome 1 are as follows:

Histological stage	Aneuploidy level	Level of significance (p value)
Gatritis	3.74	0.038
H. <i>pylori</i> gastritis	5.055	0.015
Intestinal metaplasia	6.32	0.001

3. Elderly male patients are high risk in developing gastric cancers but this study did not demonstrate any significant increase in the aneuploidy of chromosome 1 in this sub group.

4. There was no significant difference in the level of aneuploidy in chromosome 1 levels between smokers and non smokers, patients on PPI and those who are not on PPI, patients on NSAIDs and those who do not take it.
5. Aneuploidy levels between the intestinal metaplasia stage in the stomach was found to be four folds higher (6.32%) when compared with the aneuploidy levels (2.31) in the oesophagus(Barrett's oesophagus).

5.2. Findings of MAD2 and BUB1 expression levels

MAD2 and BUB1 gene expression levels were studied as these genes are considered as important genes in the mitotic check point mechanism. Biopsies from forty four patients were used to extract the RNA. The biopsies were frozen at 4° Celsius in the first 24 hours of taking the sample. RNA extraction was performed in batches and they were stored at -80°C in small aliquots. The RNA was thawed in batches to prevent frequent freezing and thawing to perform the real time PCR. The real time PCR was conducted according to the protocol described in Chapter 2 Materials and methods.

To our knowledge, the expression levels Of MAD2 and BUB1 have been studied in the gastric cancers but they have not been previously studied in all the premalignant stages of gastric cancer. It was also thought that the alterations in their expression level would correspond to the aneuploidy levels seen in the samples. The conclusions from this study are:

1. There was no difference in MAD2 and BUB1 levels between male and female patients.
2. There was no difference in the level of MAD2 and BUB1 expression levels in patients with aneuploidy. There was no difference in the expression level of MAD2 and BUB1 levels even in patients with significant aneuploidy level of 4.86% (as explained in the chapter 3) It must be stressed that the sensitivity of this study is low as only chromosome 1 was studied.

3. This study had six patients with family history of upper gastrointestinal cancers. There was a decreasing trend in the expression level of MAD2 and BUB1 levels although this did not reach statistical significance.
4. Although smoking is a well known risk factor for the development of upper gastrointestinal cancers, there was no difference in the expression of MAD2 and BUB1 levels between the smokers and the non smokers.
5. There was a significant increase in the expression levels of MAD2 and BUB1 levels in patients with *H. pylori* associated gastritis when compared with patients with normal histology. There was no difference in the expression levels of MAD2 and BUB1 levels between patients with normal histology and chronic gastritis that is not associated with *H. pylori* infection. This stresses that the infection with *H. pylori* plays an important role in the progression of the disease
6. MAD2 and BUB1 levels were analysed in all the stages of premalignant gastric cancer pathways. There was no significant difference in the levels of MAD2 and BUB1 between normal histology and *H. pylori* negative gastritis. Again there was no statistical difference in the levels of MAD2 and BUB1 levels between normal histology and intestinal metaplasia. But there was significant increase in the levels of MAD2 and BUB1 between patients with normal histology and patients with *H. pylori* associated gastritis

5.3 Limitations of the study

The strength of the study lies in the fact that it looked at a large number of patients and determined their aneuploidy levels of chromosome 1 and their MAD2 and BUB1 expression levels and included all the premalignant stages of gastric cancer, there are limitations to this study and they are:

1. Although the intention of the study was to look for the aneuploidy levels in chromosome 1 and chromosome 4, the data from chromosome 4 could not be used as it was non-specific. This is one of the major limitations of the study as only one chromosome was studied and this does decrease the sensitivity of the study.

2. The number of patients with intestinal metaplasia was low (n=4) but there is no reliable way of predicting and increasing the number of this sub group in the endoscopy.
3. Patients were given questionnaires before the endoscopy to assess their risk factors and to get details regarding their life style e.g., smoking, alcohol intake, dietary habit including the number of portions of fruits and vegetable, drugs. This was not corroborated with their primary care physician or their medical records due to the constraints on the time and resources.
4. There was no difference in the expression levels of MAD2 and BUB1 levels in patients with aneuploidy or in patients with significant aneuploidy levels (4.86%) of chromosome 1. But it must be stressed that only the aneuploidy levels of chromosome 1 was studied due to the above mentioned reason. This again decreases the sensitivity of the investigation.

5.4 Expanding work – future studies

There are number of ways this study could be taken forward and they are as follows:

1. It would be interesting to perform a follow up study in 5 years time and review individual phenotypic deterioration or progression in patients with significant aneuploidy and also the level of their aneuploidy at that time. This would help us in the assessment of aneuploidy as a predictor of gastric cancer.
2. There was one patient in the study who had significant aneuploidy levels and family history of gastric cancer. It would also be interesting to follow this patient to determine the histological progression. This could be an interesting case study – on which a future detailed study could be designed in patients with significant family history of gastric cancer.
3. Patients who have significant aneuploidy and with later stage of histology according to the stages described by Correa, can have their risk factors modified – for example testing and treating *H. pylori*, quit smoking, decrease weight and increase intake of vegetables and fresh fruits. It would be interesting to see what happens to their aneuploidy level, MAD2 and BUB1 expression levels after the risk modification. This would have to be a larger

study performed over a reasonable length of time to allow for such modification of risk to effectively affect the aneuploidy levels.

4. The biopsy taken to determine the histological stage is normally preserved as paraffin embedded tissue and is available for future use if necessary. This can be used to determine other chromosome aberrations by other methods such as immunohistochemistry. This will complement this study well as it will increase the number of chromosomes studied in the same patients.
6. This study has shown again that the background aneuploidy level is increased in normal gastric tissue when compared with the oesophageal tissue. This is due to the unique microenvironment that exists in the stomach. It would be interesting to know if this is peculiar to the stomach and if there is any difference in the aneuploidy level in different part of the gastro intestinal system. This may help to explain the difference in the incidence of cancers in different parts of the gastrointestinal system.
7. MAD2 and BUB1 gene expression levels have been studied here but it would be interesting to study other mitotic check point genes like Aurora Kinase A and Aurora Kinase B in the remaining RNA samples. Aurora KinaseA and B play an important role in the spindle check point regulation (Murata-Hori et al 2002). It has been shown that aurora kinaseB is essential for MAD2 and BUBR1 to attach to the kinetochore (Lens S et al 2010). There is a significant amount of research in determining the role of Aurora kinase inhibitors in the treatment of cancers.

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Appendices

Appendix 1.1

ANEUPLOIDY AND GENE TRANSCRIPTION LEVELS IN OESOPHAGEAL AND GASTRIC CANCERS.

**REF 07/WMW01/46
29/09/07**

VERSION 2

DR.A.SOMASEKAR, SPECIALIST REGISTRAR,

**DEPARTMENT OF GASTROENTEROLOGY, NEATH PORT TALBOT
HOSPITAL**

WHY HAVE I BEEN ASKED TO PARTICIPATE IN THE STUDY?

You are waiting for a procedure called endoscopy, which is a magic eye test looking at the gullet and stomach. There are a lot of reasons why someone will have this procedure For example, heartburn, indigestion, abdominal pain, weight loss. All patients who are waiting for this procedure are invited to participate in the study .We are interested in a certain group of patients who might have inflammation of the gullet or stomach and we plan to study the inflammation in detail.

WHO ARE DOING THE STUDY?

The study is being performed by Dr. A.Somasekar, specialist registrar in gastroenterology and this study is supervised by Prof. J.G.Williams, Consultant Gastroenterologist at the Neath Port Talbot Hospital and Dr.G.Jenkins, Molecular Biologist at the Swansea University. Dr. A. Somasekar is carrying out this study for her research degree (M.D.,) at the Swansea University.

WHAT DOES THE STUDY LOOK AT?

The study aims to look at the lining of the stomach closely by taking biopsies and brushings from the lining of the stomach.

HOW ARE THE BIOPSIES TAKEN AND BRUSHINGS TAKEN?

During the normal endoscopy we take 2-4 biopsies. They are not painful and you do not feel it. The cytology brushes are very small, flexible brushes and they are used to take scraping from the lining of the stomach and they are not painful procedures.

DOES IT EXTEND THE PROCEDURE TIME?

On an average the endoscopy takes approximately 15 minutes and if we take these additional biopsies the procedure time would be extended by another 4-5minutes.

ARE THERE ANY COMPLICATIONS WITH THE BIOPSIES?

Upper GI endoscopy with biopsies is generally a safe procedure and the complications are rare. Although it is safe procedure, there is a very small risk of bleeding or perforation that can occur during this procedure.

WHAT HAPPENS TO THE BIOPSIES AND BRUSHINGS TAKEN?

The biopsies are stored and analysed at the School of Medicine, Swansea. They would be looked at by a pathologist and certain proteins would be extracted from them to help us understand how this disease occurs. The cells from the brushes would be collected and will also be analysed for any abnormalities at the school of medicine.

WHAT HAPPENS TO THE EXTRA SPECIMEN?

The specimen would be stored during the study period and would be destroyed after this.

WHAT IF I DO NOT WANT TO PARTICIPATE IN THE STUDY?

If you choose not to participate in the study, this will not affect your procedure today or your future treatment.

WHAT IS THE BENEFIT TO ME BY THIS STUDY?

This study would help us in better understanding of the disease process which affects a significant percentage of the population but is unlikely to influence any of the treatment you are on at the moment.

WILL THE RESEARCH DIRECTLY AFFECT ME?

As this is a research looking at the progress of certain inflammatory condition, there is no need to worry regarding the results of the research as this study is aimed at improving our understanding of the disease and would not result in any change in your management at present.

WHAT WILL HAPPEN TO MY PERSONAL DETAILS?

These information would be stored in the hospital and only the research team would be eligible to access it. The biopsies and the brushings are coded so it does not bear any of your personal details.

WHO SHOULD I CONTACT TO DISCUSS REGARDING THE PROCEDURE AND TO GET THE RESULTS?

You can contact the endoscopy department on our direct line 01639 862037 or contact our specialist nurse on 01639 862551.

WOULD I BE ABLE TO OBTAIN THE RESULTS OF THE STUDY?

Yes, once the study is completed we plan to exhibit a poster with the results of the study in the endoscopy department at the Neath Port Talbot Hospital.

Appendix 1.2

ANEUPLOIDY AND GENE TRANSCRIPTION LEVELS IN OESOPHAGEAL AND GASTRIC CANCERS

Neath Port Talbot Hospital

PATIENT CONSENT FORM

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

I consent to additional biopsies and brushing during the endoscopic procedure for research purposes.

I understand and agree that the information regarding me will be held at the hospital and the information I give will not be communicated to anyone outside the research team.

PATIENTS NAME

PATIENTS SIGNATURE

DATE

23/08/2007

**ANEUPLOIDY AND GENE TRANSCRIPTION LEVELS IN OESOPHAGEAL AND
GASTRIC CANCERS**

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QUESTIONNAIRE

1.NAME

2.AGE

3. SEX

4. HOSP NO/DOB

5.WEIGHT

6.EMPLOYMENT

7.SMOKING- DURATION/AMOUNT

8. ALCOHOL – UNITS/WEEK

9. DIET

10. PMH OF PEPTIC ULCER DISEASE/GI /OTHER CANCERS

11. F/H GI CANCERS

12. PREVIOUS OGD

13. ENDOSCOPIC FINDINGS (CURRENT)

Appendix 1.2

Raw data for the endoscopy brushings for chromosome 1:

Patient Number	2Signal	1Signal	3Signal	>3Signal
1	193	4	2	0
2	151	3	3	3
3	105	3	3	1
4	194	8	0	1
5	259	8	2	2
6	209	7	3	2
7	183	5	0	0
8	252	8	1	3
9	351	13	1	1
10	347	7	1	1
11	256	19	1	5
12	214	10	1	0
13	402	3	1	2
14	391	8	3	3
15	500	9	2	7
16	120	5	0	0
17	222	5	0	1
18	149	7	0	4
19	145	3	3	0
20	299	3	2	0
21	141	6	2	1
22	131	3	0	1
23	65	3	0	0
24	255	8	0	3
25	231	2	1	1
26	266	2	3	1
27	129	4	0	0
28	244	14	0	1
29	232	7	1	0
30	133	4	0	1
31	255	3	2	0
32	243	2	1	1
33	243	2	1	1
34	105	1	0	1
35	193	0	3	3
36	248	8	4	0
37	263	4	1	0
38	75	0	1	0
39	94	2	0	0

40	148	3	3	3
41	231	0	1	0
42	214	2	1	0
43	49	4	0	1
44	62	1	0	0
45	315	4	2	1
46	305	4	0	2
47	91	1	1	0
48	97	1	1	1
49	213	9	4	0
50	235	1	1	10
51	59	1	0	1
52	367	6	0	0
53	247	4	1	0
54	327	2	2	0
55	217	3	1	0
56	214	8	2	1
57	244	5	1	0
58	211	3	1	1
59	102	2	0	0
60	435	23	1	0
61	141	2	0	0

Appendix 1.3

Raw Data with Patients with Gastritis

Patients	MAD2(A)	MAD2(B)	Mean MAD2	SD for MAD2	BUB1(a)	BUB1(B)	Mean BUB1	SD for BUB1
A4	7.500000	0.236000	3.870000	5.139939	0.723000	0.066500	0.723000	0.464396
A7	0.914000	0.162000	0.538000	0.531748	0.704000	0.171000	0.438000	0.376756
A8	0.929000	0.084100	0.506000	0.597140	0.348000	0.106000	0.227000	0.170910
A18	1.110000	1.110000	1.110000	0.000000	0.301000	0.301000	0.301000	0.000000
A20	3.340000	4.470000	3.910000	0.799362	1.130000	3.200000	2.170000	1.464597
A26	0.539000	3.750000	2.140000	2.267658	1.750000	1.460000	1.600000	0.204882
A32	0.533000	0.293000	0.413000	0.169631	0.185000	0.256000	0.221000	0.050586
A38	0.855000	0.855000	0.855000	0.000000	0.197000	0.197000	0.197000	0.000000
A39	1.090000	1.090000	1.090000	0.000000	0.258000	0.258000	0.258000	0.000000
A43	0.849000	0.904000	0.876000	0.038770	0.316000	0.267000	0.291000	0.035223
A56	0.402000	0.402000	0.402000	0.000000	0.133000	0.133000	0.133000	0.000000
A57	0.354000	0.354000	0.354000	0.000000	0.201000	0.201000	0.201000	0.000000
A62	2.090000	1.080000	1.580000	0.710276	0.316000	0.441000	0.379000	0.088658
A63	1.010000	0.738000	0.876000	0.193944	0.311000	0.361000	0.336000	0.035685
A72	0.906000	0.761000	0.833000	0.102570	0.215000	0.352000	0.284000	0.096602
A74	0.840000	0.761000	0.801000	0.056044	0.274000	0.253000	0.263000	0.014473
A75	0.810000	0.341000	0.575000	0.331711	0.374000	0.218000	0.296000	0.110327
A76	0.529000	0.656000	0.592000	0.089606	0.252000	0.196000	0.224000	0.038961
A77	1.030000	1.020000	1.030000	0.011341	0.510000	0.271000	0.391000	0.169122
A81	1.970000	0.826000	1.400000	0.807197	1.710000	0.419000	1.060000	0.910909
A78	1.640000	0.782000	1.210000	0.609824	0.405000	0.214000	0.310000	0.135400
A18	1.110000	1.110000	1.110000	0.000000	0.301000	0.301000	0.301000	0.000000

Appendix 1.4

Raw data for patients with *H. pylori* associated gastritis

Patient	MAD2(A)	MAD2(B)	Mean MAD2	SD for MAD2	BUB1(A)	BUB1(B)	Mean BUB1	SD for BUB1
A25	0.603000	1.690000	1.150000	0.767999	0.202000	0.215000	0.208000	0.009439
A36	1.110000	1.110000	1.110000	0.000000	0.631000	0.631000	0.631000	0.000000
A41	1.590000	1.020000	1.310000	0.402698	0.157000	0.226000	0.191000	0.048864
A54	0.857000	0.495000	0.676000	0.256129	0.608000	0.793000	0.700000	0.130464
A59	0.579000	0.579000	0.579000	0.000000	0.285000	0.285000	0.285000	0.000000
A73	0.860000	0.893000	0.877000	0.023017	0.709000	0.342000	0.526000	0.259694
A68	0.771000	1.580000	1.170000	0.571765	0.302000	0.302000	0.302000	0.000014
A35	10.900000	10.900000	1.090000	0.000000	1.450000	1.450000	1.450000	0.000000

Appendix 1.5

Raw data for patients with intestinal metaplasia

Patients	MAD(A)	MAD2(B)	Mean MAD2	SD for MAD2	BUB1(A)	BUB (B)	Mean BUB1	SD for BUB1
A61	1.260000	0.898000	1.080000	0.253637	0.462000	0.404000	0.404000	0.041001
A79	1.150000	0.914000	1.030000	0.165499	0.562000	0.563000	0.563000	0.000759
A80	0.585000	0.595000	0.590000	0.006865	0.292000	0.267000	0.267000	0.017652
A58	0.672000	0.512000	0.592000	0.112634	0.137000	0.596000	0.596000	0.324408

Appendix 1.6

Raw Data for MAD2 and BUB1 expression levels

Patient	Histology	MAD2(A)	MAD2(B)	Mean MAD2	SD for MAD2	BUB1 (A)	BUB1(B)	Mean BUB1	SD for BUB1
A5	Normal	2.179395	0.117541	1.148468	1.457951	0.299899	0.035745	0.167822	0.186785
A6	Normal	1.370909	0.065676	0.718292	0.922939	0.142079	0.05105	0.096565	0.064368
A27	Normal	0.53885	0.760475	0.649662	0.156713	0.252044	0.202372	0.227208	0.035123
A31	Normal	0.985646	0.587368	0.786507	0.281625	0.316752	0.480008	0.39838	0.11544
A33	Normal	1.149702	1.149702	1.149702	0	0.398521	0.398521	0.398521	0
A42	Normal	0.747108	0.700082	0.723595	0.033252	0.235949	0.180267	0.208108	0.039374
A46	Normal	0.405171	0.475569	0.44037	0.049778	0.116853	0.179821	0.148337	0.044525
A69	Normal	0.825936	0.825936	0.825936	0	0.567173	0.567173	0.567173	0
A67	Normal	0.573357	0.491339	0.532348	0.057996	0.319321	0.302282	0.310801	0.012048
A70	Normal	0.372005	0.372005	0.372005	0	0.208027	0.208027	0.208027	0
A12	Normal	0.673833	0.040699	0.357266	0.447693	0.200476	0.042205	0.12134	0.111915
A14	Normal	10.95467	10.95467	10.95467	0	14.99323	14.99323	14.99323	0

Publication from the research to date

Aneuploidy involving chromosome 1 may be a early predictive marker of intestinal type gastric cancer. Williams L, Somasekar A, Davies DJ, Cronin J, Doak SH, Alcolado R, Williams JG, Griffiths AP, Baxter JN, Jenkins GJ. Mutation Research 2009 Oct 2;669(1-2):104-11.

Aneuploidy as a biomarker of cancer risk in premalignant gastric tissue. Jenkins GJ, Williams L, Somasekar A, DoakSH, Williams J, Griffiths AP, BaxterJN. Mutagenesis vol. 25 No 6. 631-658.

Altered Gene expression levels of MAD2, BUB1, Aurora Kinase A and B in early gastric cancer. Somasekar A, Chatterjee A, Williams L, Griffiths AP, Williams J, Jenkins GJ. Gut 2009;59 A16.

Aneuploidy associated with H. pylori infection is common in early gastric cancer. A Somasekar, Williams J, Williams L, Davies J, Griffiths AP, Jenkins G. Plenary Poster. Gut 2009.