Aberrant DNA Methylation In Oesophageal Cancer And Barrett's Oesophagus

by

Eric Smith

Cert Med Lab Sc, Ass Dip Med Lab Sc, BSc

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ABSTRACT

Oesophageal cancer is the eighth most common cancer and the sixth most common cause of death from cancer worldwide. There are two main histological types of oesophageal cancer: squamous cell carcinoma (ESCC), adenocarcinoma (EAC). In the developing world the major histological type is ESCC, whilst in the developed world EAC is increasing rapidly in incidence and is now the major type. Both histological types have a similarly poor prognosis, with a high morbidity and mortality.

Barrett's oesophagus (BE) is considered a precursor to EAC. It is found in up to 1.5% of the general population, and in up to 12% of patients who are investigated for chronic reflux symptoms. Approximately 0.5 to 1% of patients with BE will develop EAC each year, and patients with BE have 30- to 125-fold increased risk of EAC compared to the general population. Gastro-oesophageal reflux is the major risk factor for the development of BE and EAC, and medical and surgical anti-reflux therapies are available to relieve symptoms of the reflux and prevent reflux-related complications, although it is not certain if they will prevent the development of cancer.

The development of oesophageal cancer is associated with an accumulation of genetic abnormalities, with some reports suggesting a stepwise progression of genetic changes involving the up-regulation and down-regulation of critical genes. Methylation of cytosine residues in CpG dinucleotides of the promoter regions of genes, DNA methylation, is a genomic change associated with silencing of gene expression.

In the studies described in this thesis I have developed a simple quantitative method to assess DNA methylation using the melt data obtained following amplification of bisulphite modified DNA. I identified eight genes (BNIP3, FBN2, ID4, MLF1, PRDM2, RBP4, RARRES1, TFAP2C) that had been reported methylated in other cancers, but not before in BE or EAC, and four genes (CLDN6, DCBLD2, FNBP1 and MGC16824) that had not previously been reported as methylated in any cancer. I have shown that in non-dysplastic (metaplastic) BE, methylation of APC, ID4, MGMT, RBP1, SFRP1, TIMP3 and TMEFF2 (but not RUNX3 or CDKN2A) occurs as frequently in BE as EAC, suggesting that BE is more like cancer than normal squamous mucosa. I have used DNA methylation as a surrogate measure of the

efficacy of fundoplication and proton pump inhibitor (PPI) treatment for BE. Five or more years after fundoplication there was a significant regression of BE and a reduction in the number of methylated genes in the remaining BE. In contrast, although high-dose PPI for six months significantly reduced inflammation and epithelial cell proliferation, it did not alter methylation. The reduction in methylation may be associated with a decreased risk for the development of dysplasia and adenocarcinoma. Finally, I have suggested extensions to the work published in this thesis. Further understanding of which genes are methylated in BE, EAC and ESCC, the mechanisms responsible for this aberrant methylation, and the function of the genes, would improve our insight into the underlying biology of oesophageal diseases, and potentially lead to new biomarkers or treatment options.

DECLARATION

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Eric Smith

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ABBREVIATIONS

BE	Barrett's oesophagus
cDNA	complementary DNA
COBRA	combined bisulfite restriction analysis
COX2	cyclooxygenase 2
CpG	cytosine-phosphate-guanine dinucleotide
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
EAC	oesophageal adenocarcinoma
EMR	endoscopic mucosal resection
ESCC	oesophageal squamous cell carcinoma
LOH	loss of heterozygosity
MBD	methyl-CpG-binding domain
MSP	methylation-specific PCR
NSAID	non-steroidal anti-inflammatory drug
PCR	polymerase chain reaction
PPI	proton pump inhibitor
RNA	ribonucleic acid
USA	United States of America

To Catherine and Maddie

In memory of our faithful hounds, Sha and Yasmine

CHAPTER 1: INTRODUCTION

1.1 Thesis overview

This thesis describes my research in which I investigated DNA methylation in cancer. More specifically, I focussed on improving methods to analyse DNA methylation, discovering new genes which are methylated in oesophageal squamous cell carcinoma (ESCC), oesophageal adenocarcinoma (EAC) and Barrett's oesophagus (BE), and determining the effect of surgical or medical anti-reflux therapy on the DNA methylation present in BE.

1.2 Cancer of the oesophagus

1.2.1 Introduction

Oesophageal cancer is the eighth most common cancer (4.2% of the all cancers) and the sixth most common cause of death from cancer worldwide (5.7% of all deaths from cancer)(Parkin *et al.* 2005). There are two main histological types of oesophageal cancer: ESCC, and EAC. In the developing world the major histological type is ESCC, whilst in the developed world EAC is increasing rapidly in incidence and is now the major type. Both histological types have a similarly poor prognosis, with a high morbidity and mortality.

1.2.2 Oesophageal squamous cell carcinoma

Oesophageal squamous cell carcinoma probably develops through a progressive sequence from normal squamous epithelium to basal cell hyperplasia, increasingly severe degrees of squamous cell dysplasia, carcinoma-situ, and invasive carcinoma (Shimizu *et al.* 2007). It can occur anywhere along the length of the oesophagus, but most commonly in the middle third.

Squamous cell carcinoma is the most common form of oesophageal cancer worldwide. There is striking geographical variation in incidence of ESCC, both between and within countries. In China ESCC is the fourth most frequent cancer overall (Li *et al.* 1996). Within China there are pockets of very high incidence, such as in parts of Hebei Province where the disease is the most common cancer, with an incidence which is the highest in the world at 199 per 100,000 population (Liu *et al.* 2004). My studies on ESCC were in collaboration with thoracic surgeons at the Fourth Hospital of the Hebei Medical University and Hebei Provincial Tumor Hospital (primarily Professor Jun Feng Liu, Director of the Department of Thoracic Surgery) and used resection tissues from their hospital.

Increased risk for ESCC has been associated with a range of carcinogens. Tobacco or alcohol are the main known risk factors involved in the low incidence areas including the West (Pandeya *et al.* 2008; Pandeya *et al.* 2009). Consumption of red meat is associated with an increase, and consumption of fruits, vegetables and olive oil with a reduction, in the cancer in a recent large European study (Lagiou *et al.* 2009). Tobacco (smoking or chewing), betel quid chewing, and alcohol are considered the main risk factors in the high incidence areas (Lee *et al.* 2005), but diet and other factors have also been suggested to be important. Thus maté consumption, particularly drunk scalding hot, is associated with increased risk in South America (Goldenberg *et al.* 2003; Sewram *et al.* 2003). The consumption of pickled vegetables, nitrosamine rich, mycotoxin or silica contaminated foods, and opium or pipe stem residues have also been implicated. Deficient dietary intake of micronutrients, including vitamins A and C, molybdenum, copper and zinc, are thought to contribute to the high risk in China, central Asia, and southern Africa (e.g., (Yang *et al.* 1984; Lu *et al.* 2006)). Genetic predisposition may contribute to the high rates of ESCC in Japan, with polymorphisms of the alcohol-metabolising enzymes, alcohol dehydrogenase 2 and aldehyde dehydrogenase 2, increasing the risk of ESCC synergistically (Yokoyama *et al.* 2002).

1.2.3 Oesophageal adenocarcinoma

Oesophageal adenocarcinomas are generally considered to arise in the glandular tissue of BE (discussed in Section 1.2.5), although it has been suggested that they can arise independently of BE, possibly from oesophageal glands. They are most commonly found in the lower third of the oesophagus, which is where the glands are found. The incidence of EAC has increased more rapidly than any other common cancer over the last three to four decades in Western Europe, North America and Australia (Lord *et al.* 1998; Botterweck *et al.* 2000; Pohl & Welch 2005; van Blankenstein *et al.* 2007). In the USA, EAC has increased 460% in males and 335% in females between 1975 to 2004 (Brown *et al.* 2008), while the relative rates of most common cancers, such as breast, colon or lung have remained stable or declined. In contrast, there have been no observed trends in the incidences of ESCC or gastric cancers, making it unlikely that a change in diagnostic classification is responsible for the increase in EAC. At the time of diagnosis more than 50% of patients will either have unresectable tumours or detectable metastases, and the overall 5 year survival rate for all patients is less than 15% (Daly *et al.* 1996; Torres *et al.* 1999; Polednak 2003).

The most important risk factors for EAC are BE and gastro-oesophageal reflux (Lagergren *et al.* 1999; Shaheen & Ransohoff 2002; Demeester 2009; Falk 2009). However, symptomatic gastro-oesophageal reflux disease is infrequent or absent in up to 48% of patients who develop EAC (Reid *et al.* 2010). Other risk factors include drugs which relax the lower oesophageal sphincter (e.g., calcium channel blockers, nitrates, beta-blockers, progesterone), obesity, fat intake, tobacco and alcohol (Pera *et al.* 2005). Protective factors include Helicobacter pylori (especially CagA positive strains), and dietary factors such as selenium, antioxidants and fruits and vegetables (Pera *et al.* 2005). The most likely explanation for the increase in incidence of EAC seems to be the increasing prevalence of BE, possibly as a consequence of gastro-oesophageal reflux becoming more common with increasing incidence of obesity.

1.2.4 Treatment of oesophageal cancer

Treatment for oesophageal cancer may be either curative or palliative, depending on the stage of the disease and the patient's condition. The main predictor of survival is the extent of lymph node metastases, which in turn is predicted by the depth of tumour invasion. If the lymph node spread is limited, even moderately advanced tumours may be cured by surgery. The earliest stages of disease, ranging from high-grade dysplasia to cancer contained within the mucosa, may be treated by oesophagectomy, with a high expectation of cure. Mortality rates following oesophagectomy are now less than 10% as a result of improvements in surgical technique and perioperative care. However, this relatively high mortality and the associated morbidity has encouraged the development of ablation or endoscopic resection as a curative therapy for these conditions, with the advantage that the oesophagus is preserved.

The poor outcome from surgery alone for so many patients prompted surgeons to incorporate either adjuvant or neo-adjuvant therapy into the treatment regime. While no benefit has been found from adjuvant chemotherapy or radiotherapy, some studies claim a benefit from neoadjuvant therapy. Problems with interpreting some of the published trials include the pooling of all histological types and insufficient study size or duration. DeMeester, in a recent review, claimed that there was no advantage for EAC patients given neoadjuvant chemoradiotherapy followed by surgery compared to surgery alone (Demeester 2009). However, from a meta-analysis of randomised trial data, Gebski concluded that there was a

significant survival benefit for patients with EAC given neoadjuvant therapy (Gebski *et al.* 2007).

Oesophageal cancer is often not discovered until it is too advanced for resection with curative intent. The aim of treatment then becomes palliation. If the tumour itself is resectable the best palliation is generally obtained by surgery, which provides relief from dysphagia in 90% of patients. For unresectable tumours or where distant metastases are present, survival is much shorter and excisional surgery is rarely justified. Under these circumstances, treatment is aimed at restoring swallowing, controlling symptoms and improving the quality of and prolonging life, rather than curing the disease. There are many options available for this, including oesophageal dilatation and intraluminal stents to keep the oesophagus open, and a range of options to debulk the tumour (Javle *et al.* 2006).

1.2.5 Barrett's oesophagus

Barrett's oesophagus is the replacement of the normal stratified squamous epithelium of the oesophagus with a columnar epithelium in which there are acid mucin-containing goblet cells. Since Norman Barrett first described it in the 1950s (Barrett 1950), the definition and hence the diagnosis of BE has been controversial, and has evolved with our understanding of the condition.

The current definition adopted by the American College of Gastroenterology and its Practice Parameters Committee is that there is "a change in the distal esophageal epithelium of any length that can be recognized as columnar type mucosa at endoscopy and is confirmed to have intestinal metaplasia by biopsy of the tubular esophagus" (Wang & Sampliner 2008). This definition draws the distinction between specialised intestinal metaplasia (which contains acid mucin-containing goblet cells), and other types of columnar metaplasia such as cardiac or fundic type epithelium. It is based on the belief that only the intestinal type of columnar metaplasia has the potential to give rise to adenocarcinoma of the oesophagus or the oesophagogastric junction. However, the British Society of Gastroenterology, in their definition of BE, requires only the presence of glandular mucosa, with or without intestinal metaplasia (Playford 2006). The rationale for this definition is that sampling errors, due to the patchy distribution of intestinal metaplasia, may result in small areas of intestinal metaplasia being missed, especially within short lengths of columnar lined oesophagus. In

my thesis BE is defined as columnar metaplasia with histological evidence of goblet cells, in accordance with the American College of Gastroenterology definition.

1.2.5.1 Incidence and risk factors for Barrett's oesophagus

Barrett's oesophagus is found in up to 1.5% of the general population (Ronkainen *et al.* 2005; Zagari *et al.* 2008), and in up to 12% of patients who are investigated for chronic reflux symptoms (Lord 2003). Factors associated with an increased risk of BE include increased acid exposure, especially nocturnal, older age, male, Caucasian ethnicity, obesity and hiatus hernia (Koek *et al.* 2008). Factors possibly or weakly associated with increased risk include tobacco smoking, alcohol and family history of BE or EAC, while Helicobacter pylori, especially CagA positive strains, is possibly associated with a decreased risk (Corley *et al.* 2008).

Chronic gastro-oesophageal reflux is present in most patients with BE, usually in conjunction with shortened length and/or reduced pressure in the lower oesophageal sphincter, and correlates with the length of columnar lined oesophagus (Fass *et al.* 2001). The composition of the refluxate is important. Reflux can be measured by 24-hour pH testing which measures the acidity of the refluxate, and by devices such as the Bilitec 2000, which has a fiberoptic spectrophotometric probe to measure absorption in the bilirubin spectrum as a marker for the presence of duodenal contents. Studies using these measuring devices suggest that acidity and other gastro-duoduodenal components, either independently or jointly, are risk factors for BE (Kauer *et al.* 1995; Nehra *et al.* 1999; Campos *et al.* 2001; Koek *et al.* 2008).

Acid exposure, even if brief, significantly increases cell proliferation and possibly decreases apoptosis in BE (Souza *et al.* 2008). Increased epithelial proliferation in patients with BE has been associated with a stepwise progression of dysplasia to adenocarcinoma (Peters *et al.* 2000; Chen *et al.* 2001). The role for components other than acid is supported by reports of BE in patients following total gastrectomy (Westhoff *et al.* 2004), and patients who reflux both gastric and duodenal secretions having a higher prevalence of BE than patients refluxing mainly gastric contents (Fein *et al.* 1997; Oberg *et al.* 1998).

It is not surprising that duodenal components are harmful to the oesophageal epithelium. Bile acids are known promoters of gut cancers *in vivo* and cell transformation *in vitro*. The bile acids deoxycholic acid and chenodeoxycholic acid stimulate cell division, induce mutagenesis

and DNA damage, promote invasiveness, angiogenesis and cytokine release, suppress TP53 (p53) function, cause cell death and apoptosis, and activate growth regulatory genes in normal oesophageal and metaplastic epithelia as well as cell lines (Zhang *et al.* 1998; Kaur *et al.* 2000; Tselepis *et al.* 2003). It is widely accepted that increased cyclooxygenase 2 (COX2) expression and increased production of prostaglandin E2 can be important in the development of cancer, including EAC (Raj & Jankowski 2004; Greenhough *et al.* 2009). There are many reports of bile and acid exposure inducing COX2 over-expression in the early transformation of oesophageal epithelium in BE and in the transition from low- to high-grade dysplasia and adenocarcinoma (Shirvani *et al.* 2000; Song *et al.* 2007; Bajpai *et al.* 2008; Park *et al.* 2008; Looby *et al.* 2009).

1.2.5.2 Barrett's oesophagus – a precursor to oesophageal adenocarcinoma

Approximately 0.5 to 1% of patients with BE will develop adenocarcinoma each year, and patients with BE have 30- to 125-fold increased risk of adenocarcinoma compared to the general population (Shaheen *et al.* 2000; Murray *et al.* 2003; Yousef *et al.* 2008). The progression from BE to EAC is thought to proceed via the histological stages of low- and high-grade dysplasia. Progress along this pathway is associated with an accumulation of genetic abnormalities, with some reports suggesting a stepwise progression of genetic changes.

The exact sequence of molecular events is not known and probably multiple molecular pathways interact in the malignant progression of BE (Koppert *et al.* 2005). Several changes in DNA structure and expression of certain genes or proteins have been reported to correlate with the development of cancer, although none of them have been proven prospectively to predict the onset of cancer. Reduced CDKN2A (p16) expression, seen in Barrett's metaplastic tissue (Eads *et al.* 2000; Bian *et al.* 2002), followed by altered TP53 expression, generally reported in dysplastic tissue, has been associated with the transition (Keswani *et al.* 2006; Galipeau *et al.* 2007), along with loss of heterozygosity (LOH) and aneuploidy. Alterations in the expression of many other genes have also been described at different stages of the progression to cancer (Chatelain & Flejou 2003; Tannapfel 2004; Peters & Fitzgerald 2007; Zagorowicz & Jankowski 2007; Zhang *et al.* 2008). In the context of this thesis, one mechanism for a reduction in gene expression is DNA methylation, which is discussed in Section 1.3.

1.2.5.3 Treatment of Barrett's oesophagus

Because up to 1% of patients with BE will develop EAC per year, it is important to consider treatments which will prevent this progression. The current approaches to improve survival rely on detection of dysplasia or EAC early, while it is potentially curable. This can be achieved by increased screening of the population to detect more patients with BE and endoscopic surveillance of those with BE. While guidelines exist for endoscopic screening and surveillance of patients with BE, this approach is inherently expensive and inefficient. Stratification of patients into risk groups may improve the efficiency of these programmes (discussed in Section 1.3.8.1).

Gastro-oesophageal reflux is the major risk factor for the development of BE and EAC, and medical and surgical anti-reflux therapies are available to relieve symptoms of the reflux and prevent reflux-related complications. In practice surgery tends to be considered when medical treatment has failed. Destruction or removal of the columnar mucosa by ablation or mucosal resection, combined with medical or surgical treatment to control reflux, can result in healing and repopulation with squamous epithelium. These approaches are discussed in the following sections.

1.2.5.3.1 Medical treatment of Barrett's oesophagus

In Australia, general practitioners and gastroenterologists prescribe proton pump inhibitors (PPIs) to such an extent that they are in the top ten drugs, by prescription counts and cost. The most common reason for prescribing these drugs is reflux. They provide rapid and effective control of the symptoms of reflux disease and dyspepsia, with few adverse effects.

The increased proliferation and expression of key cell cycle regulatory genes, which occurs early in the neoplastic progression associated with BE, is at least in part reduced by treatment with PPIs (Umansky *et al.* 2001; Lao-Sirieix *et al.* 2006). There is conflicting evidence that high doses may decrease the length of BE (Peters *et al.* 1999; Srinivasan *et al.* 2001; Lanas 2005), but no evidence that they completely reverse the condition (Peters *et al.* 2000; Fennerty 2002). However, the major objective of treatment probably should not be the reversal of the BE, but the prevention of EAC.

Certainly PPI therapy has not prevented recent increases in the incidence of EAC. Nevertheless there are claims that acid suppression with a PPI alone may prevent dysplasia and reduce cancer risk (Peters *et al.* 2000; Falk 2002; El-Serag *et al.* 2004; Hillman *et al.* 2004, 2008). In an abstract presented at Digestive Diseases Week, 2007, Khurana reported a retrospective analysis of the Veterans Administration database. Patients with reflux disease who were PPI users were about half as likely to develop oesophageal cancer compared to those who were not (Khurana *et al.* 2007). These studies do not always distinguish between low- and high-grade dysplasia, nor take account of other lifestyle modifications which may occur in patients who commit to daily medication.

Because of the role of COX2 in carcinogenesis, COX2 inhibitors have been investigated as potential chemopreventive agents. There is significant epidemiological evidence that aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of a number of cancer types, including EAC. For example, Corley found a protective association between aspirin and NSAIDs usage and oesophageal cancer (for both EAC and ESCC). Despite the number of reports, it is suggested that there are insufficient data on dose, duration, subpopulation effects and the risks versus the benefits to make positive recommendations on their use (Peters & Fitzgerald 2007; Cuzick *et al.* 2009). The most serious side effect of these drugs is cerebral haemorrhage. The most common side effect is peptic ulceration, but this may be less of an issue when given with a PPI (Fennerty 2002). The combination of esomeprazole and aspirin is currently being tested in the Aspirin Esomeprazole Chemoprevention Trial in Great Britain, the largest trial of its type to date in BE (Leedham & Jankowski 2007).

In this thesis I measured DNA methylation in a group of BE patients before and after 6 months taking high-dose (40 mg twice-daily) esomeprazole to determine if aberrant DNA methylation, present in BE and EAC, is reversed by this treatment over this period of time (Chapter 9; Appendix B9).

1.2.5.3.2 Surgical treatment of Barrett's oesophagus

Anti-reflux surgery (fundoplication) aims to prevent gastro-oesophageal reflux by surgically creating a functional lower oesophageal barrier. This barrier is usually more effective than

the normal sphincter, permitting only low levels of acid and bile reflux (Watson *et al.* 1999). In contrast, PPIs and histamine receptor antagonists reduce the acidity and volume of gastric secretions but do not prevent non-acidic reflux. There are two basic forms of fundoplication, but 24-hour pH studies at 6 months post-surgery showed no difference in the amount of reflux between total and partial fundoplications (Wong *et al.* 2008).

Fundoplication is effective in controlling clinical symptoms in the majority of patients (Watson *et al.* 1999; Ozmen *et al.* 2006; Zaninotto & Rizzetto 2007), and endoscopic and histological regression of BE has been reported. Regression was reported to be more common in short- than long-segment BE , and when it occurred did so within 5 years of the fundoplication. Anti-reflux surgery also reverses some of the molecular changes that are thought to promote carcinogenesis or reflect damage to the epithelium. These include reductions in oxidative stress as assessed by chemiluminescence, lipid peroxidation and levels of superoxide dismutase (Wetscher *et al.* 1995) and reductions in the expression of IL-8 (Oh *et al.* 2007), COX2 (Vallbohmer *et al.* 2006) and Cdx2 (Zaninotto *et al.* 2005), and an increase in apoptosis (Chen *et al.* 2002).

Progression from intestinal metaplasia to dysplasia and EAC following anti-reflux surgery has been reported, but it predominantly occurs in patients with long-segment BE and a documented recurrent increased 24-hour pH study (Csendes *et al.* 2004; O'Riordan *et al.* 2004). In a recent meta-analysis Chang concluded that while anti-reflux surgery was associated with regression of BE and/or dysplasia, it was less clear if it differed from medical treatment in preventing the development of EAC (Chang *et al.* 2007). The conclusion from the review of all the studies combined was that anti-reflux surgery was associated with a reduced incidence rate of cancer. However, many of the published studies were not controlled. Analysis of just the controlled trials and cohort studies failed to show a difference between surgical and medical therapy. Interpretation of these trials is difficult because surgical patients generally suffer more severe reflux and may be considered to have a higher risk of progression to cancer, and in these studies it is not common to determine objectively if the anti-reflux surgery remains functional.

In this thesis I measured DNA methylation in a group of patients 5 or more years after fundoplication (which was proven by a 24-hour pH study to still be functional) to determine if the operation affected aberrant DNA methylation (Chapter 8; Appendices B8, B14, and B15).

1.2.5.3.3 Ablation and mucosal resection for Barrett's oesophagus

In patients with BE and no dysplasia, resection is not recommended as the risk of death due to surgery exceeds the overall risk of malignant transformation. Instead medical, surgical (fundoplication) and ablative treatments are used. Resection is only recommended after confirmation of high-grade dysplasia, as studies have shown that these patients may harbour an intramucosal or early stage invasive oesophageal carcinoma.

There are currently a number ablative options for the treatment of BE, including focal endoscopic mucosal resection (EMR), complete eradication of metaplastic epithelium by EMR, endoscopic submucosal dissection, photodynamic therapy, radiofrequency ablation and cryotherapy (Watson 2008). The destruction of columnar mucosa in the oesophagus in an acid free environment is likely to be followed by regeneration of a squamous-type mucosa, irrespective of the method used to destroy the mucosa. Current concerns include complications during or after the endoscopic procedure, persistent islands of columnar mucosa or glandular structures within or beneath the regenerated squamous mucosa, and if the risk of cancer is completely prevented.

Five year survival rates are only now being reported for some of these techniques, and these suggest that endoscopic ablative therapy is a safe and curative approach to treat BE and high-grade dysplasia or intramucosal carcinoma (Overholt *et al.* 2005; Pech *et al.* 2008). The role of these techniques for the treatment of low-grade dysplasia and non-dysplastic BE is yet to be defined (Waxman & Konda 2009).

1.2.5.4 Risk stratification in Barrett's oesophagus

The current best practice to prevent EAC is regular endoscopic surveillance of patients with reflux disease or BE to identify patients with early, potentially treatable dysplasia or cancer. Patients with dysplasia are at the highest risk of progression to EAC (Odze 2008). However, there may be difficulties in reliably identifying these patients as there is significant inter- and intra-observer variation amongst pathologists in identifying and grading dysplasia.

There is another inherent problem with a surveillance-based strategy to prevent EAC. Current recommendations for the appropriate BE follow-up interval are as follows: two initial annual endoscopies, followed by a 3 year interval for BE without dysplasia, or less than 1 year for BE with low-grade dysplasia until dysplasia is no longer found (Sampliner 2002). The problem with this is the magnitude of the task. Shaheen (Shaheen & Ransohoff 2002) analysed this in the context of the USA. There are over 77 million people older than 50 years. Based on the proportion of these estimated to have gastro-oesophageal reflux disease, around 10 million patients would require surveillance endoscopy. The estimate of serious endoscopy complications (death, cardiovascular collapse, or perforation) from this number of endoscopies was 10,000. At that time there were about 7,200 oesophageal cancers per year. Shaheen concluded that there was insufficient evidence to endorse routine endoscopic screening of patients with chronic reflux symptoms.

A potential solution to these problems is the use of molecular biomarkers to stratify patients for their risk of progression to cancer. A biomarker is defined as a biological variable, such as a genomic change, that correlates with a biological outcome. Many biomarkers are being evaluated, mostly in cross-sectional studies with limited power. Some biomarkers studied include cyclin D1, APC, Bcl-2, VEG-F, ECAD, MUC2, Rab11, CEA, K-ras, Ki67 nuclear antigen, CDKN2A (p16), TP53, NF-kappa-B, COX2, Cdx2, anueploidy by flow cytometry, and promoter methylation. The sensitivity and specificity, and how well they correlate with disease progression, mostly remain to be determined.

Progress is being made, but slowly. Galipeau (Galipeau *et al.* 2007) reported that a combination of 17p or 9p LOH, and DNA content abnormalities provided better prediction of progression to EAC than any single TP53, CDKN2A, or DNA content lesion alone. This panel was a much more discriminate predictor of the likelihood of cancer than the presence of dysplasia. For a patient with none of these markers the chance of progression to EAC within 6 years was virtually zero, while for a patient with all 3 markers there was an 80% chance of EAC.

Current screening and surveillance are limited by poor risk stratification. In this thesis I searched for novel genes which are methylated in BE and EAC. While not part of my thesis, a longer term aim of this aspect of my project was to determine if methylation of some of

these genes may provide a biomarker which could be used to stratify patients with BE in terms of the risk of progression to EAC.

1.3 DNA Methylation

The progression to cancer in a tissue requires the stepwise up-regulation or down-regulation of critical genes. Mutation, translocation, LOH, small and large deletions have each been shown to contribute toward these alterations in gene expression. Methylation of the promoter regions of genes is one genomic change which is associated with silencing of gene expression and can alter cell phenotype.

1.3.1 DNA methylation is an epigenetic change

Appropriate methylation of cytosines in the genome is important for the normal functioning of the cell, while aberrant methylation is implicated in a number of disease processes. Because it does not involve changes in the underlying DNA sequence, methylation is an epigenetic change, and in normal cells it is generally copied faithfully in the daughter cells at cell division.

In eukaryotic organisms DNA methylation is the addition of a methyl group to the number 5 carbon of the cytosine pyrimidine ring, forming 5-methylcytosine. In vertebrates DNA methylation occurs almost exclusively at the cytosine residue of a cytosine-phosphate-guanine dinucleotide (CpG). The majority of CpGs, approximately 80%, are lone CpGs found within intergenic and intronic regions of DNA, particularly within repeat sequences and transposable elements. Typically, these lone CpGs are methylated in normal cells, and unmethylated in disease (Cedar & Bergman 2009).

The majority of the remaining CpGs are in CpG dense regions, called CpG islands, which can be found at the 5' regulatory region (promoter) of around 70% of known human genes (Saxonov *et al.* 2006). In normal cells the CpG islands are largely unmethylated. Methylation in normal cells contributes to the maintenance of DNA stability and the control of gene expression. It is responsible for the silencing of repetitive elements in the genome, including transposons and retroviruses, imprinting, and X-chromosome inactivation. In many diseases, particularly cancer, the CpG islands associated with the promoters of some genes are

aberrantly methylated, which is associated with heritable transcriptional repression of that gene.

1.3.2 Mechanisms of DNA methylation-mediated transcriptional repression

The mechanisms responsible for transcriptional repression of a methylated gene are not fully understood. Two modes of repression can be envisaged, and it is likely that both are biologically relevant. First, the methylation of DNA may itself physically impede the binding of transcriptional proteins, thus blocking transcription. Many factors are known to bind to CpG-containing sequences, and some of these fail to bind when the CpG is methylated. Strong evidence for this mechanism of gene regulation comes from studies of the role of the CCCTC-binding factor (CTCF) protein in imprinting at the H19/IGF2 locus (Hark *et al.* 2000; Holmgren *et al.* 2001; Ideraabdullah *et al.* 2008). The CTCF protein is associated with transcriptional domain boundaries (Bell & Felsenfeld 2000) and can insulate a promoter from the influence of remote enhancers. The maternally derived copy of the IGF2 gene is silent owing to the binding of CTCF proteins to the unmethylated CpGs of a CpG island between the IGF2 promoter and a downstream enhancer. These CpGs are methylated on the paternal strand which prevents CTCF binding, enabling the downstream enhancer to activate IGF2 expression.

The second mechanism, and probably more important, is that methylated DNA can attract proteins known as methyl-CpG-binding domain (MBD) proteins. At least five known MBD proteins have been identified (Nan *et al.* 1993; Cross *et al.* 1997; Nan *et al.* 1997; Hendrich & Bird 1998), of which four - MBD1, MBD2, MBD3 and Methyl-CpG-binding Protein 2 (MeCP2) - have been implicated in methylation-dependent repression of transcription. These MBD proteins play a role directly in modifying chromatin structures that facilitate both gene repression as well as gene activation (Dhasarathy & Wade 2008). They can also recruit additional proteins, such as histone deacetylases and other chromatin remodelling proteins that can modify histones, promoting the formation of compact, inactive chromatin (Fuks *et al.* 2003; Clouaire & Stancheva 2008; Dhasarathy & Wade 2008).

1.3.3 DNA methyltransferases catalyse DNA methylation

In mammals DNA methylation is the function of DNA methyltransferases (DNMTs), which catalyse the covalent addition of a methyl group to cytosine. At present three biologically active DNA methyltransferases that methylate cytosine in DNA have been identified - DNMT1 (Bestor *et al.* 1988), DNMT3A and DNMT3B (Okano *et al.* 1998). The current model proposes that DNMT3A and DNMT3B are de novo methyltransferases that establish DNA methylation patterns early in development (Okano *et al.* 1999), while DNMT1 maintains the established methylation pattern in the newly synthesized strand after DNA replication during cell division (Robert *et al.* 2003) and DNA repair (Mortusewicz *et al.* 2005). DNMT3L is homologous to the other DNMT3s but has no catalytic activity. It assists the de novo methyltransferases by increasing their ability to bind to DNA and stimulating their activity (Hu *et al.* 2008). It may play a role in cancer, as over-expression of DNMT3L in cell lines induces changes expected in carcinogenesis, including increased cellular proliferation and anchorage-dependant growth, as well as changes in the expression of many other key genes (Gokul *et al.* 2009). Finally, DNMT2 (TRDMT1), identified as an "enigmatic" DNA methyltransferase homolog, contains all ten sequence motifs common to all DNA methyltransferases (Yoder & Bestor 1998). It can bind to but does not methylate DNA, but does methylate aspartic acid transfer RNA (Goll *et al.* 2006).

There have been attempts to re-express methylated genes by inhibiting the DNMTs in dividing cells. The cytosine analogues, 5-azacytidine and its deoxy derivative 5-aza-2'-deoxycytidine (decitabine), are demethylating agents. These drugs can substitute for cytosine and become incorporated into the genome of the cell by the DNA replication machinery. The azacytosine-guanine dinucleotides are recognised by DNMTs, which initiate the methylation reaction. This traps the DNMTs in a covalent complex consisting of the enzyme and the drug, resulting in its degradation and cellular depletion (Stresemann & Lyko 2008). This results in demethylation of the genome.

1.3.4 Altered DNA methylation in cancer

Both aberrant methylation at select gene promoters and global hypomethylation have been found in all cancers (Wilson *et al.* 2007). A link between DNA methylation and cancer was first demonstrated in 1983, when it was shown that the genomes of cancer cells are

hypomethylated relative to their normal counterparts (Feinberg & Vogelstein 1983a). The mechanism for hypomethylation in cancer is unknown. It affects more of the genome than aberrant methylation, and is primarily due to the loss of methylation from repetitive regions of the genome (Wilson *et al.* 2007; Bollati *et al.* 2009; Park *et al.* 2009). DNA hypomethylation has several effects with implications for carcinogenesis (Dunn 2003). First, hypomethylation of repetitive DNA has been linked to genomic instability, a hallmark of tumour cells (Tsuda *et al.* 2002; Calvisi *et al.* 2007; Richards *et al.* 2009). Second, hypomethylation of CpG islands associated with the promoters of proto-oncogenes has been observed in some tumours relative to adjacent normal tissues, and could result in oncogene activation (Feinberg & Vogelstein 1983b). Third, hypomethylation of other genes which are methylated in the normal tissue may result in their expression and contributing to the behaviour of the tumour (Hibi *et al.* 2009; Kato *et al.* 2009; Lee *et al.* 2009). Fourth, hypomethylation of proviral sequences may lead to viral reactivation of human endogenous retroviruses (Ogasawara *et al.* 2003) or oncogenic viruses such as Epstein-Barr virus (Salamon *et al.* 2003), Kaposi's sarcoma-associated herpesvirus (Pantry & Medveczky 2009) or papillomavirus (de la Cruz-Hernandez *et al.* 2007).

In 1986 Baylin reported for the first time aberrant methylation of a promoter CpG island in cancer, with relative silencing of expression of the gene (Baylin *et al.* 1986). Since then there has been an explosion of interest in the silencing of gene expression by methylation and the cataloguing of genes which are methylated in cancer (Esteller *et al.* 2001). The mechanisms regulating the establishment of aberrant methylation of promoter CpG islands remain poorly understood. It is unclear for which genes methylation and silencing is a critical step in the evolution of the cancer, and for which methylation is an epiphenomena as a result of dysregulation of the methylation machinery in the cell. Methylation of individual CpGs or a number of CpGs in a particular region associated with a gene does not necessarily result in silencing of the gene. Many reports of methylation in the region of the promoter of a particular gene provide no experimental evidence that the gene was silenced as a result of the methylation. Nevertheless, there are clearly many tumour suppressor genes in which one or both alleles are silenced by methylation in cancer. If only one allele is silenced by methylation, then the other may be silenced by deletion, mutation or LOH. Many genes which are methylated in cancer are found in regions of frequent copy number alteration. In a period of little more than 20 years the importance of DNA methylation in cancer has been recognised.

1.3.5 Measurement of DNA methylation

The presence of 5-methylcytosine in nucleic acid was first reported in 1925 by Johnson and Coghill, who identified it in the crystalline picrate hydrolysis product of DNA from tuberculosis bacilli (Johnson & Coghill 1925). Twenty-five years later Wyatt, using paper chromatography, reported for the first time the presence of 5-methylcytosine in the DNA of higher plants and animals (Wyatt 1950). Subsequent progression in the study of DNA methylation has been dependent on the development of suitable analytical methods.

There are currently a number of qualitative and quantitative methods for the analysis of DNA methylation, each with its own advantages and disadvantages (Fraga & Esteller 2002; Wong 2006). The first techniques measured global levels of DNA methylation, but then came methods to measure methylation of individual CpGs. Many of these methods rely on treating the DNA with sodium bisulphite (Grigg & Clark 1994). Unmethylated cytosines are deaminated to uracils while methylated cytosines remain unchanged, resulting in DNA template with sequences that differ between the unmethylated and methylated forms. Regions can be amplified by polymerase chain reaction (PCR) and, depending on the design of the assay, the methylation status of individual CpGs, or of a region containing a number of CpGs, can then be obtained. Techniques which measure the methylation at each CpG within a target region include bisulphite sequencing, pyrosequencing, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. They are relatively expensive, require multiple steps, are time consuming, or require expensive hardware not readily available in many laboratories.

The commonly used methylation-specific PCR (MSP) (Herman *et al.* 1996) does not suffer from these disadvantages, but requires primers that are specific for either methylated, or unmethylated, bisulphite modified DNA. These primers are more demanding to design. This technique relies on 3′ mismatching of the PCR primers for specificity. False positives can occur if the primers are poorly designed, or the PCR is run at too low a temperature, or possibly for too many cycles. The method is sensitive, but only measures methylation of one or two CpGs located near the 3′ end of the primers, and is not quantitative. At the beginning of my project I established MSP to measure methylation. I only had access to a conventional end point PCR thermocycler. It soon became apparent that PCR bias was a problem, and optimization of the PCR conditions was critical. Generally optimization is carried out using

DNA isolated from cell lines which by their nature have relatively uniform methylation. I investigated optimization methods, and observed that optimization procedures which are appropriate when working with DNA prepared from cell populations with uniform methylation patterns, are not appropriate when analyzing DNA from tissues in which the subsets of cells have a mixture of methylation patterns. I developed an approach to overcome some of these issues (Chapter 2).

In the early part of my studies I also established another method for measuring methylation which was less susceptible to bias, was semi-quantitative, and gave information on the methylation of a number of CpGs within a region: combined bisulfite restriction analysis (COBRA). I used this method in the study of methylation of MT3 in ESCC (Chapter 4; Appendix B3).

For many purposes an estimate of the overall methylation of all CpGs within a region is as useful as knowing the methylation at specific CpGs. Two years into my research I gained access to a real time PCR thermocycler, which enabled the development of a method for measuring the overall methylation of all CpGs within a PCR amplified region of DNA using melt curve analysis, a technique first described by Ririe to differentiate between desired and undesired products of a PCR . The background to this approach, and its advantages over other methods, are described in Chapter 3. The PCR primer sets and conditions used specifically amplify bisulphite modified DNA, irrespective of the amount and pattern of methylation, but do not amplify unmodified DNA. Fully methylated and unmethylated bisulphite modified reference DNA are included as controls. The PCR amplified DNA, in the presence of a double-strand specific fluorescent dye, is then heated over a temperature range and the fluorescence intensity measured. There are two approaches to analysis. The common approach was and is to compare the negative first derivative of fluorescence with respect to temperature (-dF/dT) plots of the sample and the unmethylated and methylated references. A sample is considered methylated when its plot is shifted to the right of the unmethylated reference plot. In Chapter 3 I showed that the raw fluorescence data can be normalised. From this normalised data the temperatures at which melting begins and finishes can be calculated, which reflect the less and more methylated template molecules present respectively. Also the *T50*, the temperature at which half the amplicons are melted, can be calculated. This represents the summative methylation of all the CpGs in the template mixture. These parameters describe the methylation characteristics of the region amplified in the original

sample. This robust, rapid and simple in-tube method to analyse methylation was the basis of my later studies.

1.3.6 Identifying genes with aberrant DNA methylation

There are estimated to be about 27,800 CpG islands in the human genome, and around 70% of all 15,880 human RefSeq genes have a high CpG content at or near their start of transcription (Saxonov *et al.* 2006). Early approaches to identifying differentially methylated genes used a candidate gene approach – the examination of CpG islands associated with known tumour suppressor genes, or genes found in regions of frequent LOH or deletion in cancer, or investigation of genes or regions previously found to be methylated in other diseases. These approaches rely on prior information suggesting that a gene falls into a group with a higher than average chance to be methylated, and so are limited in scope to current knowledge.

Identification of CpG islands or individual CpGs that are differentially methylated in disease has been, until recently, limited by the lack of robust, widely available, genome wide, high throughput methods. One of the first genome wide approaches to identify novel differentially methylated genes was to use gene expression microarrays to discover genes which were up-regulated in cell lines treated with a demethylating agent. The cytosine analogue 5-aza-2'-deoxycytidine inhibits DNMTs, resulting in the progressive demethylation of dividing cells, which may reactivate expression of genes silenced by DNA methylation (Suzuki *et al.* 2002). This approach has been used to discover methylated genes in oesophageal (Yamashita *et al.* 2002) as well as many other tumour cell lines.

There are several potential disadvantages with this approach. It uses cell lines, not primary cancer tissues, so it is critical to select cell lines which represent the spectrum of the primary tumours of interest. Established cell lines have been reported to have increased CpG island methylation compared to primary cancer tissues (Smiraglia *et al.* 2001). This could cause bias toward detection of genes that are either methylated only in cell lines but not in the primary tumours, or for which the methylation is present in normal tissues as well as tumours. For some tumour types, such as EAC, there are few established cell lines. Furthermore, cell lines can be mischaracterized (e.g., (Boonstra *et al.* 2007)).

Several studies have identified genes up-regulated after treatment of cells with 5-aza-2'-deoxycytidine, despite the fact that their promoters seemed to be unmethylated (Soengas *et al.* 2001; Suzuki *et al.* 2002). There are a number of possible explanations for this observation. Methylation of upstream genes, such as those encoding transcription factors, could secondarily result in activation of the downstream genes. Inhibitors of DNMTs, such as 5-aza-2'-deoxycytidine, might affect the enzyme in ways other than simply blocking their methylating capacities. Studies showed that DNMTs have the potential to repress transcription independently of their methylating activities, both directly and through interaction with histone deacetylases and other corepressor proteins (Fuks *et al.* 2000; Robertson *et al.* 2000; Rountree *et al.* 2000; Bachman *et al.* 2001; Fuks *et al.* 2001). Another possible explanation is that methylation was assessed in the wrong region of the gene, a region not associated with silencing of expression. Demethylating drugs are toxic, and so may induce the expression of detoxifying and damage response genes. Thus for genes up-regulated in response to 5-aza-2'-deoxycytidine, their methylation status must be validated by independent methods. Despite the shortcomings, many differentially methylated genes, in a range of tumours, have been identified by this method.

Recently more powerful genome wide techniques have become available. The Illumina Infinium Methylation Assay (HumanMethylation27BeadChip), which requires knowledge of promoters and genes with CpG islands, uses a single nucleotide polymorphism approach to measure methylation of 27,500 individual CpGs distributed across more than 14,000 genes. Deep sequencing of bisulphite modified DNA is a relatively new and expensive technique, but has the distinct advantage that prior knowledge of CpG island location is not required.

One aim of my thesis was to discover new genes which are aberrantly methylated in ESCC, EAC and BE. At the time that I started these experiments, gene expression microarrays were relatively new. Oligonucleotide gene expression microarrays were printed here in Australia, and suffered from problems of variability, poor quality control, and incorrect annotation. In my first experiments the Barrett's associated EAC cell line OE33, and the ESCC cell line TE7, were treated with 5-aza-2'-deoxycytidine or vehicle, and differentially expressed genes were identified using the Queensland Institute of Medical Research 4.5K cDNA microarrays (Appendix B4). This approach lead to the discovery that MT3 was frequently methylated in ESCC primary tumours (Chapter 4; Appendix B3), and ID4 was frequently methylated in primary EAC and BE (Chapter 7; Appendix B7). However, the results of this study were

disappointing as very few of the genes which were up-regulated on the expression array could be independently validated as methylated. Thereafter the University of New South Wales provided a service for measuring gene expression on the Affymetrix GeneChip HG-U133 Plus 2.0 arrays. I repeated the demethylation experiments and measured up-regulated genes in OE33 treated with 5-aza-2'-deoxycytidine or vehicle using this platform. It proved more reliable, and a significantly higher number of candidate genes were validated as methylated in EAC or ESCC (Chapter 6; Appendices B10, B11, and B12).

1.3.7 DNA methylation in oesophageal cancer and its precursors

There has been a rapid accumulation of data on aberrant DNA methylation in oesophageal cancers and their precursor lesions since the late 1990s (e.g., (Sato & Meltzer 2006; Wu *et al.* 2006)). These studies vary from single to multiple gene analyses. Compared to the number of CpG islands, or CpG island-associated genes in the human genome, the total number of genes studied remains small. To date there has not been a study published of DNA methylation in EAC using genome-wide methods.

1.3.7.1 DNA methylation in oesophageal adenocarcinoma and Barrett's oesophagus

The progression to EAC is generally accepted to proceed via oesophagitis, BE, low-grade and then high-grade dysplasia. These histological changes are thought to mirror the stepwise accumulation of genomic changes, and up-regulation and down-regulation of the expression of many genes (Wijnhoven *et al.* 2001; Koppert *et al.* 2005; di Pietro *et al.* 2008). Down-regulation may result from mutation, translocation, loss of small or large regions of the genome, or DNA methylation. There is accumulating evidence that aberrant DNA methylation occurs early in the progression of Barrett's metaplasia to dysplasia and cancer.

At the commencement of my candidature (2002) there were seven publications describing frequent aberrant DNA methylation of eight different genes in BE. Wong and colleagues in Brian Reid's laboratory in Seattle, Washington, were the first to report aberrant methylation in BE (Wong *et al.* 1997). They observed methylation of the tumour suppressor gene CDKN2A (p16/INK4A) in the flow-sorted aneuploid cell population from patients with BE without cancer, and concluded that promoter methylation was a common mechanism for inactivation of CDKN2A in the pathogenesis of EAC. Reid's laboratory were interested in using

methylation as a tool to study the evolution of genomic aberrations in cancer. In subsequent studies they concluded that most Barrett's metaplasia contains genetic and/or epigenetic CDKN2A lesions and cells have the ability to undergo clonal expansion. This creates a field in which other abnormalities can arise in the setting of altered CDKN2A that can lead to EAC (Wong *et al.* 2001), and that the clonal evolution is more complex than predicted by linear models (Barrett *et al.* 1999). The finding of frequent methylation of CDKN2A in BE was later confirmed by others (Klump *et al.* 1998; Eads *et al.* 2000).

In addition to CDKN2A, Eads (Eads *et al.* 2000; Eads *et al.* 2001) demonstrated frequent methylation of APC, CALCA, ESR1, MGMT, MYOD1 and TIMP3 in BE tissues from a small cohort of patients without or with concurrent high-grade dysplasia or EAC. Kawakami (Kawakami *et al.* 2000) confirmed the finding that APC is frequently methylated in EAC and Barrett's metaplasia but not in matching normal oesophageal tissues.

1.3.7.2 DNA methylation in oesophageal squamous cell carcinoma

The progression from normal squamous epithelium to ESCC probably occurs through a progressive sequence of basal cell hyperplasia, increasingly severe degrees of squamous cell dysplasia, carcinoma-situ and invasive carcinoma (Shimizu *et al.* 2007). Similar to EAC, the histopathological progression in ESCC is accompanied and perhaps caused by a stepwise acquisition of a different set of genetic and epigenetic alterations (Mandard *et al.* 2000; Kuwano *et al.* 2005).

Aberrant DNA methylation has been observed in the precursor lesions of ESCC, and a number of studies suggest that its prevalence increases from normal squamous tissue through basal cell hyperplasia and squamous cell dysplasia to ESCC. Some of these studies have been with single genes (Wang *et al.* 2003), most with a number of genes (Nie *et al.* 2002; Guo *et al.* 2006; Roth *et al.* 2006; Ishii *et al.* 2007). In a study of resection specimens, the cancer tissue adjacent to dysplastic tissue had the same epigenetic alterations as the less advanced lesions but often had methylation of other genes in addition (Guo *et al.* 2006).

The findings that aberrant DNA methylation is prevalent in precancerous lesions have prompted the evaluation of methylation in oesophageal balloon cytology specimens, for the detection of oesophageal squamous cell dysplasia and early ESCC. A panel of four genes,

AHRR, p16INK4a, MT1G, and CLDN3, resulted in sensitivity and specificity of 50% and 68%, respectively (Adams *et al.* 2008). While promising, this study suggests that more sensitive methylation markers will be required for development of a clinically useful screening test based on detection of methylation in balloon cytology samples.

When I began my PhD project the following genes had been reported to be methylated in ESCC: APC, CDH1, FHIT, HLA-A, HLA-B, HLA-C, MTS1, p14, p15 and CDKN2A. In a review in 2006 Sato reported that only eight genes had been reported to be methylated in ESCC in more than one study (Sato & Meltzer 2006) compared to 11 genes in EAC. Over 70 genes have been reported in at least one study since then. The search for methylated genes has lead to a number of genes that appear to have a role in cancer. One of the aims of my project was to discover new genes methylated in ESCC by looking for genes up-regulated in cell lines grown with the demethylating drug 5-aza-2'-deoxycytidine (Chapter 6).

1.3.8 Clinical applications of DNA methylation

1.3.8.1 DNA Methylation and Predicting Progression to Cancer

In Section 1.2.5.4 I discussed risk stratification of patients using genomic biomarkers, and the need for biomarkers which will identify the subset of patients at highest risk of progression to cancer and those who need rigorous surveillance or perhaps early intervention. This has kindled an interest in the potential of DNA methylation as a biomarker.

There are many cross-sectional studies in which DNA methylation of a gene is measured in a range of tissues, and when more methylation is noted in dysplastic and cancer tissues it is concluded that methylation of the particular gene might have value as a biomarker (e.g., (Zou *et al.* 2005a; Zou *et al.* 2005b; Hamilton *et al.* 2006; Jin *et al.* 2007a; Jin *et al.* 2007b; Jin *et al.* 2008)). Several retrospective studies have analysed the incidence of methylation of a limited number of specific loci or regions of genes in a small numbers of patients without dysplasia that either did or did not progress to high grade dysplasia or EAC over a known follow-up period, but they did not report the predictive value of this methylation (Klump *et al.* 1998; Clement *et al.* 2006).

Recently S.J. Meltzer's group has examined the utility of measuring methylation of specific genes as a predictor of progression from Barrett's metaplasia to high-grade dysplasia or adenocarcinoma (Schulmann *et al.* 2005; Sato *et al.* 2008; Jin *et al.* 2009). In their first study they found that methylation of CDKN2A (p16), RUNX3, and HPP1 (TMEFF2) occurred early in Barrett's-associated neoplastic progression and predicted progression risk (Schulmann *et al.* 2005). Risk of progression was elevated up to, but no earlier than, two years preceding neoplastic progression. They then proposed that a combination of columnar lined oesophagus segment length, histological grade, methylation of CDKN2A, RUNX3 and HPP1, could successfully stratify patients into low-, intermediate, and high-risk of Barrett's-associated neoplastic progression (Sato *et al.* 2008). Most recently they have reported that an eight marker methylation panel (CDKN2A, RUNX3, HPP1, NELL1, TAC1, SST, AKAP12, and CDH13) can predict neoplastic progression in BE patients and is suitable for risk stratification (Jin *et al.* 2009). Despite intense interest, there are no validated biomarkers for use clinically in BE.

1.3.8.2 Methylated DNA in serum or plasma from oesophageal cancer patients

The detection of tumour-specific biomarkers in body fluids could provide a minimally- or non-invasive method for early diagnosis of cancer. The use of PCR based techniques permits detection of very small amounts of nucleic acids in body fluids, opening up the possibility of DNA or RNA markers for early cancer detection, diagnostics, and follow-up.

It has been known for decades that tumour derived DNA is present in body fluids of patients with cancer and used to measure cancer specific mutations or allelic imbalances. Whereas mutations might lie anywhere along the gene, aberrant methylation is generally confined to relatively small regions. To detect methylated DNA a set of primers that amplify part of that region would generally be sufficient, while to detect the presence of a mutation in a gene many primers and PCR amplifications may be required. As methylated DNA can be detected with a very high degree of sensitivity there is interest in diagnostic or prognostic assays based on measuring cancer specific methylation changes in cell-free DNA from body fluids.

There are a number of studies measuring methylated DNA in the blood of patients with oesophageal cancer or disease. Hibi reported that the CDKN2A promoter methylation present in the ESCC tumour tissue could be detected in the serum of the same patient, and that 23%

of the patients had the same methylation changes in the serum as in the tumour (Hibi *et al.* 2001). In a similar study CDKN2A methylation was detected in the serum of two of 38 (5.2%) patients with ESCC compared to 20% of the primary tumours (Fujiwara *et al.* 2008).

Jin measured circulating levels of methylated TAC1 in EAC (Jin *et al.* 2007b), and Kawakami reported that the levels of methylated APC in the plasma may be a useful biomarker of biologically aggressive disease in EAC (Kawakami *et al.* 2000). Hoffmann reported that methylated DAPK and APC promoter DNA detection in peripheral blood was significantly associated with apparent residual tumor and outcome. Each of these genes was significantly associated with unfavourable prognosis, but the combination of both markers significantly increased the sensitivity and specificity (Hoffmann *et al.* 2009). Vasavi measured methylation of MLH1 in BE and EAC. In only 14.5% of cases was methylation measured in both the tissue and blood, indicating that its could not be used as a noninvasive tool to investigate oesophageal disease (Vasavi *et al.* 2006).

These assays have not yet translated into clinically useful tests. Specificity appears to vary depending on the gene and/or the method used for detection. A further complication is that in some genes DNA methylation may be present in apparently normal cells.

1.3.8.3 DNA methylation as a predictor of survival in oesophageal cancer

Attempts to stage cancer using genetic techniques have been hindered by two main problems. First, only a few genes are somatically mutated in solid tumours and, second, because cell populations of primary neoplasms are heterogeneous, it is probable that no single marker can accurately predict the behaviour of the tumour. Emerging evidence suggests a possible prognostic value of gene promoter methylation in oesophageal cancers. Methylation of UCHL1 (Mandelker *et al.* 2005), TIMP3 (Ninomiya *et al.* 2008) and TAC1 (Jin *et al.* 2007b) has been associated with a significant reduction in overall patient survival in ESCC. Some markers appear to be stage specific. Thus methylation of FHIT was associated with a poor prognosis for stage I-II ESCC (Lee *et al.* 2006) and methylation of CDH1 and ITGA4 were associated with a high risk of recurrence and a poor recurrence-free survival after surgery of stage I and stage II ESCC respectively (Lee *et al.* 2008). Methylation of APC is related to superior prognosis in terms of recurrence and to a lower number of metastatic lymph nodes in ESCC (Kim *et al.* 2009).

In EAC methylation of NELL1 was associated with poor prognosis in patients with stages I-II but not stages III-IV disease (Jin *et al.* 2007a). Measurement of multiple genes appears to provide more power. In a study of APC, CDH1, MGMT, ESR1, CDKN2A, DAPK and TIMP3 in EAC, methylation of some genes individually showed only trends toward diminished survival, whereas patients whose tumours had greater than 50% of these genes methylated had both significantly poorer survival and earlier tumour recurrence (Brock *et al.* 2003).

Together this suggests that measurement of methylation for multiple genes may be of use for predicting oesophageal tumour behaviour, and that methylation patterns may identify patients with a worse prognosis.

1.3.8.4 Demethylation as a therapeutic intervention

Given the role of DNA methylation in carcinogenesis, there has been interest in the use of demethylating drugs in cancer treatment. The major application to date has been the use of decitabine for the treatment of haematological cancers, although it is being trialled in solid tumours (Stewart *et al.* 2009). Its use is limited by bone marrow toxicity, and concern the surviving daughter cells may carry mutations (Issa & Kantarjian 2009). Another possible approach is the use of antisense RNA to inhibit DNMTs by degrading their mRNAs and preventing their translation. However, it is currently unclear if targeting a single DNMT alone will be sufficient to reactivate tumour suppressor genes silenced by DNA methylation. At present there are no known trials of DNA demethylating drugs in patients with oesophageal cancer or BE.

1.4 Aims of the Study

The propose of this study was to investigate DNA methylation in cancer, particularly EAC and ESCC.

The specific aims were to:

(1) Establish robust methods to qualitatively and quantitatively measure DNA methylation,

(2) Discover novel aberrantly methylated genes that are associated with transcriptional silencing in BE, EAC and ESCC, and

(3) Assess the effect of treating gastro-oesophageal reflux by either fundoplication or proton pump inhibitors on aberrant DNA methylation in patients with BE.

CHAPTER 2: METHOD FOR OPTIMIZING METHYLATION-SPECIFIC PCR

Eric Smith[1], Tina Bianco-Miotto[1], Paul A. Drew[2], David I. Watson[3]

[1]Discipline of Surgery, The University of Adelaide, Adelaide, 5005, Australia;
[2]School of Nursing and Midwifery, Flinders University, Bedford Park, 5042, Australia;
[3]Flinders University Department of Surgery, Flinders Medical Centre,
Bedford Park, 5042, Australia.

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CHAPTER 3: QUANTITATION OF DNA METHYLATION BY MELT CURVE ANALYSIS

Eric Smith[1], Michael E. Jones[2], Paul A. Drew[3]

[1]Discipline of Surgery, The University of Adelaide, Adelaide, 5005, Australia;
[2]School of Medicine, Flinders University, Bedford Park, South Australia, 5042, Australia;
[3]School of Nursing and Midwifery, Flinders University, Bedford Park, 5042, Australia.

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Quantitation of DNA methylation by melt curve analysis

Eric Smith[1], Michael E Jones[2] and Paul A Drew*[3]

Address: [1]Discipline of Surgery, School of Medicine, The University of Adelaide, Royal Adelaide Hospital, South Australia 5005, Australia, [2]School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia and [3]School of Nursing and Midwifery, Flinders University, Bedford Park, South Australia 5042, Australia

Email: Eric Smith - eric.smith@adelaide.edu.au; Michael E Jones - michael.e.jones@flinders.edu.au; Paul A Drew* - paul.drew@flinders.edu.au

* Corresponding author

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Abstract

Background: Methylation of DNA is a common mechanism for silencing genes, and aberrant methylation is increasingly being implicated in many diseases such as cancer. There is a need for robust, inexpensive methods to quantitate methylation across a region containing a number of CpGs. We describe and validate a rapid, in-tube method to quantitate DNA methylation using the melt data obtained following amplification of bisulfite modified DNA in a real-time thermocycler.

Methods: We first describe a mathematical method to normalise the raw fluorescence data generated by heating the amplified bisulfite modified DNA. From this normalised data the temperatures at which melting begins and finishes can be calculated, which reflect the less and more methylated template molecules present respectively. Also the *T50*, the temperature at which half the amplicons are melted, which represents the summative methylation of all the CpGs in the template mixture, can be calculated. These parameters describe the methylation characteristics of the region amplified in the original sample.

Results: For validation we used synthesized oligonucleotides and DNA from fresh cells and formalin fixed paraffin embedded tissue, each with known methylation. Using our quantitation we could distinguish between unmethylated, partially methylated and fully methylated oligonucleotides mixed in varying ratios. There was a linear relationship between *T50* and the dilution of methylated into unmethylated DNA. We could quantitate the change in methylation over time in cell lines treated with the demethylating drug 5-aza-2'-deoxycytidine, and the differences in methylation associated with complete, clonal or no loss of MGMT expression in formalin fixed paraffin embedded tissues.

Conclusion: We have validated a rapid, simple in-tube method to quantify methylation which is robust and reproducible, utilizes easily designed primers and does not need proprietary algorithms or software. The technique does not depend on any operator manipulation or interpretation of the melt curves, and is suitable for use in any laboratory with a real-time thermocycler. The parameters derived provide an objective description and quantitation of the methylation in a specimen, and can be used to for statistical comparisons of methylation between specimens.

Background

Methylation of DNA is a common epigenetic change which is important for the normal functioning of the cell. Methylation occurs almost exclusively on cytosines in the setting of a CpG dinucleotide. Most CpGs are methylated in the genome, except for those in the majority of CpG dense regions (CpG islands) found at the 5' end of around 50% of mammalian genes. Abnormal methylation is implicated in a number of disease processes. This applies particularly in cancer where there is genome wide hypomethylation together with hypermethylation of many CpG islands, which can silence tumor suppressor genes. There is great interest in assessing methylation because it may have diagnostic or prognostic value, or be a predictive marker for therapy. It is therefore important to have simple, accurate and inexpensive techniques for measuring methylation, which are suitable for use in a range of laboratories.

There are a number of qualitative and quantitative methods for the analysis of methylation, each with its own advantages and disadvantages [1,2]. Many of these methods rely on treating the DNA with sodium bisulfite. This deaminates unmethylated cytosines to uracils while methylated cytosines remain unchanged, resulting in templates that differ in sequence between the unmethylated and methylated forms. Regions can be amplified by PCR using primers specific for bisulfite modified DNA and, depending on the design of the assay, the methylation status of individual CpGs, or of a region containing a number of CpGs, can then be obtained. Techniques which measure the methylation at each CpG within a target region include bisulfite sequencing [3], pyrosequencing [4], and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [5]. These techniques are relatively expensive to perform, require multiple steps, are time consuming, or require expensive hardware that is not readily available in many laboratories. The commonly used methylation-specific PCR (MSP) [6] does not suffer from these disadvantages, but the primers are more demanding to design, and the assay only measures methylation of CpGs located near the 3' end of the primers.

For many purposes an estimate of the overall methylation of all CpGs within a region is as useful as knowing the methylation at specific CpGs. We describe an objective assessment of overall methylation using melt curve analysis, a technique first described by Ririe to differentiate between desired and undesired products of a PCR [7]. In melt curve analysis PCR products are slowly heated in the presence of double-stand DNA (dsDNA) specific fluorescent dyes such as SYBR Green I, LCGreen, SYTO9 or EvaGreen. With increasing temperature the dsDNA denatures (melts), releasing the fluorescent dye with a resultant decrease in the fluorescent signal. The temperature at which dsDNA melts is determined by factors such as nucleotide sequence, length and GC/AT ratio. A methylated sequence of DNA, following bisulfite modification, will maintain a higher GC/AT ratio and so melt at a higher temperature than its unmethylated equivalent. Melt curve analysis can detect a single base difference [8]. Worm applied this principle to DNA methylation analysis [9], and a number of variations have since been described, such as methylation-sensitive high resolution melting [10] and dissociation analysis [11]. In each of these methods methylation is assessed visually. In melting curve analysis-methylation (MCA-Meth) the ratio of methylated to unmethylated amplicons was calculated from the respective heights of the derivative peaks (-dF/dT), but this method of quantitation cannot be applied to samples containing partially methylated molecules [12].

We describe a simple mathematical approach to generate normalised melt curves from the raw fluorescence melt data obtained following PCR. We can then calculate from the normalised melt curve the melt temperature (*T50*), the temperature at which 50% of the molecules in the PCR product are melted, which reflects the average methylation of all the CpGs in the region amplified, and the temperatures at which melting begins and is complete, which reflect the heterogeneity of methylation of the alleles within the amplified region. These parameters provide an objective description and quantitation of the homogeneous and heterogeneous methylation in a specimen, and can be used to compare methylation between specimens.

Methods

Primers and oligonucleotides

Primers (Table 1), and oligonucleotide sequences representing the bisulfite modified fragments of the CDKN2A promoter (Table 2), were synthesized by GeneWorks (Thebarton, SA, Australia). Primers for melt curve analysis were designed to amplify both methylated and unmethylated bisulfite modified DNA, but not unmodified DNA. Our primer design guidelines were as follows.

1. They should contain at least one T corresponding to a non-CpG C within the last three nucleotides at the 3'-end of the primer, to increase the likelihood of amplification of only bisulfite modified template.

2. They should not contain CpGs, but when this is unavoidable the number of CpGs are minimised and placed as far as possible toward the 5'-end of the primer. In these cases primers are designed with either an inosine or a degenerate base (C/T in the forward primer, or G/A in the reverse primer) so that templates with both methylated and unmethylated CpGs would be amplified.

Table 1: Primers used in this study

Gene	Primer sequences 5'-3'	Location[a]	Size[b]	CpGs[c]
CDKN2A	Forward-GAAGAAAGAGGAGGGGTTGGTTGGTTATT Reverse-ACCTACTCTCCCCCTCTCCGCAA	chr9:21964847 – 21964930	84	6
TIMP3	Forward-GGYGGTATTATTTTTTATAAGGATTTG Reverse-AAACCCCRCCTCRAACTATTAAA	chr22:31527488 – 31527645	158	10
MGMT	Forward-IGIGTTTIGGATATGTTGGGATAGTT Reverse-ACIAAACIACCCAAACACTCACCAAA	chr10:131155461 – 131155570	110	12

Y – C/T
R – A/G
I – inosine
[a]Genome location, as determined by UCSC Genome Browser Database (GBD, http://genome.ucsc.edu) Human March 2006 (hg18) assembly.
[b]Size of the PCR product.
[c]Number of CpGs in the PCR product between the primer binding sites.

3. There should be limited self-complementarity and limited complementary sequences between primer pairs. These parameters can be assessed using freely available software tools including Oligo Calc: Oligonucleotide Properties Calculator to test for self-complementarity http://www.basic.northwestern.edu/biotools/oligocalc.html) and Amplify (version 3.1.4, http://engels.genetics.wisc.edu) to test for potential primer-dimers.

4. They should amplify a product which is approximately 80 – 160 bp in length.

5. The primers should be approximately 20 – 30 bp in length.

6. The primer pairs should have melting temperatures as similar as possible, but differing by no more than 2°C.

Reference DNA

Bisulfite modified genomic DNA prepared from the lymphocytes of healthy donors was used as unmethylated reference [13]. For the methylated reference, 2 μg of lymphocyte genomic DNA was treated with 10 U of M.SssI CpG Methylase (New England BioLabs Inc., Beverly, MA, USA) for 16 h at 37°C in a 50 μL reaction volume containing 160 μM S-adenosylmethionine and NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9). Methylase treated DNA was precipitated with 150 μL of 100% ethanol and centrifuged for 15 min at 4°C. The ethanol was removed and the DNA pellet was air-dried under vacuum. The DNA was resuspended in 18 μL of ultra-pure water (UPW, Fisher Biotec Australia, West Perth, WA, Australia), and bisulfite modified.

Cell culture

The esophageal adenocarcinoma cell line OE33 was cultured in RPMI 1640 supplemented with 10% foetal bovine serum, at 37°C, in air enriched with 5% CO_2. Triplicate cultures of OE33 were grown for 24 h, then treated with either 1 μM 5-aza-2'-deoxycytidine (aza) (Sigma-Aldrich, Saint Louis, MO, USA) or vehicle (0.0027% v/v final concentration acetic acid) for 48, 72 or 120 h. The cells were incubated for a further 24 h in fresh medium not containing aza or vehicle and harvested. The DNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Colorectal carcinoma tissue specimens

For each colorectal carcinoma (CRC) tissue, two 10 μm formalin fixed paraffin embedded (FFPE) sections were de-waxed with xylene, washed with 100% ethanol, rehydrated with UPW and air-dried under vacuum. Sections were then digested in a solution consisting of 20 μL 10 mg/ml proteinase K (Promega, Madison, WI, USA) and 200 μL 100 mM NaCl, 10 mM Tris-HCl, 10 mM EDTA and 0.5% (w/v) sodium dodecyl sulfate for 48 h at 55°C, add-

Table 2: Oligonucleotide sequences representing a fragment of the CDKN2A promoter

Oligonucleotide	Sequence
Unmethylated	gaagaaagaggagggg**T**ggT**T**ggt**T**aT**T**agagggtgggg**TG**ga**T****TGTG**tg**TG**T**t****TG**g**TG**gT**t**gCGgagaggggagagTaggT
Partially methylated	gaagaaagaggagggg**T**ggT**T**ggt**T**aT**T**agagggtgggg**TG**ga**T****TGTG**tg**CG**T**t****CG**g**CG**gT**t**gCGgagaggggagagTaggT
Fully methylated	gaagaaagaggagggg**T**ggT**T**ggt**T**aT**T**agagggtgggg**CG**ga**T****CGCG**tg**CG**T**t****CG**g**CG**gT**t**gCGgagaggggagagTaggT

The oligonucleotide sequences representing a bisulfite modified fragment of the CDKN2A promoter that were either unmethylated, partially methylated, or fully methylated. The sequence to which the primers bind is underlined. The location of the six CpGs between the primer binding sites are in bold. Thymines which are generated following bisulfite modification and amplification are capitalised.

ing 20 μL fresh 10 mg/ml proteinase K after the first 24 h. Protein was removed by precipitation with 80 μL of 6 M NaCl. The DNA was precipitated with 700 μL of 100% ethanol, washed with 70% ethanol, air-dried under vacuum and resuspended in 100 μL UPW. This study was approved by the Research Ethics Committee of the Royal Adelaide Hospital. The study complied with the appropriate institutional guidelines.

Bisulfite modification

Genomic DNA (2 μg) was bisulfite modified as previously described [14,15]. Bisulfite modified DNA from lymphocytes and cell lines were resuspended in UPW at a volume of 100 μL, and from FFPE tissue in a volume of 20 μL.

PCR and melt analysis

Bisulfite modified DNA (1 μL) was amplified using QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) containing a final concentration of 0.5 μM of each primer (Table 1) in a final reaction volume of 15 μL. The primers and PCR conditions were specific for bisulfite modified DNA, and did not amplify unmodified DNA. The PCR was performed using a Rotor-Gene 3000 (RG3000, Corbett Life Science, Sydney, NSW, Australia) with a 95°C activation step for 15 min; 95°C for 30 s, 55°C for 60 s for 45 cycles; and a final extension step of 72°C for 4 min. The melt of the PCR product was performed from 60 to 90°C, rising in 0.5°C increments, waiting for 30 s at the first step and for 5 s at each step thereafter, and acquiring fluorescence at each temperature increment.

Statistics

Two groups were compared using the Wilcoxon-Mann-Whitney test, and three groups were compared using the Kruskal-Wallis one-way analysis of variance by ranks [16]. More than three groups were compared with one-way analysis of variance with Tukey multiple comparison post-test. All statistics were considered significant when the two tailed P = 0.05.

Results

Algorithm: analysis of melt curves from raw melt data

A graph of the raw fluorescence plotted against temperature for a typical melt of dsDNA PCR products using the RG3000 is shown in Figure 1A. The magnitude of the starting fluorescence often varies significantly between samples, making direct comparisons difficult. To facilitate comparisons, the raw fluorescence data can be normalised, as described below. Figure 1B shows that the graph of raw melt data for a sample has three distinguishable phases. In Phase 1 there is a linear decrease in fluorescence with increasing temperature. This decrease can be represented mathematically. There is a constant background amount of fluorescence, and added to this is a linearly decreasing amount which can be calculated from the regression line which best fits the fluorescent data over the linear reduction phase (represented as a solid blue line in Figure 1B). In Phase 2, the rapid reduction in fluorescence is a combination of the linear decrease and the melting of dsDNA. During this phase the observed fluorescence ($F(T)obs$) is less than the fluorescence expected from the Phase 1 alone ($F(T)max$). In Phase 3, the final plateau phase, the dsDNA is fully melted and there is no further decline in fluorescence.

At each temperature point it is possible to calculate how much fluorescence is lost as a result of melting of the dsDNA. The amount of fluorescence in the sample at any given temperature, if the dsDNA does not melt and assuming that the reduction in fluorescence is linear with increasing temperature, can be calculated from the extrapolation of the regression line of the Phase 1 decline. The reduction in fluorescence ($F(T)melt$) due to the melting of the dsDNA at any given temperature, is the difference between the fluorescence predicted at that temperature from the regression line ($F(T)max$) and the observed value ($F(T)obs$), or $F(T)melt = F(T)max - F(T)obs$.

The normalised fluorescence at any given temperature is the ratio of the amount of fluorescence observed to the amount of fluorescence predicted if no melting of the dsDNA had occurred ($F(T)obs/F(T)max$), expressed as a percentage. Normalised fluorescence values can then plotted against temperature as normalised melt curves (Figure 1C). The initial phase is a flat line, as there is no reduction in fluorescence due to specific dsDNA melting. Reduction in fluorescence due to specific melting of the dsDNA begins at the take-off temperature (Tto) and finishes at the touch down temperature (Ttd), where the fluorescence reaches background. We calculated these as the 95% and 5% percentile fluorescence respectively, but other values could be easily used.

Normalising the raw melt data facilitates comparisons between melt curves of different samples, and to unmethylated and fully methylated DNA references. Two parameters which describe the methylation of the population of DNA templates for the region amplified can be determined from the normalised curve. The temperature range over which melting occurs, the difference between take-off and touchdown temperatures ($Ttd - Tto$), will be greater if there is a mixture of methylated and unmethylated molecules than if there is a homogeneous population, making this a measure of methylation heterogeneity. The extent of methylation of the population can be described by the temperature at which half the dsDNA molecules are melted and half are intact ($T50$). The higher the $T50$, the more the methylation in the DNA population. The normalised melt curve and the individual

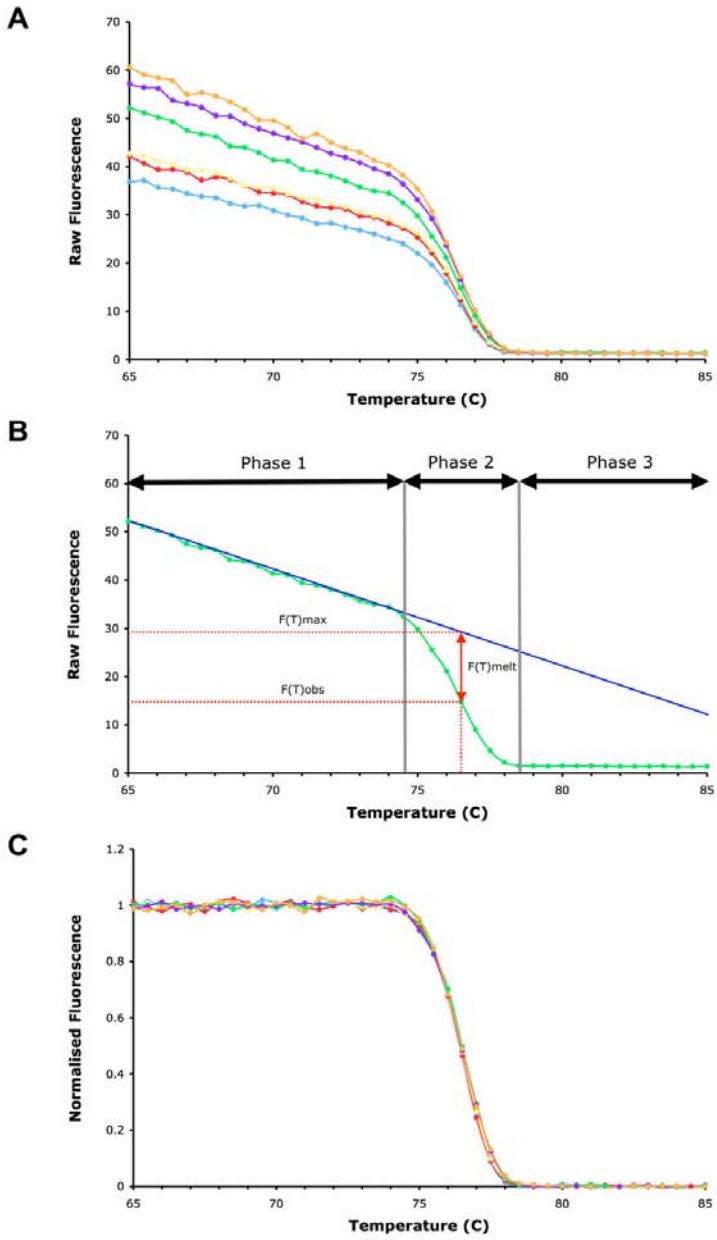

Figure 1

Raw and normalised melt curves. A) Typical examples of raw fluorescence melt curves. Six replicates of an oligonucleotide representing a fragment of the unmethylated CDKN2A promoter were amplified using the CDKN2A primers and QuantiTect SYBR Green PCR Kit, then melted by increasing the temperature from 60 to 90°C, rising in 0.5°C increments, waiting for 30 s at the first step and for 5 s at each step thereafter, acquiring fluorescence at each increment. Raw fluorescence was plotted against temperature. The plots show that the magnitude of the starting fluorescence may vary significantly even between replicates. B) Graph of raw fluorescence plotted against temperature from one sample from Figure 1A shows that there are three distinguishable phases. In Phase 1 there is a linear decrease in fluorescence with increasing temperature (represented as a solid blue line). In Phase 2 there is a rapid reduction in fluorescence which is a combination of the linear decrease and the melting of dsDNA. During Phase 2 the observed fluorescence (*F(T)obs*) is less than the fluorescence expected from the Phase 1 alone (*F(T)max*). The reduction in fluorescence due to the melting of the dsDNA alone at any given temperature (vertical dotted red line) is the difference between *F(T)max* and *F(T)obs*, *F(T)melt* (solid red line with arrow heads). In Phase 3 the dsDNA is fully melted and there is no further decline in fluorescence. C) Normalised melt curves for the six replicates in Figure 1A. The normalised fluorescence was calculated by the formula *F(T)obs*/*F(T)max* at every given temperature increment. Normalised fluorescence expressed as a percentage was then plotted against temperature.

parameters derived from it can be simply computed using the raw fluorescence data exported from the real-time thermocyler or other microvolume fluorometer integrated with a rapid temperature cycler.

Melt curve analysis of synthesised oligonucleotides

We tested the application of this analysis using synthesized oligonucleotides of known methylation. Three different oligonucleotides were synthesised, each representing a bisulfite modified DNA fragment of the same region of the CDKN2A promoter but differing in the extent of methylation, the number of methylated CpGs in the sequence. These CDKN2A oligonucleotide sequences are shown in Table 2. The region was 84 nucleotides long, and contained seven CpGs in total, six CpGs between the primer binding sites. In the oligonucleotide representing unmethylated bisulfite modified CDKN2A, the cytosines of the six CpGs were substituted with thymines. For the methylated oligonucleotide these cytosines remained as cytosines. For the partially methylated oligonucleotide three of the cytosines were substituted with thymines, the other three remained as cytosines.

Figure 2A shows the derivative peaks (-dF/dT), and Figure 2B the normalised melt curves, calculated as described, for the PCR products amplified from each of the three oligonucleotides. The general shapes of the -dF/dT plots and the normalised curves are similar, but they are hotter in proportion to the extent of methylation. The melt curve for the partially methylated oligonucleotide (three cytosines and three thymines) is between that of the unmethylated and methylated oligonucleotides. The *T50*, *Tto* and *Tdo* significantly increased with the extent of methylation (Table 3; $P < 0.005$ for all comparisons), demonstrating that differences could be measured between oligonucleotides which differed by as few as three methylated CpGs per molecule. The normalised melt curves did not differ whether the PCR products were melted immediately at the end of the PCR, or on a subsequent day, or had been melted a number of times before, even though the magnitude of the fluorescence intensity may have decreased over the time (data not shown).

Next, we investigated the ability of the method to analyse a mixture of two different oligonucleotides. Unmethylated oligonucleotide was spiked with either partially or fully methylated oligonucleotide, amplified and then melted. Spiking the unmethylated oligonucleotide with either partially methylated (U/P) or fully methylated (U/M) oligonucleotide significantly altered the shape of the -dF/dT (Figure 2C) and the normalised (Figure 2D) melt curves. The spiking did not significantly alter *Tto*, but did significantly increase *T50* and *Ttd* in proportion to the extent of methylation of the spiked mixture (Table 3; $P < 0.005$ for all comparisons).

Melt curve analysis of mixtures of methylated and unmethylated cell DNA

To determine the relationship between *T50* and the ratio of methylated to unmethylated molecules, we made dilutions of methylated reference DNA into unmethylated reference DNA. The dilutions were amplified using TIMP3 primers, the products melted, and the melt data normalised (Figure 3A). A visible shift to the right of the unmethylated reference was observed when 5% of the template molecules were methylated. There was a linear correlation between the percentage of methylated molecules in the mixture and the *T50* (Figure 3B). Similar results were observed for regions of the CDKN2A and MGMT genes, except that shifts in the melt curves could be detected when 1% of the template molecules were methylated (data not shown).

Melt curve analysis of cell line DNA

To validate melt curve analysis in cell line DNA we looked for a correlation between the melt curve parameters and the reduction in methylation in a cell line cultured with the demethylating agent 5-aza-2'-deoxycytidine (aza). We have previously shown that TIMP3 is methylated in the OE33 cell line, and that with aza treatment TIMP3 methylation is reduced and expression increased [14,15]. The results in Figure 4 show the normalised melt curves for DNA isolated from cells treated with vehicle or aza for 48, 72 or 120 h. Compared to unmethylated and methylated reference, all normalised melt curves for vehicle treated

Table 3: *T50*, *Tto*, *Ttd* and *Ttd – Tto* values for known oligonucleotides

Oligonucleotide	*T50*	*Tto*	*Ttd*	*Ttd – Tto*
Unmethylated	79.07 ± 0.06	78.48 ± 0.05	80.60 ± 0.06	2.12 ± 0.07
Partially methylated	80.00 ± 0.26	78.85 ± 0.53	82.39 ± 0.17	3.54 ± 0.47
Fully methylated	82.10 ± 0.00	81.13 ± 0.05	84.29 ± 0.03	3.16 ± 0.15
U/P	79.27 ± 0.21	78.30 ± 0.05	81.47 ± 0.18	3.17 ± 0.15
U/M	80.17 ± 0.15	78.45 ± 0.11	83.36 ± 0.19	4.90 ± 0.21

U/P – unmethylated oligonucleotide spiked with partially methylated oligonucleotide.
U/M – unmethylated oligonucleotide spiked with fully methylated oligonucleotide.
All values are the mean ± standard deviation of 3 replicate reactions.

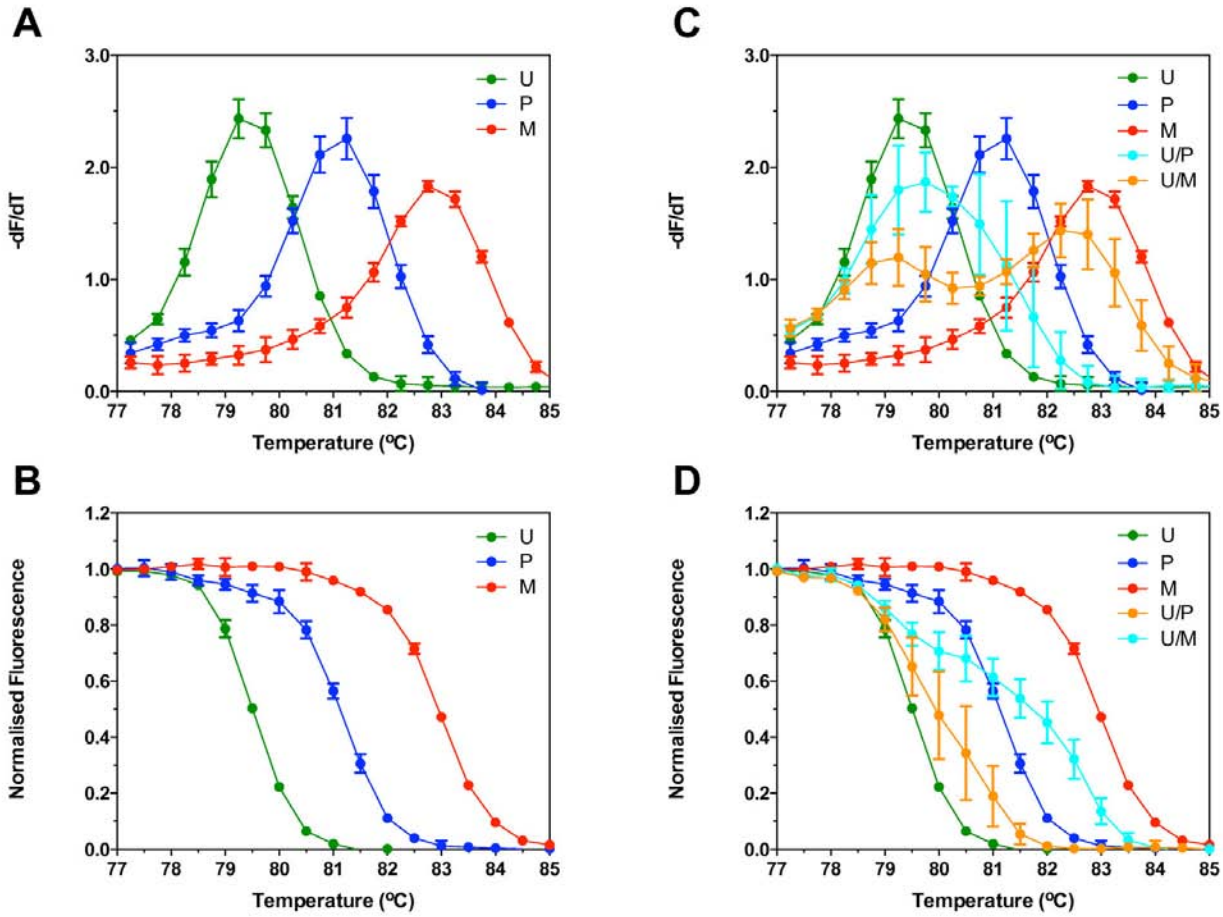

Figure 2
Melt curves for CDKN2A oligonucleotides. Oligonucleotide sequences representing a fragment of bisulfite modified unmethylated (U), partially methylated (P) or fully methylated (M) CDKN2A promoter (Table 2) were amplified using the CDKN2A primers and melted. The raw fluorescence data was converted to either the negative first derivative of the fluorescence with respect to temperature (-dF/dT) (A), or normalised (B), and plotted against temperature. Unmethylated oligonucleotide was spiked with either partially (U/P) or fully methylated (U/M) oligonucleotide, amplified using the CDKN2A primers and melted. Raw fluorescence data was either converted to -dF/dT (C), or normalised (D) and plotted against temperature. Data shown are the mean ± standard deviation of triplicate reactions.

OE33 gave an intermediate melt, suggesting that OE33 was partially methylated. Incubating the cells with vehicle for increasing lengths of time did not alter the normalised melt curves, nor the *T50*, *Tto*, or *Ttd* (Table 4). Treatment with aza shifted the normalised melt curves to the left, with the degree of shift increasing with the length of treatment (Figure 4). The shifts in the normalised curves corresponded to a significant reduction in *T50*, *Tto* and *Ttd* when compared to vehicle. The *Tdo – Tto*, a measure of methylation heterogeneity, decreased with length of aza treatment (Table 4). Methylation was confirmed by COBRA [13]. Similar shifts in the normalised melt curves were observed for MGMT (data not shown).

Melt curve analysis of tissue DNA

To demonstrate the utility of our melt analysis for detecting different patterns of methylation in tissue samples, methylation was measured in bisulfite modified DNA prepared from whole sections of FFPE CRC tissue with variable expression of MGMT as demonstrated by immunohistochemistry. Methylation of MGMT is associated with a reduction of its mRNA and protein expression in cancer cell lines and tissues [15,17]. Sections were taken from a single block of tissue that had either no, clonal, or complete loss of MGMT expression in the tumor cells. The normalised melt curves for a region of the MGMT promoter are shown in Figure 5. The CRC with no

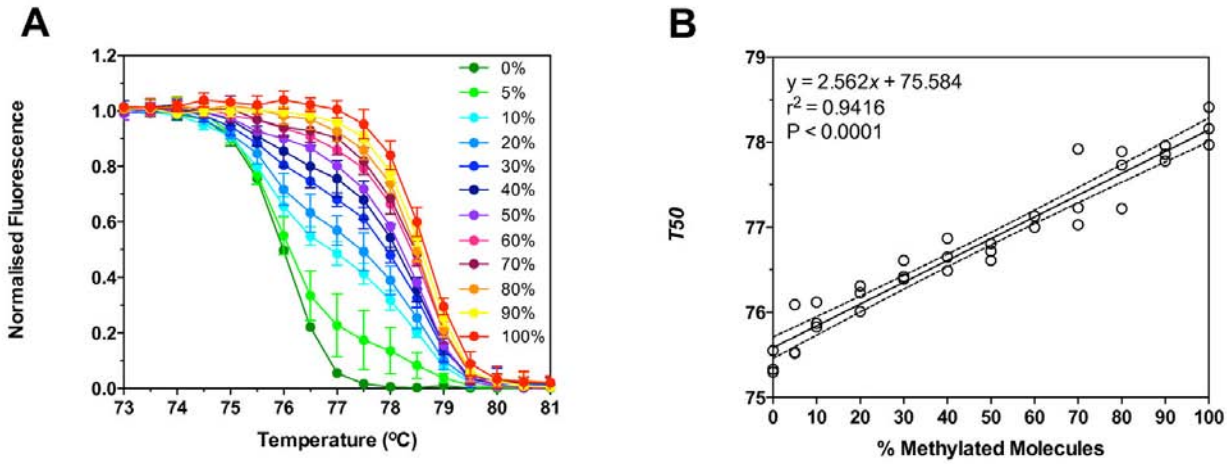

Figure 3
Correlation between percentage of methylated reference and *T50*. Unmethylated reference (bisulfite modified normal donor lymphocyte DNA) and methylated reference (bisulfite modified CpG methylase treated normal donor lymphocyte DNA) were mixed so that the final percentage of methylated reference in the unmethylated reference was 0%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%. The mixtures were amplified using the TIMP3 primers and melted. A) Graph of normalised fluorescence plotted against temperature. Data shown are the mean ± standard deviation of triplicate reactions. B) Linear correlation between percentage of methylated reference and *T50*.

detectable loss of MGMT expression by immunohistochemistry melted at the lowest temperature, that with complete loss the highest temperature, and that with clonal loss melted at an intermediate temperature. The *T50*, *Tto*, *Ttd* and *Ttd – Tto* are presented in Table 5. The *T50* significantly increased with increasing loss of expression. There was no significant difference in the *Tto* for unmethylated reference and any CRC tissue. The *Tto* for the unmethylated reference and each of the CRC tissues were significantly less than the methylated reference, indicating the presence of unmethylated molecules in each of the CRC tissues. This would be expected due to the presence of stromal and other non-tumor cells in the tissue section. The *Ttd* for the methylated reference and each of the CRC tissues was significantly more than the unmethylated reference, indicating the presence of methylated molecules in all the CRC tissues, including the tissue with no detectable loss of MGMT protein expression. The patterns of methylation were confirmed by direct bisulfite sequencing (Additional File 1).

Discussion

We describe a simple method to quantitate DNA methylation using the raw fluorescence melt data obtainable following PCR amplification of bisulfite modified DNA. The method, which provides a summative measure of methylation at all the CpGs in the PCR product, is reproducible, sensitive, and informative whether the methylation in the region amplified is homogeneous or heterogeneous. While the differences due to methylation were apparent visually, key descriptive parameters (*T50*, *Tto*, *Ttd and Ttd – Tto*) could be calculated mathematically, which permits statistical analysis of results and eliminates the subjectivity of visual assessments. We validated the utility of the method with synthesised oligonucleotides and DNA prepared from fresh cells and formalin fixed paraffin embedded tissues.

In the PCR of bisulfite modified DNA, what were unmethylated cytosines in the genomic DNA will be amplified as thymines, and only methylated cytosines will be ampli-

Table 4: *T50*, *Tto*, *Ttd* and *Ttd – Tto* values for TIMP3 in OE33 cells treated with vehicle or 5-aza-2'-deoxycytidine

Sample	*T50*	*Tto*	*Ttd*	*Ttd – Tto*
U ref	75.52 ± 0.07	74.74 ± 0.16	77.36 ± 0.08	2.62 ± 0.24
M ref	78.07 ± 0.23	77.26 ± 0.37	79.83 ± 0.02	2.57 ± 0.35
veh 48 h	76.87 ± 0.18	75.63 ± 0.29	79.37 ± 0.04	3.74 ± 0.29
veh 72 h	76.80 ± 0.11	75.56 ± 0.17	79.33 ± 0.07	3.77 ± 0.19
veh 120 h	76.89 ± 0.18	75.70 ± 0.33	79.34 ± 0.08	3.65 ± 0.36
aza 48 h	75.40 ± 0.66	73.84 ± 1.15	78.90 ± 0.09	5.05 ± 1.12
aza 72 h	75.54 ± 0.42	74.34 ± 0.75	78.69 ± 0.06	4.35 ± 0.74
aza 120 h	75.49 ± 0.33	74.38 ± 0.57	78.45 ± 0.19	4.07 ± 0.64

U ref – unmethylated reference.
M ref – methylated reference.
veh – OE33 esophageal adenocarcinoma cell line treated with vehicle for 48, 72 or 120 h.
aza – OE33 esophageal adenocarcinoma cell line treated with 1 μM 5-aza-2'-deoxycytidine for 48, 72 or 120 h.
Data shown for veh or aza treated OE33 are the mean ± standard deviation of triplicate reactions for each of triplicate cultures. Data shown for U ref and M ref are the mean ± standard deviation of triplicate reactions.

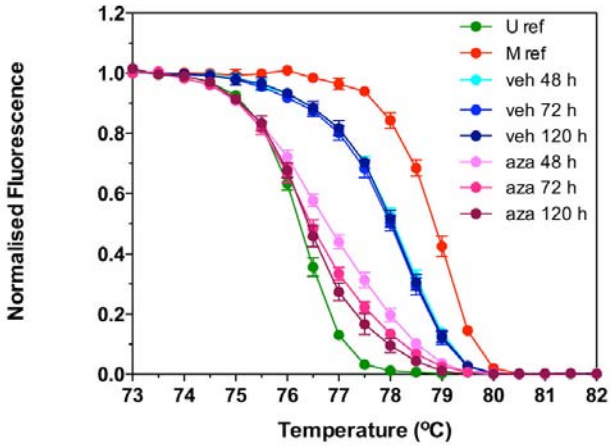

Figure 4
Normalised melt curves of TIMP3 in OE33 cells treated with vehicle or 5-aza-2'-deoxycytidine. Triplicate cultures of esophageal adenocarcinoma cell line OE33 were treated with either vehicle (veh) or 1 μM 5-aza-2'-deoxycytidine (aza) for 48, 72, or 120 h. Bisulfite modified DNA from treated OE33, unmethylated reference (U ref) and methylated reference (M ref) was amplified using the TIMP3 primers, melted, and the fluorescence normalised. Data shown for veh or aza treated OE33 are the mean ± standard deviation of triplicate reactions for each of triplicate cultures. Data shown for U ref and M ref are the mean ± standard deviation of triplicate reactions.

fied as cytosines. These sequence differences can be distinguished by the melt curves of the amplified products. The temperature range over which melting occurs and the shape of the curve is a function of the length, sequence and GC content of the product. Products derived from DNA template containing methylated cytosines will have a higher GC content, and so a higher melt temperature and touch down temperature, than otherwise equivalent products from DNA with unmethylated cytosines. Similarly, products from unmethylated DNA will have a cooler melt temperature and lower take off temperature than otherwise equivalent methylated DNA. The difference between the take off and touch down temperatures will reflect the heterogeneity (or uniformity) of methylation in the template molecules. Melt curve analysis, unlike gel electrophoresis, can distinguish between products which are of the same length but have different GC/AT ratios.

Several groups have used the melt curves from PCR products to detect mutations, polymorphisms or methylation. Worm [9], Akey [18] and Guldberg [19] detected methylation by comparing the melting temperature (*T*m) from the derivative peaks (-dF/dT) of the melt curves of the PCR products. While populations containing only methylated or unmethylated molecules are easily distinguishable by the difference in *T*m, heterogeneous mixtures of unmethylated, partially methylated and fully methylated molecules are difficult to analyse or compare. Lorente [12] described a technique called MCA-Meth in which they calculated a ratio of the relative height of the -dF/dT peaks for unmethylated to methylated molecules. The MCA-Meth is semi-quantitative, and cannot be used if partially methylated molecules are present.

Wittwer described a normalisation of the raw data from high resolution melts made possible with the development of the Idaho HR-1 instrument for genotyping and mutation [8]. To derive the normalised curves the experimenter had to select the 100% and 0% fluorescence values by eye. Sequence alterations, such as due to methylation, were identified visually by changes in the shape or position of the normalised curve. Wojdacz [10] used high resolution melting to measure methylation following amplification in the Rotor-Gene 6000 with SYTO9 dye. Mixtures of fully methylated or fully unmethylated DNA molecules, but not partially methylated molecules, were used in the validation of their method. In the analysis the raw melt curves were adjusted, in a manner not described, so that all the samples had the same starting and ending fluorescent signal. In these high resolution melt methods methylation differences between samples was assessed subjectively, not quantitatively. To the best of our knowledge, we are the first to describe and validate a method to quantitate DNA methylation from melt curves that can be used for samples that contain any mixture of methylated, unmethylated and/or partially methylated molecules, and does not depend on visual analysis.

Our method is based on normalising the raw melt fluorescence data using a simple algorithm which is easily computerised. It is easy to assess methylation visually from the normalised melt curve, but the power of the method is that it permits a mathematical analysis of the methylation. From the normalised melt data we calculate four parameters which describe the methylation status of the amplified region. The *Tto*, the temperature at which melting begins, reflects the least methylated amplicons present. The *Ttd*, the temperature at which the product is completely melted, reflects the most methylated amplicons present. The *Ttd* – *Tto* reflects the heterogeneity of the amplicons with respect to methylation. If *Ttd* – *Tto* is small, most alleles have a similar amount of methylation, if it is large then some alleles are high in methylation, others low. The *T50* is the temperature at which half of the amplicons are melted, and reflects the sum of all methylation of all the CpGs in the amplified region. The more the methylation in a region, the higher the *T50*. Because these parameters are derived mathematically, the subjectivity of other melt curve analytical methods is eliminated.

To validate our method we first used synthesised oligonucleotides representing a bisulfite modified sequence from

Table 5: *T50*, *Tto*, *Ttd* and *Ttd – Tto* values for MGMT in FFPE CRC tissues

Sample	*T50*	*Tto*	*Ttd*	*Ttd – Tto*
U ref	74.17 ± 0.04	73.64 ± 0.04	75.54 ± 0.04	1.90 ± 0.03
M ref	78.70 ± 0.25	78.06 ± 0.37	80.43 ± 0.15	2.36 ± 0.25
No	74.18 ± 0.09	73.54 ± 0.06	77.24 ± 1.01	3.71 ± 0.95
Clonal	75.84 ± 0.21	73.62 ± 0.17	81.12 ± 0.37	7.50 ± 0.31
Complete	76.17 ± 0.07	73.70 ± 0.09	80.32 ± 0.45	6.62 ± 0.40

U ref – unmethylated reference.
M ref – methylated reference.
No, Clonal and Complete – whole sections of FFPE CRC tissue with either no, clonal or complete loss of MGMT protein expression in tumor cells, as determined by immunohistochemistry.
All values are the mean ± standard deviation of 6 replicate reactions.

the CDKN2A promoter in its methylated, unmethylated and partially methylated form to show that the shape of the normalised melt curve, and the temperature at which the melting commenced and was completed, reflected the degree of methylation of the template alleles. We then demonstrated the utility of the method in more the complex situation of analysing DNA preparations from fresh cells and formalin fixed paraffin embedded tissues. Using lymphocyte DNA methylated with CpG methylase diluted into unmethylated lymphocyte DNA we showed a linear relationship between *T50* and the percentage of methylated amplicons in the mixture. The method could unambiguously detect methylation in samples containing between 1–5% of methylated DNA, depending on the target sequence. We were able to quantitate the anticipated reduction in methylation (*T50*) and methylation heterogeneity (*Ttd – Tto*) over time in a cell line treated with the demethylating agent 5-aza-2'-deoxycytidine. Finally, we could quantitate differences in methylation associated with complete, clonal or no loss of expression of MGMT expression in formalin fixed paraffin embedded tissues. Our melt analysis of methylation was confirmed by bisulfite sequencing.

The melt curve analysis is a valuable addition to the methods available for measuring methylation. The most common method for the analysis of methylation is methylation specific PCR (MSP), which uses primers that are specific for either methylated, or unmethylated, bisulfite modified DNA. This technique relies on 3' mismatching of the PCR primers for specificity. False positives can occur if the primers are poorly designed or the PCR is run at too low a temperature, or possibly for too many cycles. The method is sensitive, but only measures methylation of one or two CpGs located near the 3' end of the primers and is not quantitative. Tumors can be classified as methylated when only a minor percentage of cells are methylated, or if the bisulfite conversion of the DNA is incomplete (with some unmethylated cytosines remaining as cytosines, not being converted to uracils). Pyrosequencing accurately quantifies DNA methylation levels for multiple CpG sites within the PCR product, but it requires more expensive, biotin labeled primers, and a pyrosequencer in addition to a PCR thermocycler. MethyLight is an extremely sensitive and quantitative assay which uses TaqMan technology to measure methylation, utilising the cleavage of a dual-labeled fluorogenic hybridization probe by the 5' nuclease activity of Taq polymerase during the PCR amplification. The probes are expensive, and are specific for a particular methylation pattern within the amplified region. Generally the probes are designed to detect the fully methylated or fully unmethylated allele only, not partially methylated alleles. Bisulfite sequencing is the gold standard for methylation analysis, but is time consuming and expensive, and its accuracy is limited by the number of clones which are sequenced. Melt curve analysis is rapid and cost-effective method to quantitate DNA methylation when information about the summative methylation of the amplified region is required. Unlike MSP, it is quantitative and does not generate false positives. Unlike pyrosequencing it does not require equipment other than a PCR thermocycler, and unlike MethyLight it resolves heterogeneous methylation and can quantitate mixtures of variably methylated molecules in the same reaction, and does not require specific probes. It is much quicker than bisulfite sequencing, but does not provide the same detail about the pattern of methylation. It is particularly suited to measuring methylation in CpG rich regions, such as CpG islands associated with the promoter regions of genes, where it is generally sufficient to measure average CpG methylation levels rather than the level for every single CpG [20]. If high resolution methylation detail is required, such as can be provided by bisulfite sequencing, melt curve analysis can be used to screen for samples or clones which are unmethylated and do not require further analysis.

Two modifications have the potential to improve the performance of the method, although the principles of the analysis would not change. Fluorescent dyes such as SYTO9, LC Green or Eva Green which do not redistribute during melting may improve the detection of minor unmethylated populations, increasing sensitivity. Instruments which acquire fluorescence data with greater accuracy and over smaller temperature increments (e.g., 0.01°C compared to 0.5°C with the RG3000 used in this study) would also be expected to improve the sensitivity of the assay.

Conclusion

We have developed a rapid, reproducible and cost-effective in-tube assay to quantitate DNA methylation which yields significant information about the methylation in the template DNA. It resolves heterogeneous methylation, quantitating the total methylation when there are mix-

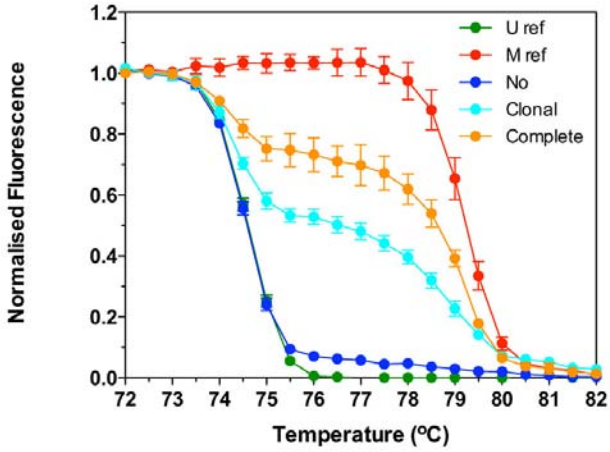

Figure 5
Normalised melt curves of MGMT in formalin fixed paraffin embedded colorectal carcinoma tissues. DNA was isolated from whole sections of FFPE CRC tissues that had either no, clonal, or complete loss of MGMT expression as demonstrated by immunohistochemistry. Bisulfite modified DNA from CRC tissues, unmethylated reference (U ref) and methylated reference (M ref) was amplified using MGMT primers, melted and the fluorescence normalised. Data shown are the mean ± standard deviation of six replicate reactions.

tures of fully methylated, partially methylated and unmethylated molecules in the same reaction, and requires no extra experimental processing after the PCR reaction. The primers are as easy to design as for a conventional PCR reaction, and expensive labelled probes are not required. The mathematical analysis does not rely on proprietary algorithms or software, is simple to computerise, and eliminates the imprecision in methods which require visual manipulation and interpretation of melt curves. The method is sensitive and reproducible for measuring methylation using SYBR Green I in a commonly used real-time thermocycler, and so is well suited to most research or routine laboratories.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
ES undertook the laboratory component of the study and participated in the design of the study, the analysis of the results, and the drafting the manuscript. MEJ participated in the design of the study and wrote the software for the melt analysis. PAD participated in the design of the study, the analysis of the results and the drafting of the manuscript. Each author read and approved the final manuscript.

Additional material

Additional file 1
***Direct bisulfite sequencing of MGMT PCR product**. Following amplification and melting, the MGMT PCR products of the CRC tissues with no, clonal or complete loss of expression, unmethylated reference (U ref) and methylated reference (M ref) were electrophoresed on 2% agarose gels. The presence of a single product and its size were confirmed by staining the gel with ethidium bromide. Each of the bands from the six replicate reactions for each sample were excised from the gel, the replicate bands combined, and purified using QIAquick Gel Extraction Kit (Qiagen), following the manufacturers instructions. The purified products (1 – 4 ng) were sequenced using BigDye Terminators v 3.1 (Applied Biosystems Inc, Foster City, CA) and both forward or reverse MGMT primers.*
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CHAPTER 4: METALLOTHIONIEN 3 EXPRESSION IS FREQUENTLY DOWN-REGULATED IN OESOPHAGEAL SQUAMOUS CELL CARCINOMA BY DNA METHYLATION

Eric Smith[1], Paul A. Drew[2], Zi-Qing Tian[3], Neville J. De Young[1], Jun-Feng Liu[3], George C. Mayne[4], Andrew R. Ruszkiewicz[5], David I. Watson[4], Glyn G. Jamieson[1]

[1]Discipline of Surgery, The University of Adelaide, Adelaide, 5005, Australia;
[2]School of Nursing and Midwifery, Flinders University, Bedford Park, 5042, Australia;
[3]Department of Thoracic Surgery, Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, P.R. China;
[4]Flinders University Department of Surgery, Flinders Medical Centre, Bedford Park, 5042, Australia;
[5]Division of Tissue Pathology, Institute of Medical and Veterinary Science, Adelaide, South Australia, 5000, Australia.

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Metallothionien 3 expression is frequently down-regulated in oesophageal squamous cell carcinoma by DNA methylation

Eric Smith*[1], Paul A Drew[2], Zi-Qing Tian[3], Neville J De Young[1], Jun-Feng Liu[3], George C Mayne[4], Andrew R Ruszkiewicz[5], David I Watson[4] and Glyn G Jamieson[1]

Address: [1]Department of Surgery, The University of Adelaide, Royal Adelaide Hospital, Adelaide, South Australia, 5005, Australia, [2]School of Nursing and Midwifery, Flinders University, Bedford Park, South Australia, 5042, Australia, [3]Department of Thoracic Surgery, Fourth Hospital of Hebei Medical University, Shijiazhuang, Hebei, P.R. China, [4]Department of Surgery, Flinders University, Bedford Park, South Australia, 5042, Australia and [5]Division of Tissue Pathology, Institute of Medical and Veterinary Science, Adelaide, South Australia, 5000, Australia

Email: Eric Smith* - eric.smith@adelaide.edu.au; Paul A Drew - paul.drew@adelaide.edu.au; Zi-Qing Tian - tizq12@vip.163.com; Neville J De Young - neville.deyoung@adelaide.edu.au; Jun-Feng Liu - liujf@heinfo.net; George C Mayne - mayn0020@flinders.edu.au; Andrew R Ruszkiewicz - andrew.ruszkiewicz@imvs.sa.gov.au; David I Watson - david.watson@flinders.edu.au; Glyn G Jamieson - glyn.jamieson@adelaide.edu.au

* Corresponding author

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Abstract

Background: Metallothionein 3 (MT3) inhibits growth in a variety of cell types. We measured *MT3* gene expression by RT-PCR, and DNA methylation in the *MT3* promoter by combined bisulphite restriction analysis, in four oesophageal cancer cell lines and the resected oesophagus from 64 patients with oesophageal squamous cell carcinoma (SCC).

Results: *MT3* expression was not detected in one of the four oesophageal cell lines. The *MT3* promoter was methylated in all of the oesophageal cell lines, but the degree of methylation was greater in the non-expressing cell line. After treatment with 5-aza-2'-deoxycytidine there was a reduction in the degree of methylation, and an increase in *MT3* expression, in each of the cell lines ($p < 0.01$). Methylation was detected in 52% (33 of 64) of primary SCC and 3% (2 of 62) of histologically normal resection margins. *MT3* expression was measured in 29 tumours, 17 of which had methylation of *MT3*. The expression of *MT3* was significantly less in the methylated tumours compared to either the unmethylated tumours ($p = 0.03$), or the matched margin ($p = 0.0005$). There was not a significant difference in *MT3* expression between the tumour and the margin from patients with unmethylated tumour. No correlations were observed between methylation of *MT3* and survival time, patient age, gender, smoking or drinking history, tumour stage, volume, or lymph node involvement.

Conclusion: We conclude that *MT3* expression is frequently down-regulated in oesophageal SCC, by DNA methylation, but that this is not a prognostic indicator.

Background

The metallothioneins (MT) are a group of low molecular weight, cysteine-rich intracellular proteins that are involved in maintaining intracellular metal homeostasis by binding transition metals such as zinc and copper. There are 10 functional isoforms of MTs described, which are divided into 4 classes, designated MT1 – 4, on the basis of small differences in protein sequence and charge characteristics [1,2]. The MTs have been proposed to play an important role in protecting against DNA damage, apoptosis and oxidative stress [3].

Metallothionein 3 (MT3) was first identified as a growth inhibitory factor, expressed in normal brain, which inhibited the survival of neurones in culture and also neurite formation [4]. Subsequent studies using glial [5] or tumour[6-8] cells, stably transfected with *MT3*, showed that its endogenous over-expression could inhibit cell growth. More recently, down-regulation of *MT3* was reported as one of 17 changes in gene expression which was most likely to be associated with metastasis and poor clinical outcome in a range of solid tumours [9]. One mechanism for reducing gene expression is methylation of the CpG island when present in the promoter region of the gene [10]. Methylation of the *MT3* promoter has been observed and has been suggested to cause reduced expression in gastric cancer [11].

The levels of *MT3* expression, the frequency of *MT3* methylation, and correlations between *MT3* methylation and clinical parameters, have not been investigated in oesophageal squamous cell carcinoma (SCC). In this study we used combined bisulfite restriction analysis (COBRA) [12] to estimate the frequency of methylation at specific sites within the *MT3* promoter in oesophageal cancer cell lines, and investigated the relationship between methylation and its expression by quantitative real-time RT-PCR. We then measured *MT3* gene expression and the frequency of *MT3* methylation in primary oesophageal SCCs and, when available, the histologically normal, proximal resection margin from those patients.

Results

Methylation analysis of MT3 *in normal lymphocytes and oesophageal cell lines*

The methylation status of the *MT3* CpG island was measured by COBRA within 3 overlapping regions (Figure 1). Complete digestion of the COBRA PCR product indicated methylation at one or more of the BstUI sites within that region of all alleles. Incomplete digestion indicated methylation at one or more of the BstUI sites within that region of some but not all alleles.

COBRA was performed on the bisulphite modified lymphocyte DNA from 19 different normal donors without any known cancers or other genetic abnormalities. In each of these donor lymphocytes samples an average of two percent (± 1% SD) of molecules were methylated at site 12 in the R1 region, located in intron 1. There was no evidence of methylation at any of the other sites analysed by COBRA in the R1 and the R2 regions (data not shown).

COBRA was performed on the oesophageal cancer cell lines OE19, OE21, OE33 and TE7, cultured with or with-

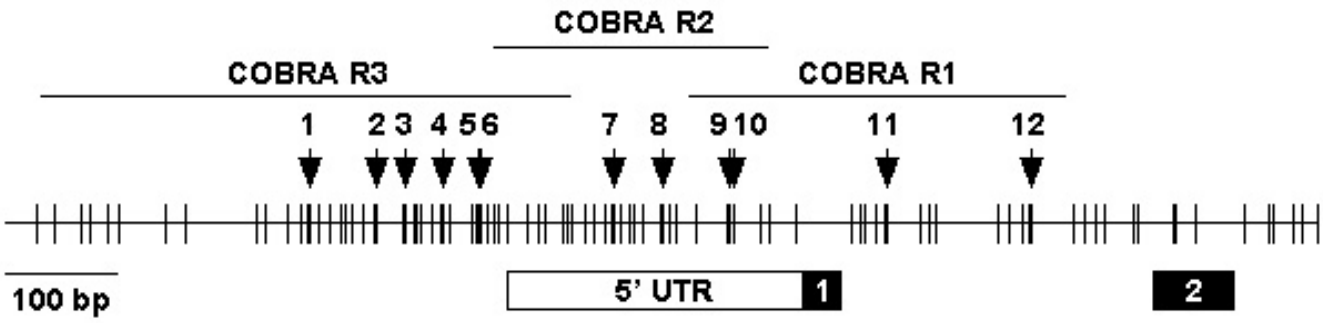

Figure 1

Schematic diagram of the *MT3* promoter. The diagram was generated by downloading the *MT3* CpG island genetic element with the addition of 200 bases upstream from http://genome.ucsc.edu/, using the Human July 2003 Freeze. The location of 5' UTR (□), exon 1 and exon 2 (■) of *MT3* (NM_005954) are shown. Each vertical line represents a CpG. The arrows represent the location of the 12 BstUI restriction enzyme sites analysed. The regions amplified for the 3 COBRA PCRs, R1, R2 and R3, are shown.

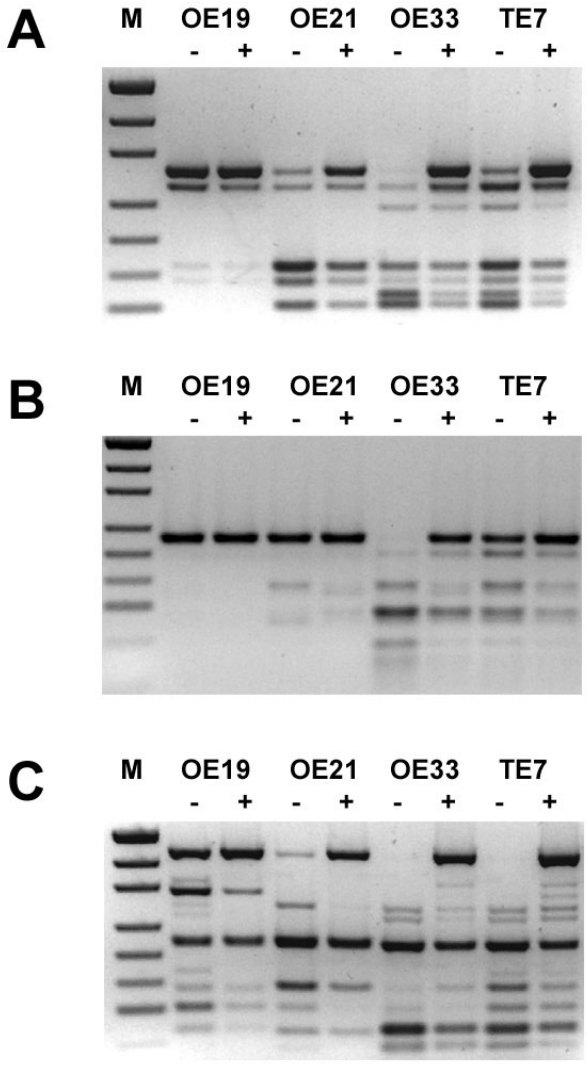

Figure 2
The *MT3* COBRA on regions 1 (A), 2 (B) and 3 (C) for the oesophageal cell lines with or without treatment with Aza-dC. The COBRA was performed on the oesophageal cell lines following 72 to 96 hrs of treatment with either 1 μM aza-dC (+) or vehicle (-). M, molecular weight marker.

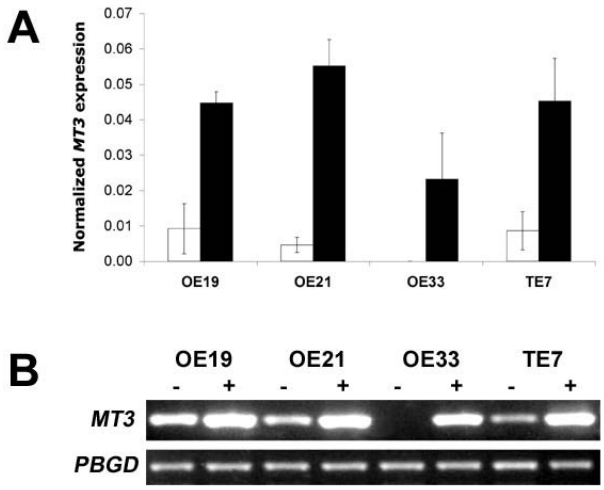

Figure 3
The expression of *MT3* in oesophageal cell lines. A) *MT3* expression in the oesophageal cell lines grown for 72 to 96 h with either 1 μM aza-dC (■) or vehicle (□). Results are the average of triplicate values ± SD, normalised to that of *PBGD*. B) Representative example of gel electrophoresis of the *MT3* and *PBGD* RT-PCR products for the cell lines following treatment with either 1 μM aza-dC (+) or vehicle (-).

out the DNA demethylation drug 5-aza-2'-deoxycytine (aza-dC) (Figure 2). Complete digestion of each of the 3 regions was observed for OE33. For TE7, incomplete digestion was observed in the R1 and R2 regions, whilst complete digestion was observed in R3 region. Incomplete digestion was observed in each of the 3 regions for OE21. For OE19, no digestion was observed in the R2 region, but incomplete digestion was observed in the R1 and R3 regions. The frequency of methylation at any BstUI site within each of the regions, R1, R2 or R3, was estimated in the oesophageal cell lines (Table 1). The frequency of methylation in each region varied from cell line to cell line, and the frequency of methylation in a given cell line varied from region to region. We then estimated the frequency of methylation at each BstUI site within a region (Table 2). We observed that the frequency of methylation at each BstUI site within a region varied for a given cell line and varied between cell lines.

Re-expression of the MT3 *after 5-aza-2'-deoxycytine treatment*

To determine the relationship between methylation and transcription of the *MT3* gene, we measured the expression of *MT3* by RT-PCR in 4 oesophageal cell lines, OE19, OE21, OE33 and TE7, cultured in the presence or absence of aza-dC (Figure 3). *MT3* expression was undetectable in the completely methylated cell line OE33. Low levels of *MT3* were measured in the partially methylated cell lines OE19, OE21 and TE7. Demethylation treatment with aza-dC reduced the frequency of methylation both within and between different regions for all cell lines provided that the intial frequency of methylation was ≥ 10 %. Demethylation treatment did not completely demethylate all the cells in culture. The expression of *MT3* was significantly increased following aza-dC treatment in each of the oesophageal cell lines ($p < 0.01$). The increase in *MT3* expression induced by aza-dC was significantly less in the

Table 1: The frequency of methylation of any BstUI site within a region, in oesophageal cell lines. The estimated frequency of methylation of any BstUI site within R1, R2 or R3, in the oesophageal cancer cell lines treated without (-) or with (+) with 1 μM aza-dC, where Δ is the change in methylation frequency. The results are from a single, representative experiment.

	OE19			OE21			OE33			TE7		
	-	+	Δ	-	+	Δ	-	+	Δ	-	+	Δ
R1	32%	23%	-10%	93%	57%	-37%	97%	37%	-60%	88%	33%	-55%
R2	6%	6%	0%	15%	10%	-5%	96%	32%	-64%	51%	19%	-32%
R3	73%	30%	-43%	97%	40%	-57%	95%	51%	-45%	100%	37%	-63%

OE33 cell line compared to the OE19, OE21 or TE7 (p = 0.02).

Methylation and expression of MT3 *in oesophageal SCC*

The frequency of *MT3* methylation was measured in resected primary tumour and, when available, the proximal resection margin from 64 patients with oesophageal SCC. From each sample the R1 and R2 regions of the *MT3* CpG island were amplified by PCR and then analysed for methylation by COBRA. As with the lymphocyte samples, low levels of methylation at BstUI site 12 in the R1 region were observed in all the histologically normal margins and the tumour samples from these patients. Thus, this ubiquitous low level of methylation was not considered in the subsequent analysis. Methylation of the R1 region was observed in 33 (52%) and of the R2 region in 10 (16%) of the tumour samples. All samples that were methylated in the R2 region were also methylated in the R1 region. No correlations were observed between patient age, gender, smoking or drinking history, tumour, volume, or lymph node involvement and the methylation of *MT3* (Table 3). Methylation of the R2 region was observed in none of the 62 proximal resection margin tissues available, whilst methylation of the R1 region was observed in 2 (3%).

MT3 gene expression was analysed by quantitative real-time RT-PCR in 29 of the primary tumours and their matched non-cancerous proximal resection margins for which RNA was available. *MT3* was methylated in 17 of these tumours, and unmethylated in 12. The *MT3* expression was normalized to that of *ACTB*. The expression of *MT3* was significantly less in the tumours in which the promoter was methylated compared to those in which it was unmethylated (Figure 4, p = 0.03). *MT3* expression in methylated tumours was significantly less than that of the matched margin (p = 0.0005). There was not a statistically significant difference in expression between the tumour and matched margin from patients in whom the tumour was unmethylated.

Survival data was available for 41 patients, 19 with an unmethylated tumour, 22 methylated. There was no difference between these 2 groups with respect to the time since operation (unmethylated: range 3 – 22 months, median 20 months; methylated: range 1 – 22 months, median 21 months). There were 5 deaths in the unmethylated and 4 in the methylated group (P = 0.7). There was no difference in the survival time following operation, with deaths in the unmethylated group occurring 3, 13, 13, 17 and 18 months post-operatively, compared to 1, 4, 8 and 22 months in the methylated group.

Discussion

In this study we investigated the expression of *MT3* and its control by DNA methylation in oesophageal SCC. We measured methylation of *MT3* by COBRA in three separate regions of its promoter, and gene expression by quantitative real-time RT-PCR. In the oesophageal cell line OE33, which had complete methylation at all the CpG sites analysed, there was also complete transcriptional silencing of the *MT3* gene. In a further 3 cell oesophageal cancer cell lines, each of which had partial methylation, there was a partial but not complete reduction in gene

Table 2: The frequency of methylation of any BstUI site within R1, in oesophageal cell lines. The estimated frequency of methylation at each BstUI site of R1 in the oesophageal cancer cell lines treated without (-) or with (+) with 1 μM aza-dC, where Δ is the change in methylation frequency. The results are from a single, representative experiment.

	OE19			OE21			OE33			TE7		
	-	+	Δ	-	+	Δ	-	+	Δ	-	+	Δ
9/10	4%	5%	1%	34%	18%	-16%	60%	13%	-47%	36%	9%	-27%
11	7%	6%	-1%	88%	48%	-40%	85%	16%	-69%	55%	13%	-42%
12	30%	21%	-10%	76%	48%	-29%	91%	34%	-57%	82%	30%	-52%

Table 3: The incidence of *MT3* methylation for the 64 patients with oesophageal SCC.

	n	Unmethylated	Methylated
Male	45	22	23
Female	19	9	10
Age, median (range)	57 (42 – 76)	57 (46 – 76)	58 (42 – 70)
Males	57 (42 – 70)	54 (46 – 68)	57 (42 – 70)
Females	62 (49 – 76)	63 (49 – 76)	61 (50 – 68)
Tumour volume (cm^3), median (range)	57 (4 – 300)	54 (4 – 300)	60 (4 – 225)
Histological differentiation			
Well/moderate	57	28	29
Poor	4	2	2
Not recorded	3	3	0
Tumour stage			
T1N0M0	3	1	2
T2N0M0	9	5	4
T2N1M0	3	2	1
T2N2M0	1	1	0
T3N0M0	29	13	16
T3N1M0	9	6	3
T3N2M0	1	0	1
T3N2M1	1	0	1
T4N0M0	2	2	0
T4N0M1	1	0	1
T4N1M0	2	1	1
Not recorded	3	0	3

expression. Treatment of each of these cell lines with aza-dC, which reduced methylation, resulted in an increase in gene expression.

In those the cell lines which had partial methylation, the amount of methylation could vary between contiguous CpGs in the same cell line, and could vary in particular CpGs between cell lines. Thus, a particular CpG which might be unmethylated in one cell line could be partially or completely methylated in other cell lines expressing similar levels of *MT3*. This is consistent with the reported variability of methylation of the p16 CpG island in primary human mammary epithelial cells during escape from growth arrest [13], and the O6-methylguanine-DNA methyltransferase gene in human cell lines [14]. The significance of this finding is that methods for the measurement of DNA methylation such as methylation specific PCR (MSP) which analyse methylation at one or two CpGs only may misrepresent the methylation pattern of a region. In addition these methods would mislead if they probed CpGs with low levels of methylation in all tissues analysed, such as the site 12 in intron 1 in our analysis of *MT3*. This was methylated in normal lymphocytes and normal oesophageal tissue, as well oesophageal SCC, even though other CpGs were unmethylated and gene expression was high.

In order to show the relationship between methylation within the regions studied and MT3 gene expression, we incubated cultures of the oesophageal cell lines for approximately two cell divisions with aza-dC, a potent inhibitor of DNA methyltransferase. Because the rate of division varied for each of the cell lines, we determined the length of time required for each of the cell lines to divide twice in the presence of aza-dC using PKH-26 labelling [15]. This stable red fluorescent dye inserts into the cytoplasmic membrane, and is distributed equally amongst each of the daughter cells at the time of cell division, such that the mean fluorescence intensity of the cell population is halved with each cell division. As expected, treatment with aza-dC for two cell divisions did not completely demethylate the cell lines. This is because it is only newly synthesised strands which are demethylated, so if in a cell a particular region of DNA is completely methylated on both alleles, after 2 divisions only 75% of the alleles will be demethylated. We found that there was a significant reduction in the amount of methylation at each of the methylated sites analysed following drug treatment. Interestingly, the amount of the reduction varied between different CpG sites within a cell line, and varied from cell line to cell line at a given site. The reasons for this cell line and regional variation in the extent of demethylation are unknown.

Previous studies have shown that the methylation of only a subset of available CpG sites within a region can be sufficient to reduce transcription [16-18,13], with a greater reduction in transcription as the density of CpG methyla-

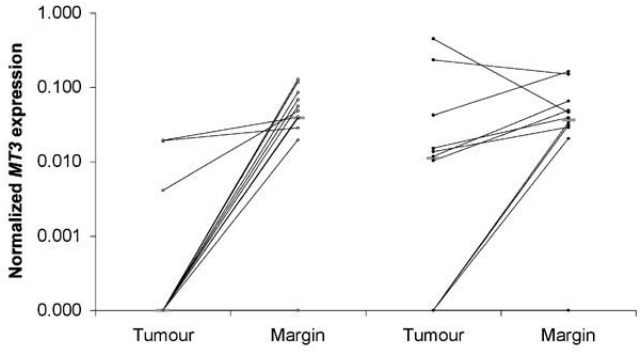

Figure 4
***MT3* expression in patient-matched tumour and non-cancerous proximal resection margin from patients with SCC of the oesophagus**. *MT3* expression in the oesophageal tissue from patients with either methylated tumour (❍) or umethylated tumour (●). Results are the average of triplicate values, normalised to that of *ACTB*. The grey bars represent the median expression for each group. The black lines link expression values of tissues from the same patient.

tion increases [19]. Complete methylation of all the sites in the regions which we studied correlated with lack of any gene expression, while methylation of only a subset of sites correlated with some *MT3* expression, and the further reduction in methylation resulting from demethylation with aza-dC always correlated with an increase in *MT3* expression. In this study we evaluated *MT3* expression only by RT-PCR, because an antibody specific for the MT3 protein was not commercially available. However, previous studies have shown that when the *MT3* transcript was able to be detected the protein was also detectable [20,21,7,8].

Our interest in *MT3* came from a report that solid tumours with a specific pattern of expression of 17 genes, including down-regulation of *MT3*, were most likely to be associated with metastasis and poor outcome [9]. Oesophageal SCC is an aggressive disease with a 5 year survival of about 20%, death most commonly due to secondaries. Molecular markers which assist in predicting metasases might help to tailor treatment options better. *MT3* was first identified as a growth inhibitory factor which decreased the survival of neuronal cultures [4]. Subsequent studies using stably transfected cell lines showed that up-regulation of *MT3* was growth inhibitory in some, but not all, cell lines [6-8].

The reported role of *MT3* in carcinogenesis is unclear. In gastric carcinomas *MT3* expression was found to be markedly reduced, but there was no indication of any relationship to outcome in this report [22]. In contrast, levels of *MT3* protein were shown to be elevated in bladder [20] and breast carcinomas [21]. This elevated expression was a poor prognostic indicator, being associated with increased tumour stage in bladder carcinoma, and poor disease outcome in some breast carcinomas. In our series of 64 patients with oesophageal SCC we found a degree of methylation in 52% of primary tumours and 3% of the histologically normal proximal resection margin. We observed that *MT3* expression was frequently down-regulated in primary oesophageal SCC when compared to normal mucosa, and significant down-regulation was most commonly observed in those tumours that were methylated for *MT3*, and not unmethylated tumours. Interestingly, 3 tumours which had methylated DNA also had normal expression of *MT3*. This might reflect a heterozygous pattern of methylation, with only one allele methylated, or a mixed tumour cell population containing some cells which were methylated and some which were not. The techniques used in this study could not distinguish between these possibilities. Also, 3 patients with unmethylated DNA had low levels of *MT3* expression, perhaps reflecting mutation or other change in the tumour. We found no relationship between methylation status and survival in the subset of patients for whom data were obtainable, suggesting that in these patients there would also be no relationship between gene expression and survival. Methylation and reduced expression of *MT3* was also not associated with tumour stage or tumour size. Thus a change in *MT3* expression by itself does not appear to favour increased tumour growth, and methylations status is not associated with survival, in patients with oesophageal SCC.

Conclusion

We have shown that the *MT3* promoter has a heterogeneous pattern of methylation in oeosphageal cancer cell lines, and is associated with a reduction in the gene expression. In resected oesophageal SCC tissue *MT3* expression was frequently down-regulated, most commonly in those tumours in which the promoter region was methylated. Methylation or down-regulation of *MT3* did not correlate with and patient age, gender, smoking or drinking history, tumour stage, volume, lymph node involvement, or patient survival. MT3 methylation does not show promise as a prognostic marker in patients with oesophageal SCC.

Methods

Cell lines and tissue samples

The oesophageal cancer cell lines OE19, OE21, OE33 and TE7 [23] were each cultured in RPMI 1640 supplemented with 10% foetal bovine serum at 37°C with 5% CO_2. Whole blood was obtained from 19 normal donors, none of whom had known cancers or genetic abnormalities. Primary tumour and, when available, non-cancerous

Table 4: Primer sequences and related information.

Primer	Genome position[a]	Primer sequence	Annealing temperature	Product size
R1-forward	+148	5'-AGTATYGTTATTTGTTGTTATTAGTTAAGT-3'	54°C	298 bp
R1-reverse	+445	5'-TAAAATACCAAATCTCCCTATTCTC-3'		
R2-forward	-8	5'-GAGYGGGTTTYGGTAGTGTATATAT-3'	52°C	217 bp
R2-reverse	+209	5'-TAAACRAACTTCTCCAAACAACTAAAC-3'		
R3-forward	+58	5'-GAAATGGAATAYGTTTTTTGTTAGTGAT-3'	60°C	427 bp
R3-reverse	-369	5'-ACTCCRACRCACRCACTACATCT-3'		
MT3-forward		5'-GACCTGCCCCTGCCCTTCTGGTGG-3'	69°C	219 bp
MT3-reverse		5'-GCTCCACACGGAGGGGTGCCTTCT-3'		
ACTB-forward		5'-TTGCCGACAGGATGCAGAAG-3'	59°C	101 bp
ACTB-reverse		5'-CTTTCCAAGCGGAGCCATGTCTGG-3'		
PBGD-forward		5'-CTTTCCAAGCGGAGCCATGTCTGG-3'	59°C	377 bp
PBGD-reverse		5'-CATGAGGGTTTTCCCGCTTGCAGA-3'		

[a] Nucleotide position relative to the transcription start site.

proximal resection margin from 64 consecutive patients undergoing oesophagectomy for SCC at the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University, were collected into RNAlater (Ambion, Austin, TX). Patient gender, age at the time of operation, smoking and drinking history and tumour type, stage, volume, and conventional histopathology were recorded. Survival data were available for 41 patients. The study complied with the appropriate institutional guidelines.

Demethylation of oesophageal cell lines by 5-aza-2'-deoxycytine treatment

Re-expression studies for *MT3* were performed on each of the oesophageal cancer cell lines. To determine the culture conditions required to achieve at least 2 cell divisions in the presence or absence of 5-aza-2'-deoxycytine (aza-dC), cells, labelled with PKH-26 (Sigma-Aldrich, Sydney, NSW, Australia), were analysed by flow cytometry as described previously [15]. The cells were treated for 72 (OE21, OE33 and TE7) or 96 (OE19) hr with culture medium containing either vehicle or 1 μM aza-dC (Sigma-Aldrich). The medium was then replaced with fresh medium not containing aza-dC, and the cells were incubated for a further 24 hr before harvesting.

Preparation of bisulphite modified DNA

Genomic DNA was isolated from normal donor lymphocytes, cultured cells, and RNAlater stabilized tissues. Lymphocytes were isolated from whole blood using Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden). The adherent cell lines were harvested by detaching the cells from culture flasks using trypsin/EDTA. RNAlater stabilized tissues were homogenised using disposable pestles (Edwards Instruments, Narellan, NSW, Australia). Next, the samples of lymphocytes, cultured cells, or RNAlater stabilised tissues were digested for 3 days with proteinase K and sodium dodecyl sulfate in TES, pH 8. The protein was removed by NaCl precipitation. The DNA was precipitated with ethanol, and resuspended in TE, pH 8. The DNA was bisulphite modified using a modification of the method previously described [24]. Briefly, 2 μg of genomic DNA was denatured by treatment with NaOH at 37°C for 15 min. The DNA was modified using a mixture of 5 M sodium bisulfite (Sigma-Aldrich) and 0.72 μM hydroquinone (Sigma-Aldrich) for 4 hr at 56°C. The samples were purified using Rapid PCR Purification System (Marligen Biosciences, Ijamsville, MD), desulphonated with NaOH for 15 min at 37°C, precipitated with ethanol and sodium acetate, and resuspended in 50 μl of TE, pH 8.

Methylation analysis of the MT3 *promoter by COBRA*

Bisulphite modified DNA was amplified using primers that amplified 3 overlapping regions, designated R1, R2 and R3 (Figure 1). The primers did not discriminate between methylated and unmethylated alleles. The primers and PCR conditions were specific for bisulphite modified DNA, and did not amplify unmodified DNA. All COBRA PCRs were performed in a volume of 50 μl containing 2 μl of bisulphite modified DNA, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of forward and reverse primer, and 0.5 U of HotStar Taq in 1 × PCR Buffer (Qiagen, Hilden, Germany). The reactions were incubated in an Eppendorf Mastercycler at 95°C for 15 min; then 45 cycles of 94°C for 1 min, 52, 54 or 60°C for 1 min, and 72°C for 1 min; and a 4 min final extension at 72°C (Table 4). The PCR products (5 μl) were digested with 5 units of BstUI restriction enzyme (New England Biolabs, Beverly, MA) in a final volume of 10 μl, for 4 hr at 60°C. BstUI specifically digests at CGCG sites that are retained after bisulphite modification when CpGs are methylated. The unmethylated cytosines are deaminated to uracil by the bisulphite reaction, then amplified as thymine in the PCR reaction, and so are not digested by BstUI. The digested PCR products were resolved on 2% agarose gels and stained with ethidium bromide. The intensity of each band was quantified and converted to nanograms of DNA

using Kodak ID Image Analysis Software (Kodak, Rochester, NY). For each gel, 250 ng of the molecular weight marker pUC19/Hpa II (GeneWorks, Adelaide, SA, Australia) was used as a standard to determine DNA fragment size and mass. Restriction maps for each digest were used to determine the length of all possible fragments, and the number of molecules in each band were determined by multiplying the mass of DNA in each band by its' fragment length. The frequency of methylation at each BstUI site was then estimated by dividing the number of molecules for each fragment by the total number of molecules in the digest.

Analysis of MT3 *expression by quantitative real-time RT-PCR*

Cell line RNA was isolated using the RNeasy kit (Qiagen) with an on-column DNase I digestion. Tissue RNA was isolated using Trizol (Invitrogen, Mount Waverly, VIC, Australia) and treated with DNase I (Ambion). The cDNA was synthesised from 2 μg of RNA using an M-MLV kit (Invitrogen). Quantitative real-time RT-PCR was performed on a RotorGene 2000 PCR thermocycler (Corbett Research, Sydney, NSW, Australia). Triplicate reactions were done using the appropriate PCR primers (Table 4) and the Quantitect Sybr-Green PCR mix (Qiagen). The PCR products were electrophoresed on 1.5 % agarose gels and stained with ethidium bromide. The expression of *MT3* was normalized to that of either *ACTB* or *PBGD* [25].

Data analysis

Statistical analysis of expression of *MT3* before and after aza-dC treatment for each cell line was performed using unpaired T-tests. Comparisons of the levels of *MT3* between cell lines with or without aza-dC treatment were performed using ANOVA with Tukey-Kramer multiple comparisons test. The comparison of the volume of methylated and unmethylated tumours was performed using a Kruskal-Wallis Test. The comparison of patients with or without positive lymph nodes and methylation status was performed using a Chi-squared test. The Mann-Whitney test was used to compare *MT3* expression in methylated and unmethylated tumour. Comparisons of *MT3* expression in tumour and margin were performed using Wilcoxon matched-pairs sign-rank tests. Survival data were analysed using Fisher's Exact Test. All statistics were performed using InStat version 3.0a (GraphPad Software, San Diego, CA).

List of abbreviations

aza-dC, 5-aza-2'-deoxycytidine; COBRA, combined bisulphite restriction analysis; MT3, metallothionein 3; RT-PCR, reverse transcriptase-polymerase chain reaction; SCC, squamous cell carcinoma.

Authors' contributions

ES isolated the DNA and RNA, performed the bisulphite modifications, COBRAs and RT-PCRs, designed the study, performed the statistical analysis and coordinated and drafted the manuscript. GCM aided with the RT-PCRs. ZQT and JFL collected the oesophagectomy material and clinical data. NJD performed the cell culture and edited the manuscript. AR confirmed the pathology. PD, DIW and GGJ supervised the work and edited the manuscript. All authors read and approved the final manuscript.

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CHAPTER 5: METHYLATION OF TIMP3 IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

Eric Smith[1], Neville J. De Young[1], Zi-Qiang Tian[2], Maria Caruso[3], Andrew R. Ruszkiewicz[3], Jun-Feng Liu[2], Glyn G. Jamieson[1], Paul A. Drew[4]

[1]Discipline of Surgery, The University of Adelaide, Adelaide, 5005, Australia;
[2]Department of Thoracic Surgery, 4th Hospital of Hebei Medical University, Hebei, Shijiazhuang 050011, P.R. China;
[3]Division of Tissue Pathology, Institute of Medical and Veterinary Science, Adelaide, South Australia, 5000, Australia;
[4]School of Nursing and Midwifery, Flinders University, Bedford Park, 5042, Australia.

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CHAPTER 6: METHYLATION OF CLDN6, FBN2, RBP1, RBP4, TFPI2, AND TMEFF2 IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

Shigeru Tsunoda[1,2], Eric Smith[1], Neville J. De Young[1], Xian Wang[1], Zi-Qing Tian[3], Jun-Feng Liu[3], Glyn G. Jamieson[1], Paul A. Drew[4]

[1]Discipline of Surgery, The University of Adelaide, Adelaide, 5005, Australia;
[2]Department of Surgery, Graduate School of Medicine, Kyoto University, Kawaracho 54, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan;
[3]Department of Thoracic Surgery, 4th Hospital of Hebei Medical University, Hebei, Shijiazhuang 050011, P.R. China;
[4]School of Nursing and Midwifery, Flinders University, Bedford Park, 5042, Australia.

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CHAPTER 7: SIMILARITY OF ABERRANT DNA METHYLATION IN BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA

Eric Smith[1], Neville J. De Young, Sandra J. Pavey[2], Nicholas K. Hayward[2], Derek J. Nancarrow[2], David C. Whiteman[3], B. Mark Smithers[4], Andrew R. Ruszkiewicz[5], Andrew D. Clouston[4], David C. Gotley[4], Peter G. Devitt[1], Glyn G. Jamieson[1], Paul A. Drew[6]

[1]Discipline of Surgery, The University of Adelaide, Adelaide, 5005, Australia;
[2]Oncogenomics, Queensland Institute of Medical Research, Brisbane, 4006, Australia;
[3]Division of Population Studies and Human Genetics, Queensland Institute of Medical Research, Brisbane, 4006, Australia;
[4]School of Medicine, University of Queensland, Brisbane, Queensland, 4072, Australia;
[5]Surgical Pathology, SA Pathology, Adelaide, 5000, Australia;
[6]School of Nursing and Midwifery, Flinders University, Bedford Park, 5042, Australia.

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Similarity of aberrant DNA methylation in Barrett's esophagus and esophageal adenocarcinoma

Eric Smith[1], Neville J De Young[1], Sandra J Pavey[2], Nicholas K Hayward[2], Derek J Nancarrow[2], David C Whiteman[3], B Mark Smithers[4], Andrew R Ruszkiewicz[5], Andrew D Clouston[4], David C Gotley[4], Peter G Devitt[1], Glyn G Jamieson[1] and Paul A Drew*[6]

Address: [1]Discipline of Surgery, School of Medicine, The University of Adelaide, Royal Adelaide Hospital, Adelaide, South Australia, 5005, Australia, [2]Oncogenomics, Queensland Institute of Medical Research, Brisbane, 4006, Australia, [3]Division of Population Studies and Human Genetics, Queensland Institute of Medical Research, Brisbane, 4006, Australia, [4]School of Medicine, University of Queensland, Brisbane, Queensland, 4072, Australia, [5]Division of Tissue Pathology, Institute of Medical and Veterinary Science, Adelaide, South Australia, 5000, Australia and [6]School of Nursing and Midwifery, Flinders University, Bedford Park, South Australia, 5042, Australia

Email: Eric Smith - eric.smith@adelaide.edu.au; Neville J De Young - neville.deyoung@adelaide.edu.au; Sandra J Pavey - sandraP@qimr.edu.au; Nicholas K Hayward - nickH@qimr.edu.au; Derek J Nancarrow - derekN@qimr.edu.au; David C Whiteman - daveW@qimr.edu.au; B Mark Smithers - M.Smithers@mailbox.uq.edu.au; Andrew R Ruszkiewicz - andrew.ruszkiewicz@imvs.sa.gov.au; Andrew D Clouston - andrewclouston@envoi.com.au; David C Gotley - D.Gotley@mailbox.uq.edu.au; Peter G Devitt - peter.devitt@adelaide.edu.au; Glyn G Jamieson - glyn.jamieson@adelaide.edu.au; Paul A Drew* - paul.drew@flinders.edu.au

* Corresponding author

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Abstract

Background: Barrett's esophagus (BE) is the metaplastic replacement of squamous with columnar epithelium in the esophagus, as a result of reflux. It is the major risk factor for the development of esophageal adenocarcinoma (EAC). Methylation of CpG dinucleotides of normally unmethylated genes is associated with silencing of their expression, and is common in EAC. This study was designed to determine at what stage, in the progression from BE to EAC, methylation of key genes occurs.

Results: We examined nine genes (APC, CDKN2A, ID4, MGMT, RBP1, RUNX3, SFRP1, TIMP3, and TMEFF2), frequently methylated in multiple cancer types, in a panel of squamous (19 biopsies from patients without BE or EAC, 16 from patients with BE, 21 from patients with EAC), BE (40 metaplastic, seven high grade dysplastic) and 37 EAC tissues. The methylation frequency, the percentage of samples that had any extent of methylation, for each of the nine genes in the EAC (95%, 59%, 76%, 57%, 70%, 73%, 95%, 74% and 83% respectively) was significantly higher than in any of the squamous groups. The methylation frequency for each of the nine genes in the metaplastic BE (95%, 28%, 78%, 48%, 58%, 48%, 93%, 88% and 75% respectively) was significantly higher than in the squamous samples except for CDKN2A and RBP1. The methylation frequency did not differ between BE and EAC samples, except for CDKN2A and RUNX3 which were significantly higher in EAC. The methylation extent was an estimate of both the number of methylated alleles and the density of methylation on these alleles. This was significantly greater in EAC than in metaplastic BE for all genes except APC, MGMT and TIMP3. There was no significant difference in methylation extent for any gene between high grade dysplastic BE and EAC.

Conclusion: We found significant methylation in metaplastic BE, which for seven of the nine genes studied did not differ in frequency from that found in EAC. This is also the first report of gene silencing by methylation of ID4 in BE or EAC. This study suggests that metaplastic BE is a highly abnormal tissue, more similar to cancer tissue than to normal epithelium.

Background

The incidence of esophageal adenocarcinoma (EAC) is increasing rapidly and patient outcomes remain poor. Known risk factors for EAC include obesity, gastro-esophageal reflux, and the presence of Barrett's esophagus (BE). Repeated injury from gastro-duodenal reflux is thought to result in the replacement of the esophageal squamous mucosa with a metaplastic columnar lined epithelium. The presence of goblet cells within columnar epithelium is diagnostic for BE. Approximately 0.5 – 1% of patients with BE will develop EAC each year and patients with BE have a 50- to 100-fold increased risk of EAC compared to the general population [1,2].

The progression from BE to EAC is generally accepted to proceed via the histological stages of low-grade and high-grade dysplasia. Progress along this pathway appears to mirror the accumulation of genetic abnormalities, with a number of reports suggesting a stepwise progression of genetic changes. Abnormalities in CDKN2A, seen in BE metaplastic tissue, followed by altered TP53 expression, generally reported in dysplastic tissue, have been associated with the transition from BE to EAC [3,4]. Alterations in the expression of many other genes have also been described at different stages of the progression to cancer [5,6].

Genes can be aberrantly down-regulated as a result of genomic alterations such as mutation, deletion, or DNA methylation. In humans, methylation of cytosines generally occurs in the context of a CpG dinucleotide. Regions of relatively high CpG content, termed CpG islands, are found in the promoter region of many genes. Methylation within these regions is associated with suppression of the expression of certain genes, for example the switching off of key developmental genes in adult tissues. However, aberrant methylation of normally unmethylated CpG islands is a common cause of altered gene expression in cancer. As esophageal biopsies are easily obtained at endoscopy, it is possible to determine at what stage methylation occurs during the progression to cancer. In this study we compared the methylation of nine genes, frequently methylated in other cancers, between squamous, BE and EAC tissues.

Results

Gene methylation and expression in esophageal cancer cell lines

Methylation and expression of the nine genes listed in Table 1 were measured in triplicate cultures of the esophageal cancer cell lines OE33 and TE7, following treatment with either 5-aza-2'-deoxycytidine (aza-dC) or vehicle (Figure 1). Methylation was observed in all genes in OE33, except APC and CDKN2A, while in TE7 it was only observed in ID4, RBP1, SFRP1, and TMEFF2. Treatment with aza-dC reduced methylation and significantly increased the expression of all methylated genes except TMEFF2, which was undetected in either cell line. CDKN2A was not detected in TE7 by methylation PCR or quantitative real-time reverse-transcription PCR (qRT-PCR), suggesting that the gene was deleted in this cell line. Following treatment with aza-dC, transcript levels were not significantly increased for any unmethylated genes, except for APC in OE33.

Methylation in esophageal adenocarcinoma and Barrett's esophagus

The results in Figure 2 show the methylation frequency, the percentage of samples that had any extent of methylation, for each of the nine genes, in squamous tissues from either patients with no known history of BE (S), or patients with BE (S-BE) or patients with adenocarcinoma (S-EAC), in metaplastic Barrett's from patients with BE but no dysplasia or adenocarcinoma (BE), in high grade dysplastic Barrett's from patients with adenocarcinoma (D-EAC), and in adenocarcinoma (EAC). In squamous tissues the methylation frequency for each gene did not differ significantly between S and S-BE, while in S-EAC methylation was significantly higher for SFRP1 (compared to either S or S-BE) and ID4 (compared to S) and lower for APC (compared to S-BE). For all nine genes the methylation frequency in BE and D-EAC was significantly higher than each of the squamous tissues (S, S-BE and S-EAC) with the exception of CDKN2A (no difference

Table 1: The primer sequences and annealing temperatures for methylation analysis

Gene	Forward primer	Reverse primer	Annealing temperature (°C)
APC	GAAGYGGAGAGAGAAGTAGTTG	ACRAACTACACCAATACAACCACATA	55
CDKN2A	TYGGYGGYGGGGAGTAGTATGGAGTTT	RTTAAACAACRCCCCCRCCTCCAACAA	60
ID4	GGGGYGTAYGGTTTTATAAATATAGTTG	TAATCACTCCCTTCRAAACTCCGACTAAA	55
MGMT	5G5GTTT5GGATATGTTGGGATAGTT	AC5AAAC5ACCCAAACACTCACCAAA	55
RBP1	TGTGYGYGTTGGGAATTTAGTTG	CRAAAAATAACTAAAACCAATTAACCACAAA	55
RUNX3	YGTYGTTTTTTGYGTTTTGAGGTT	ACTTAAATCTACRAAAATACRCATAACAA	55
SFRP1	ATTTTYGGGAGTYGGGGYGTATT	RACCAATAACRACCCTCRACCTA	57
TIMP3	TTTGAGGGGGYGGGTTTTAATAGTT	AACRACCTCCCRACGAAAAAACAAA	55
TMEFF2	TTGTTTTTTYGTYGGGTGTTATTGTTAT	AACAAACRACTTCCRAAAAACACAAA	55

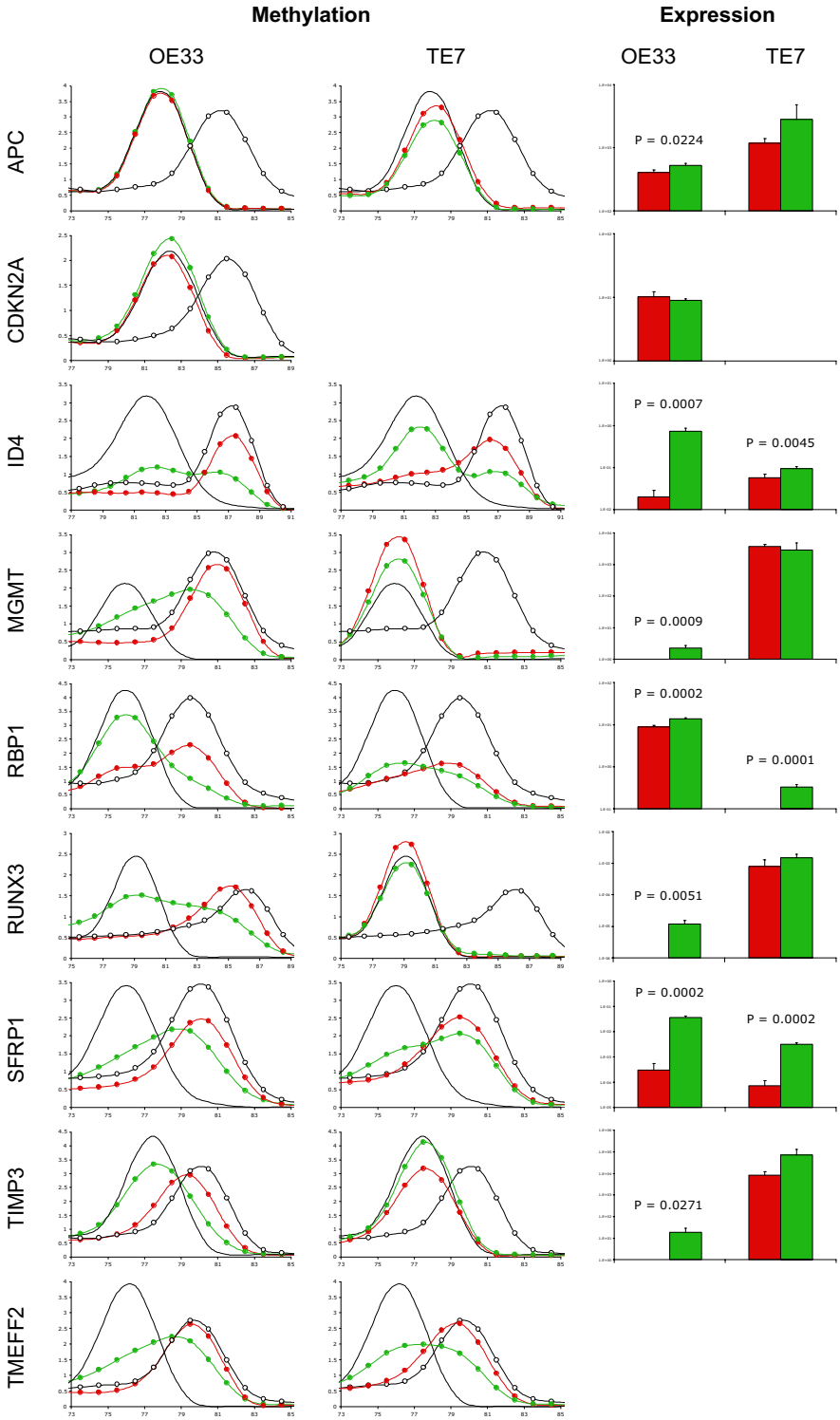

Figure 1 *(see legend on next page)*

Figure 1 *(see previous page)*
Methylation and expression of APC, CDKN2A, ID4, MGMT, RBP1, RUNX3, SFRP1, TIMP3 and TMEFF2 in esophageal cancer cell lines OE33 and TE7. The esophageal cancer cell lines OE33 and TE7 were treated with either 1 μmol/L aza-dC or vehicle for 72 hours. The medium was replaced with fresh medium only, and the cells incubated for a further 24 hours before harvesting. Bisulphite modified DNA was amplified using primers and PCR conditions (Table 2) which were specific for bisulphite modified DNA, did not discriminate between methylated and unmethylated sequences, and did not amplify unmodified DNA. The PCR products were melted by increasing the temperature from 60 to 95°C, rising 0.5 or 1°C at each step, waiting 30 seconds on the first step then 5 seconds for each step thereafter. Data was collected and analysed using the Melt Curve Analysis function of the RG-3000 Application Software. The left hand column shows the melt curves for each of the nine genes for OE33, the central column for TE7. Each plot shows the melt curves for the unmethylated (black lines) and methylated (open circles) controls and the cell lines treated with vehicle (red circles) or aza-dC (green circles). The horizontal axis represents temperature and the vertical axis -dF/dT. CDKN2A was not amplified in TE7. Interpretation of the melt curves is described in the Materials and Methods. The right hand column shows the gene expression in cell lines treated with vehicle (red columns) or aza-dC (green columns), as determined by qRT-PCR and normalised to HMBS. Data shown are the means ± SD from three independent experiments. TMEFF2 expression was below detectable limits in either cell line treated with vehicle or aza-dC.

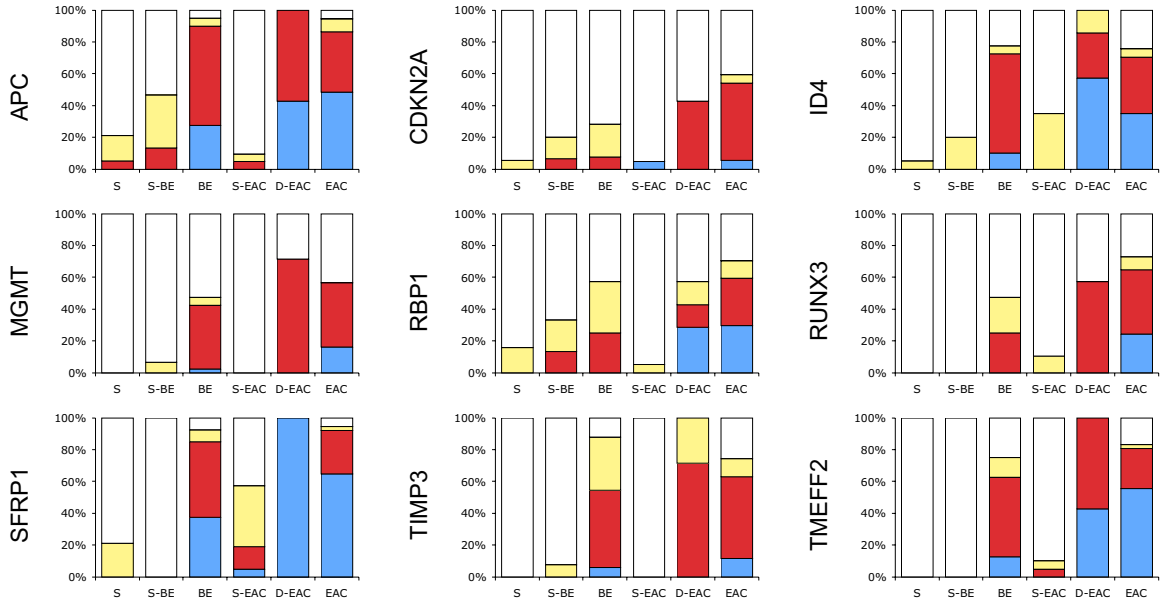

Figure 2
Methylation frequency and methylation extent in esophageal tissues. Methylation of each gene was measured in multiple biopsies of squamous mucosa (S, n = 19) from seven patients without BE, single biopsies of squamous mucosa (S-BE, n = 16) and multiple biopsies of columnar mucosa (BE, n = 40) from 18 patients with BE, single biopsies of squamous mucosa (S-EAC, n = 21), high grade dysplastic Barrett's (D-EAC, n = 7) and tumor (EAC, n = 37) from 38 patients with EAC. The methylation was graded as unmethylated (white), methylated 1 (yellow), 2 (red), or 3 (blue), as described in the Materials and Methods.

between BE or D-EAC and S or S-BE), RBP1 (BE vs S-BE and D-EAC vs S or S-BE) and SFRP1 (D-EAC vs S-EAC). There were no differences between BE and D-EAC. The methylation frequency for all nine genes was significantly higher in EAC than any squamous tissue. There were no differences between EAC and D-EAC. There were no differences between EAC and BE, except for CDKN2A and RUNX3, which were significantly higher in EAC than BE ($P = 0.0104$ and 0.0358 respectively).

The methylation extent, which reflects the combination of both the number of methylated alleles and the density of methylation of those alleles, was graded on a scale of 1–3, with 1 being low and 3 being high methylation (Figure 2). The methylation extent in EAC was significantly greater than in BE for all genes, except for APC, MGMT and TIMP3. There were no significant differences in methylation extent between EAC and D-EAC.

The number of genes methylated in each specimen is shown in Figure 3. Significantly more genes were methylated in specimens of BE, D-EAC and EAC, compared to any of the squamous tissues ($P < 0.01$ for each comparison). There were no significant differences between any of the squamous tissues, nor between BE, D-EAC and EAC.

Methylation and gene expression in esophageal tissues

We examined the relationship between methylation and gene expression in tissues from patients with EAC, from which sufficient RNA was available (Figure 4). The expression of APC, CDKN2A, MGMT, RUNX3, TIMP3 and TMEFF2 did not differ significantly between EAC, D-EAC and S-EAC. There was significantly less expression of ID4, RBP1 and SFRP1 in EAC compared to S-EAC, and of ID4 and SFRP1 in EAC compared to D-EAC (Figure. 4a).

We then looked for an association between the presence of methylation and a reduction in gene expression within the EAC tissues. There was no significant difference in APC, CDKN2A, ID4, RBP1, RUNX3 and TIMP3 expression levels between methylated and unmethylated tumors. There were too few SFRP1 unmethylated tissues for this analysis. There was significantly less expression of MGMT in methylated tumors compared to unmethylated tumors (Figure. 4b). Furthermore, the expression of MGMT did not differ between unmethylated squamous and unmethylated tumor tissues, suggesting that the observed reduction in EAC was primarily due to methylation, not phenotypic differences.

Discussion

Methylation is an important mechanism for the silencing of genes in the development of cancer. We compared the frequency and extent of methylation of APC, CDKN2A, ID4, MGMT, RBP1, RUNX3, SFRP1, TIMP3, and TMEFF2 in esophageal adenocarcinoma (EAC), high grade dysplastic Barrett's from patients with adenocarcinoma (D-EAC), metaplastic Barrett's from patients without dysplasia or adenocarcinoma (BE), and histologically normal esophageal squamous epithelium. All nine genes showed an increase in frequency of methylation in EAC compared to squamous epithelium. We found that for seven of these genes there was no difference in the frequency of methylation between the BE and EAC. For only CDKN2A and RUNX3 was there a significant increase in frequency of methylation in EAC compared to BE. The extent of methylation in EAC was significantly greater than in BE for six of the nine genes (CDKN2A, ID4, RBP1, RUNX3, SFRP1 and TMEFF2) but was not different to D-EAC for any gene. This suggests that methylation of these genes can occur early in the development of Barrett's metaplasia, and for only some of these genes does the extent of methylation increases during the progression to cancer.

In the technique we used to analyse methylation, bisulphite modified DNA is amplified using primers and conditions that do not discriminate between methylated and unmethylated sequences. Amplification is therefore independent of the presence or extent of methylation within the region being assessed. Unlike two common methods for measuring methylation, Methylation-Specific PCR [7] or MethyLight [8], the method we used will detect small amounts of methylation anywhere along the target sequence. Thus the methylation frequency we measure may differ from that reported by others. The methylation extent reflects a combination of the percentage of the alleles that are methylated, and the density of methylation on each allele. To determine if there was a relationship between the methylation measured in our assays and transcriptional silencing we measured gene expression in esophageal cell lines treated with the demethylating drug aza-dC. There was an increase in expression of ID4, MGMT, RBP1, RUNX3, SFRP1 and TIMP3, suggesting an association between methylation and silencing of expression for these genes. A relationship could not be determined for APC or CDKN2A, which were unmethylated, or TMEFF2, for which we could not measure any transcript in either cell line. For these three genes we have shown using cell lines established from other cancers that methylation of the regions we amplified are associated with gene silencing (unpublished observations). In our EAC tissues only methylation of MGMT was associated with a significant reduction in mRNA expression. The lack of a significant difference in gene expression for the other genes may be due to methylation of only one allele, or the presence of unmethylated cells in the tissue, such as clonal variants of the tumor, or stromal or infiltrating cells. Methylation in the cancer tissues was always greater than in the squamous epithelium of patients without cancer, so even if

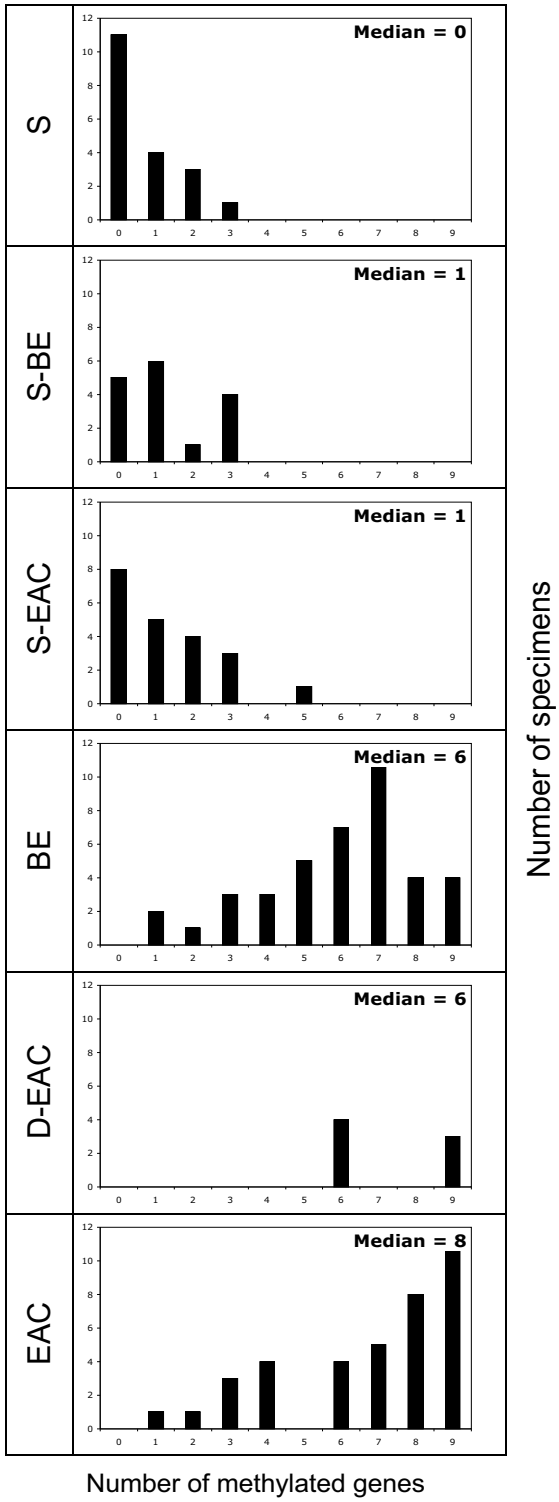

Figure 3
Number of methylated genes in esophageal tissues. The number of methylated genes in specimens of squamous mucosa from patients without BE (S), squamous mucosa (S-BE) and columnar mucosa (BE) from patients with BE, and squamous mucosa (S-EAC), high grade dysplastic Barrett's (D-EAC) and adenocarcinoma (EAC) from 38 patients with EAC.

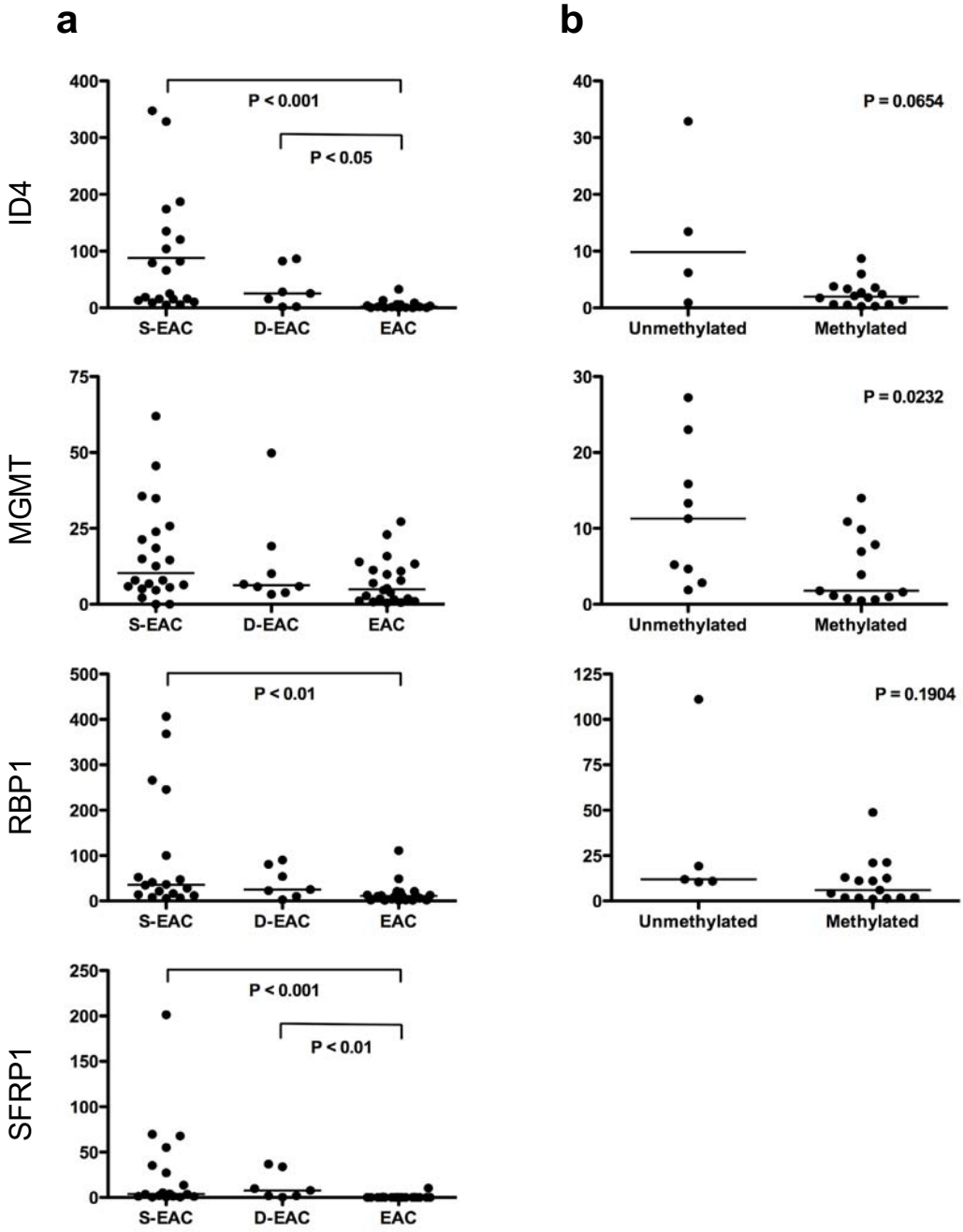

Figure 4

Expression and methylation in esophageal tissues. (a) Normalised mRNA expression of ID4, MGMT, RBP1, and SFRP1 in squamous (S-EAC), high grade dysplastic Barrett's (D-EAC) or tumor (EAC) tissues from patients with EAC. (b) Normalised expression of ID4, MGMT, and RBP1 in tumors unmethylated or methylated for the gene. The horizontal bar represents the median expression.

methylation did not result in gene silencing, it was still associated with cancer.

The processes involved in the transition from squamous epithelium to Barrett's metaplasia are unknown, although chronic gastro-esophageal reflux is widely believed to be the main trigger, with approximately 10% of gastro-esophageal reflux patients having BE [9]. It is accepted that BE is a pre-malignant condition, which over time, as a result of the accumulation of multiple genetic and epigenetic modifications, can progress through dysplasia to EAC in a percentage of patients. A large number of such molecular changes have been reported in BE and EAC, although the sequence of these changes appear not to be as predictable as in some other cancers such as in the colon [6].

It is not clear from the literature when aberrant DNA methylation occurs during the progression to EAC. There may be differences in molecular profile of BE tissue obtained from cancer resection specimens, compared to BE tissue obtained from patients with metaplasia only. Our samples of BE were obtained from patients with no detectable dysplasia or cancer, and were compared to cancer tissue from different patients. High levels of methylation in such BE specimens have been reported by others, such as methylation of SFRP1 being as frequent in BE (81 – 100% of samples) as it is in EAC (93 – 100%) [10,11]. In several studies high levels of methylation have been found in BE for many genes, but a few have reported low methylation in BE and then high in EAC tissues. Eads et al examined a single biopsy of metaplastic tissue from six patients with BE without associated EAC and reported methylation of APC, MGMT and TIMP3 in BE and also in cancers from other patients, but they did not compare the amounts of methylation in each [12]. They also reported four genes, CDKN2A, ESR1 and MYOD1 and CALCA as unmethylated in these BE patients, and methylated in the cancers. Schulmann et al, using MethyLight, measured methylation in 93 biopsies of BE from an undisclosed number of patients [13]. Methylation was as frequent in samples of EAC (n = 77) as in BE for APC, MGMT, RBP1 and TIMP3, but was significantly greater in EAC compared to BE for CDKN2A, RUNX3 and TMEFF2. Furthermore, the methylation level, their measure of the number of extensively methylated or hypermethylated molecules in the specimen, increased significantly from BE to dysplasia for these three genes. Clement reported that APC, TIMP3 and TERT were significantly more methylated in BE of patients who progressed to EAC compared to patients with BE which had not progressed during a follow-up of between four to ten years [10]. Of the nine genes which we studied, only methylation of CDKN2A and RUNX3 appeared to increase in frequency in the progression from BE to cancer, and only six of the nine genes increased in the extent of methylation.

The underlying mechanisms that cause aberrant DNA methylation in Barrett's metaplasia and cancer are unknown. Our study confirms that aberrant methylation of multiple genes is an early event, and mostly occurs independently of dysplasia or EAC. Thus, we speculate that methylation of some genes occurs at or shortly after the transition from squamous to columnar metaplasia. To date there are no longitudinal studies investigating methylation changes during metaplasia.

This is the first report of ID4 methylation in BE or EAC. The other genes in this study have previously been reported to be methylated in esophageal disease. ID4 is a member of the inhibitor of DNA binding (ID) family of proteins that inhibit the binding of basic helix-loop helix transcription factors to DNA. It regulates the transcription of genes important in development and differentiation, and is a candidate tumor suppressor gene [14,15]. Methylation of ID4 has been reported in and associated with the silencing of gene transcription and loss of protein expression in lymphoma [16], and gastric [17], colorectal [18] and breast carcinoma [19,20]. Methylation of ID4 correlated with increased risk of lymph node metastasis in T1 stage breast cancer [20], and histopathological tumor grade and poorer prognosis in colorectal carcinoma [18]. Our findings that ID4 is frequently methylated in BE and EAC but not in the normal squamous mucosa and that demethylation of cancer cell lines significantly increases expression, suggests loss of ID4 expression is important in the neoplastic progression of BE, supporting its role as a tumor suppressor.

DNA methylation is not the only abnormality reported in the genome of metaplastic Barrett's epithelium of patients without dysplasia or EAC. Using gene expression microarrays, Wang et al reported that the gene expression profile of BE more closely resembled EAC than esophageal squamous epithelium. Rather than BE being a benign tissue, they concluded that it was biologically closer to cancer than to normal squamous epithelium [21]. Genomic loss, chromosomal gains and amplifications, mutations, and aneuploidy are observed in BE [22,23], and loss of heterozygosity of CDKN2A is reported in 47 – 75% of patients with BE, in the absence of dysplasia or EAC [24-26]. In metaplastic epithelium from patients with dysplasia or EAC, 5q (APC), 13q (RB1), 17p (TP53), and 18q (DCC) are commonly lost, whilst 8q, 8p, and 6p are frequently gained [27-29]. In contrast, mutations in BE are relatively uncommon. Mutations in CDKN2A have been reported in up to 7% of patients with metaplasia [24,30], but most reports suggest that mutations are absent in BE from patients without dysplasia or EAC [31].

In this study of methylation in esophageal disease we have measured little methylation in any esophageal squamous epithelium, but in BE tissues there was significant methylation which for all but two of the nine genes examined did not increase in frequency in the progression from BE to EAC. We have also reported methylation of ID4 for the first time in BE or EAC. Together these findings confirm that BE is a precancerous tissue, and that aberrant promoter methylation occurs early in metaplasia before histological evidence of progression towards cancer, and that metaplastic BE is nearly as abnormal epigenetically as EAC.

Methods

Patient samples

Single samples of primary esophageal adenocarcinoma (EAC, n = 37), dysplastic Barrett's (D-EAC, n = 7) and histologically normal squamous mucosa from the proximal resection margin (S-EAC, n = 21) from 38 patients with EAC were collected into liquid nitrogen or into RNAlater (Ambion, Austin, TX, USA). Multiple biopsies every 2 cm from within circumferential columnar lined esophagus (BE, n = 40) and a single biopsy of squamous mucosa proximal to the squamo-columnar junction (S-BE, n = 16) from 18 patients with BE were collected into RNAlater. The presence of goblet cells in at least one biopsy from the columnar lined esophagus was confirmed in all patients with BE. Up to three biopsies of squamous mucosa (S, n = 19) from each of seven patients without a known history of BE, but who had undergone a fundoplication for gastro-esophageal reflux disease more than five years earlier, were collected into RNAlater. Clinicopathological details of all patients are summarised in Table 2, and details of each patient are described in Additional Files Tables S1 – S3. The study complied with the appropriate institutional guidelines.

Demethylation of cell lines with 5-aza-2'-deoxycytidine

To study the effects of demethylation, triplicate cultures of the esophageal cancer cell lines OE33 and TE7 [32] were grown in RPMI 1640 supplemented with 10% foetal bovine serum at 37°C in 5% CO_2. The OE33 cell line was established from a Barrett's associated adenocarcinoma of the lower esophagus, and the TE7 is thought to be derived from a squamous cell carcinoma of the esophagus [33]. Cells were seeded into flasks and cultured for 24 hours before they were treated with either 1 μmol/L 5-aza-2'-deoxycytidine (aza-dC, Sigma-Aldrich, Saint Louis, MO) or vehicle (0.0027% v/v final concentration acetic acid). Following a further 72 hours incubation, time for the cells to undergo at least two cycles of division [34], the medium was replaced with fresh medium not containing either aza-dC or vehicle, and the cells incubated for a further 24 hours before harvesting.

Isolation of RNA and DNA from cell lines and patient samples

Tissues were disrupted using either disposable pestles (Edwards Instruments, Narellan, NSW, Australia) or TissueLyser with 5 mm Stainless Steel Beads (Qiagen, Hilden, Germany). RNA and DNA were isolated from cell lines and tissues from patients with EAC using Trizol (Invitrogen, Carlsbad, CA). RNA and DNA were isolated from all other biopsies using either the RNA/DNA Kit or the All-Prep DNA/RNA Mini Kit (Qiagen).

Methylation analysis

Bisulphite modified DNA was prepared as described previously [34,35], and amplified using primer sets which did not discriminate between methylated and unmethylated sequences. The PCR primers (GeneWorks, Thebarton, SA, Australia) and conditions were specific for bisulphite modified DNA, and did not amplify unmodified DNA. All methylation analysis PCRs were performed using the QuantiTect SYBR Green PCR Kit (Qiagen) in a final volume of 15 μL, containing 1 μL of bisulphite modified DNA and a final concentration of 0.5 μmol/L of each forward and reverse primer. Bisulphite modified lymphocyte DNA, CpG methyltransferase (M.SssI) (New England Biolabs Inc., Ipswich, MA) treated lymphocyte DNA and unmodified DNA were included in each PCR run and served as unmethylated, methylated and negative controls respectively. Reactions were incubated in a Rotor-Gene 3000 (RG-3000) (Corbett Life Science, Sydney, NSW, Australia) at 95°C for 15 minutes, then 45 cycles of 95°C for 30 seconds and 60 seconds at the annealing temperature specified in Table 2, followed by a final extension of 72°C for 4 minutes. At the end of the amplification cycle the PCR products were melted by increasing the temperature from 60 to 95°C, rising 0.5 or 1°C at each step, waiting 30 seconds on the first step then 5 seconds for each step thereafter. Data was collected and analysed using the Melt Curve Analysis function of the RG-3000 Application Software v6 (Corbett Life Science) which converts the raw flu-

Table 2: Demographic characteristics of patients

	EAC	BE	Without BE or EAC
Number of patients	38	18	7
M:F	34:4	18:4	2:7
Median age, yr (range)	64 (51 – 78)	56 (38 – 71)	60 (37 – 76)

Table 3: The primer sequences and annealing temperatures for qRT-PCR

Gene	Forward primer	Reverse primer	Annealing temperature (°C)
APC	TGGAGAACTCAAATCTTCGACA	CAATCTGTCCAGAAGAAGCCATA	62
CDKN2A	GAGGCCGATCCAGGTCAT	CCAGCGTGTCCAGGAAG	62
HMBS	ACATGCCCTGGAGAAGAATG	TTGGGTGAAAGACAACAGCA	57
ID4	CCGAGCCAGGAGCACTAGAG	CTTGGAATGACGAATGAAAACG	60
MGMT	TGGAGCTGTCTGGTTGTGAG	GCTGGTGGAAATAGGCATTC	60
RBP1	AGGCATAGATGACCGCAAG	CTCATCACCCTCGATCCAC	62
RUNX3	GCAGGCAATGACGAGAACTA	CAGTGATGGTCAGGGTGAAA	57
SFRP1	TTGAGGAGAGCACCCTAGGC	TGTGTATCTGCTGGCAACAGG	60
TIMP3	CCAGGACGCCTTCTGCAAC	CCTCCTTTACCAGCTTCTTCCC	60
TMEFF2	CAATGGGGAGAGCTACCAGA	TGGACTCCATCTCCAGATCC	62

Table 4: Table S1 – Demographic details of patients with EAC.

Patient	Gender	Age(yr)	Differentiation[a]	S-EAC[b]	D-EAC[b]	EAC[b]
0226	M	53	P	+	-	+
0227	M	58	M	+	+	-
0230	M	77	M-P	+	+	+
0235	M	61	M	+	-	+
0246	F	62	W	+	+	+
0250	M	68	M-P	+	+	+
0253	M	77	M-P	+	-	+
0292	M	55	M	+	-	+
0306	M	68	P	+	-	+
0307	M	58	M	+	-	+
0316	M	69	P	+	+	+
0329	M	67	P	-	-	+
0339	F	63	P	+	-	+
0343	F	62	P	-	-	+
0347	M	56	P	+	-	+
0355	F	62	P	+	-	+
0369	M	75	M	+	-	+
0374	M	65	M	+	-	+
0375	M	72	M	+	-	+
0380	M	57	M	+	-	+
0385	M	70	M	+	-	+
0406	F	53	M	+	-	+
0410	M	62	M	+	-	+
0415	M	56	P	-	+	+
0417	M	73	P	-	+	+
40320	M	61	M	-	-	+
40323	M	60	P	-	-	+
40325	M	61	M-P	-	-	+
40328	M	65	M	-	-	+
40331	M	64	P	-	-	+
40337	M	70	P	-	-	+
40338	M	71	W	-	-	+
40341	M	78	P	-	-	+
40345	M	60	M	-	-	+
40355	M	65	M-P	-	-	+
40357	M	65	M-P	-	-	+
53145	M	51	M	-	-	+
54017	M	76	P	-	-	+

[a]W, well; M, moderate; P, poor
[b]+ tissue available; – no tissue available

Table 5: Table S2 – Demographic details of patients with BE

Patient No.	Gender	Age (yr)	S-BE[a]	BE[a]	No. of BE biopsies
1	M	71	+	+	2
2	M	51	+	+	3
3	F	70	+	+	2
4	M	65	+	+	2
5	M	61	+	+	2
6	M	43	+	+	2
7	M	38	+	+	5
8	M	54	+	+	1
9	M	53	-	+	1
10	M	70	+	+	2
11	M	64	+	+	3
12	M	68	+	+	2
13	F	54	-	+	2
14	M	52	+	+	3
15	F	50	+	+	2
16	M	49	+	+	2
17	M	58	+	+	2
18	F	58	+	+	2

[a]+ tissue available; – no tissue available

orescence data to melt curves by plotting the negative first derivative of the fluorescence with respect to temperature (-dF/dT), against temperature. The melt curve of the sample was compared to those of the unmethylated and methylated controls. A sample was considered methylated when there was a visible shift to the right of the unmethylated melt curve. The methylation extent, a function of both the number of alleles which are methylated and the density of methylation in each allele, was graded as 0 (unmethylated), 1 (low), 2 (moderate), or 3 (high methylation) according to the degree of the shift [35]. Briefly, a curve which was almost identical to the methylated control was scored as 3, a curve whose melting temperature was closer to methylated control than the unmethylated control was scored as 2, a curve which was almost identical to unmethylated control was scored 0 and the rest were scored as 1. All assessment was undertaken independently by two investigators (E.S. and P.A.D.), and if their opinions differed, consensus was reached by discussion.

Table 6: Table S3 – Demographic details of patients without BE or EAC

Patient No.	Gender	Age (yr)	No. of biopsies
1	M	49	3
2	M	76	3
3	F	66	3
4	M	37	3
5	M	37	2
6	F	60	3
7	M	69	2

Measurement of gene expression by quantitative real-time reverse-transcription PCR

To measure gene expression, cDNA was synthesised using SuperScript II (Invitrogen) from 2 μg of RNA which had been treated with the TURBO DNA-free Kit (Ambion). Quantitative real-time reverse-transcription PCR (qRT-PCR) was performed using the QuantiTect SYBR Green PCR Kit in a final volume of 10 μL, containing 1 μL of 1/5 diluted cDNA and a final concentration of 0.5 μmol/L of each forward and reverse primer. Triplicate reactions were incubated in an RG-3000 at 95°C for 15 minutes, then 45 cycles of 95°C for 15 seconds and 45 seconds at the annealing temperature specified in Table 3, followed by a final extension of 72°C for 4 minutes. Data was collected and analysed using the RG-3000 Application Software. Threshold cycle Ct values were determined on auto-threshold settings with reference to a standard dilution curve. All mRNA quantitation data was normalised to hydroxymethylbilane synthase (HMBS) [34]. Following the PCR, the products were melted to confirm specificity, and electrophoresed on 1.5% (w/v) agarose gels stained with ethidium bromide to confirm expected product size.

Statistical analyses

Statistics were performed using GraphPad InStat version 3.0a or Prism version 5.0a for Macintosh (GraphPad Software, San Diego California USA, http://www.graphpad.com). Gene expression in cell lines treated with either aza-dC or vehicle was compared using Student's t-test. Methylation frequency in tissues was compared using Fisher's exact test. The number of methylated genes in a specimen and gene expression was compared using Kruskal-Wallis with Dunn's post-test. Methylation extent and gene expression in unmethylated and methylated tissues was compared using the Mann-Whitney test. All statistics were considered significant when the two tailed $P \leq 0.05$.

Conclusion

We have measured methylation of genes in BE and EAC and found significant methylation in metaplastic BE, which for seven of the nine genes studied did not differ in frequency from that found in EAC. This study provides important confirmatory evidence to support the concept that BE is a highly abnormal tissue, more similar to cancer tissue than to normal squamous epithelium.

Competing interests

The authors declare that they have no competing interests.

Additional files

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CHAPTER 8: THE EFFECT OF LONG TERM CONTROL OF REFLUX BY FUNDOPLICATION ON ABERRANT DNA METHYLATION IN PATIENTS WITH BARRETT'S ESOPHAGUS

Eric Smith[1], John J. Kelly[1], Andrew R. Ruszkiewicz[2], Thomas Sullivan[3], Paul A. Drew[4], Glyn G. Jamieson[1]

[1]Discipline of Surgery, The University of Adelaide, Adelaide, 5005, Australia;
[2]Surgical Pathology, SA Pathology, Adelaide, 5000, Australia;
[3]Discipline of Public Health, The University of Adelaide, Adelaide, 5005, Australia;
[4]School of Nursing and Midwifery, Flinders University, Bedford Park, 5042, Australia.

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Mini-Abstract

Patients with Barrett's esophagus were assessed five years following fundoplication. Subjects with an intact fundoplication had less DNA methylation than subjects with a failed fundoplication or who had never had surgery. Reflux is associated with methylation and control of reflux reduces genomic changes associated with cancer.

Structured Abstract

Objective:

We investigated the relationship between reflux and aberrant DNA methylation, comparing methylation in the columnar epithelium following successful fundoplication to that in subjects with a failed fundoplication.

Summary Background Data:

Gastro-esophageal reflux is the main risk factor for Barrett's esophagus and adenocarcinoma. In these diseases there is a high level of DNA methylation.

Methods:

We enrolled 41 patients with Barrett's esophagus and a fundoplication at least five years earlier for a 24-hour pH study, endoscopy and collection of biopsies. Biopsies were also obtained from 17 Barrett's esophagus subjects who had not undergone esophageal surgery.

Results:

At the time of the study 31 subjects were pH normal, 10 abnormal. Columnar biopsies were collected from 21 of the pH normal and nine of the pH abnormal subjects, and all no surgery subjects. Complete regression of columnar mucosa was seen in seven pH normal subjects and one pH abnormal. The length of Barrett's esophagus did not differ between groups pre-operatively, but was significantly less at the time of the study in the pH normal compared to pH abnormal or no surgery groups. There were significantly fewer genes methylated in the pH normal than the pH abnormal or no surgery groups, which did not differ from each other. The number of methylated genes correlated with increased reflux and increased Barrett's esophagus length.

Conclusions:

Fundoplication which reduces reflux to normal levels can lead to regression of the columnar mucosa. Reflux is associated with aberrant DNA methylation and control of reflux reduces deleterious genomic changes associated with cancer.

INTRODUCTION

Gastro-esophageal reflux is extremely common in the West, and if untreated can lead to esophagitis or the replacement of the squamous epithelium with a columnar lined epithelium. Barrett's esophagus is diagnosed when goblet cells are present within this columnar lined epithelium. It is found in up to 1.5% of the general population, and in up to 12% of patients who are investigated for chronic reflux symptoms.[1]

The clinical importance of Barrett's esophagus is that it is a major risk factor for esophageal adenocarcinoma.[2] While estimates vary, a recent meta-analysis reported that approximately 0.4% of patients with Barrett's esophagus will develop adenocarcinoma each year.[3] Significantly, the incidence of adenocarcinoma in the West is increasing rapidly, in contrast to esophageal squamous cell carcinoma which is relatively static.[4]

The progression from Barrett's esophagus to cancer is generally thought to proceed via the histological stages of low-grade and high-grade dysplasia, which mirrors the accumulation of genetic abnormalities. For example, reduced CDKN2A (p16) expression, seen in Barrett's metaplastic tissue[5, 6], followed by altered p53 expression, generally reported in dysplastic tissue, have been associated with progression.[7, 8] Up- and down-regulation of many other genes has been described at different stages of the progression from metaplasia to cancer.[9] One mechanism for the down regulation of gene expression is DNA methylation, which can result in the silencing of critical genes such as tumour suppressor genes.

There is evidence for the gastric (acid) and duodenal (bile) components of the reflux, either independently or jointly, being responsible for the tissue and genomic changes.[10-12] In this study we enrolled subjects who had a fundoplication more than 5 years before and who had pre-operative endoscopic evidence and histological confirmation of Barrett's esophagus. A fundoplication is usually more effective than the normal sphincter, and is generally associated with very low levels of both acid and bile reflux.[13, 14] If a fundoplication fails reflux will resume. We investigated the relationship between reflux, as measured by a 24-hour pH study, and aberrant DNA methylation, by comparing the methylation in the columnar epithelium of these subjects who had normal levels of reflux, to that in subjects with increased reflux.

METHODS

Protocol

All subjects who had undergone laparoscopic fundoplication for gastro-esophageal reflux disease at the Royal Adelaide Hospital, Flinders Medical Centre or associated private hospitals between October 1991 and March 2000, and who also had a preoperative diagnosis of Barrett's esophagus (confirmed by histology), were identified from our prospective database. Of the 82 subjects who met the criteria, 41 agreed to return for a 24-hour pH study, a manometric study, endoscopy and collection of tissue biopsies. Biopsies were also obtained from 17 subjects who had a histological diagnosis of Barrett's esophagus, and no history of esophageal surgery (no surgery group). The protocol for this study was approved by the Research Ethics Committee of the Royal Adelaide Hospital.

All subjects underwent a 24-hour pH study and manometry, as previously described.[15] Abnormal reflux was defined as a pH < 4 for more than 4% of the total study time, and normal reflux as a pH < 4 for less than 4% of the total time.

Biopsies were collected for histology and for DNA methylation analysis at endoscopy. Four-quadrant biopsies from within circumferential, and random biopsies from within non-circumferential, columnar lined esophagus were collected at 2 cm intervals from all subjects for histology. Subjects were diagnosed as having Barrett's esophagus if goblet cells were present in at least one of these biopsies from the columnar lined esophagus.

Additional biopsies for DNA methylation analysis were collected into RNAlater (Ambion, Austin, TX, USA) or snap frozen fresh with liquid nitrogen. One biopsy from within circumferential columnar lined esophagus and one from squamous mucosa at least 2 cm proximal to the squamo-columnar junction were collected from the fundoplication subjects. In some subjects with longer length Barrett's more than one biopsy was collected, separated by at least 2 cm along the length of the columnar lined epithelium. Biopsies were collected similarly from within circumferential columnar lined esophagus of the subjects in the no surgery group.

Isolation and Bisulfite Modification of DNA

Biopsies were disrupted using either disposable pestles (Edwards Instruments, Narellan, NSW, Australia) or 5 mm stainless steel beads in a TissueLyser (Qiagen, Hilden, Germany). The DNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. DNA was bisulfite modified as described previously, except that the bisulfite modified DNA was purified using an UltraClean PCR Clean-up DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA, USA), and was resuspended in 100 μL ultra pure water (Fisher Biotec, Wembley, WA, Australia).[16]

Methylation Analysis

To measure methylation, bisulfite modified DNA was amplified and melted as described previously.[17] The polymerase chain reaction (PCR) primer sets and conditions (Table 1) used did not discriminate between methylated and unmethylated DNA, and did not amplify unmodified DNA. Bisulfite modified lymphocyte DNA (unmethylated reference), M.SssI CpG methyltransferase (New England Biolabs Inc., Ipswich, MA) treated lymphocyte DNA (methylated reference) and unmodified DNA (negative control) was included in each PCR. A Rotor-Gene 3000 was used for the PCR amplification and melting of the PCR product, and the melt data was analysed using the Melt Curve Analysis function of the Application Software v6 (Corbett Life Science, Sydney, NSW, Australia). The -dF/dT plot of the sample was compared to the unmethylated and methylated references. A sample was considered methylated when its plot was shifted to the right of the unmethylated reference plot.[16]

Statistical Analyses

Statistical analyses were performed using GraphPad InStat version 3.0a for Macintosh (GraphPad Software, San Diego California USA) or SAS Version 9.2 (SAS Institute Inc., Cary, NC, USA). Normally distributed data were summarised using means and ranges and were compared between reflux groups using unpaired t-tests. Non-normally distributed continuous data were summarised using medians and ranges and were compared between reflux groups using Mann Whitney tests or within groups using Wilcoxon matched-pairs signed-ranks tests. Categorical data were compared between reflux groups using Fisher's exact tests. Linear mixed effects models incorporating a random subject effect were used to assess the relationship between the total number of methylated genes in a biopsy and either reflux group or Barrett's esophagus length. This analysis accounted for the inclusion of multiple biopsies from some subjects. Logistic GEE regression models were used to assess

the relationship between methylation of individual genes and either reflux group or Barrett's esophagus length. All statistics were considered significant when the two tailed $P \leq .05$.

RESULTS

Clinical Characteristics of all Fundoplication Subjects

Forty one subjects with Barrett's esophagus at the time of a fundoplication were recruited to this study. Their mean age was 55.5 years (95% CI 51.4-59.6 *y*; range 34-88), and the mean time interval between surgery and this study was 99 months (95% CI 92.6-105.3 mo; range 65-151 mo). As a group, the median length of their Barrett's esophagus at the time of the study (1.0 cm; range 0-12.0) was significantly less than before the fundoplication (4.0 cm; range 1.0-13.0) ($P < .0001$).

At the time of the study 31 of the fundoplication subjects had a pH < 4 for less than 4% of the total time (pH normal) and 10 a pH < 4 for more than 4% of the total time (pH abnormal), as determined by a 24-hour pH study (Figure 1). The ratio of males to females, patient age at time of the study, time interval between surgery and the study, and the pre-operative length of Barrett's esophagus, did not differ significantly between the pH normal and the pH abnormal subjects (Table 2). There was no dysplasia seen in any of the biopsies. The length of Barrett's esophagus at the time of the study was significantly less in the pH normal than the pH abnormal subjects (0.5 vs 2.5 cm; $P = .0404$). There was no significant difference in the length of Barrett's esophagus at the time of the study compared to the pre-operative length in the pH abnormal subjects (2.5 vs 4.0 cm), but there was a significant reduction in the pH normal subjects (0.5 vs 4.0 cm; $P < .0001$).

Clinical Characteristics of Biopsied Subjects

Biopsies of columnar mucosa were collected from 21 of the 31 pH normal and nine of the 10 pH abnormal subjects at endoscopy (subjects with columnar biopsies, Table 2). Columnar biopsies could not be collected from one pH abnormal and seven pH normal subjects because the columnar lined esophagus had completely regressed, nor from three pH normal subjects because the amount of columnar lined esophagus was too limited to biopsy confidently. The ratio of males to females, age at the time of the study, and the time interval between surgery and the study did not differ between those subjects from whom columnar biopsies were

obtained and those from whom they were not obtained. The pre-operative length of Barrett's esophagus did not differ significantly between the pH normal and the pH abnormal subjects from whom columnar biopsies were collected. There was a significant difference in the length of Barrett's esophagus at the time of the study compared to the pre-operative length in the pH normal subjects from whom columnar biopsies were collected (1.0 vs 5.0 cm; $P < .0001$), but no difference in the pH abnormal subjects (3.0 vs 5.0 cm).

Columnar biopsies were also taken from 17 subjects with Barrett's esophagus who had never had any esophageal surgery (no surgery group). There was no significant difference between the no surgery and either the pH normal or pH abnormal subjects in the ratio of males to females or their age at the time of the study (Table 2). The length of Barrett's esophagus in the no surgery subjects did not differ significantly from the pre-operative length in the pH normal or pH abnormal subjects. At the time of the study the length of Barrett's esophagus in the no surgery group did not differ from the pH abnormal subjects, but was significantly longer than in the pH normal subjects (4.0 vs 1.0 cm; $P < .0026$).

Methylation Analysis

The results in Figure 2 show the number of methylated genes in each biopsy. Methylation was measured for each of the nine genes in each biopsy, with multiple biopsies being assessed from some patients. There was no significant difference in the number of genes methylated between the squamous biopsies from pH normal and pH abnormal subjects (adjusted mean number of methylated genes per biopsy were .49 and .30 respectively). The number of genes methylated in the squamous biopsies was significantly less than in the columnar biopsies from the pH normal, pH abnormal or no surgery subjects (adjusted means: 3.65, 5.32 and 6.04 respectively) ($P < .0001$ for all columnar/squamous comparisons).

There were significantly fewer genes methylated in the columnar biopsies from pH normal compared to pH abnormal subjects ($P = .0067$). There was no statistical difference in methylation between the pH abnormal and no surgery subjects, but there were significantly fewer methylated genes in the columnar biopsies from pH normal compared to the no surgery subjects ($P < .0001$). Methylation of TIMP3 was significantly more common in columnar biopsies from reflux abnormal than reflux normal subjects (OR = 5.2493, $P = .0228$), but there was no significant difference for any of the other genes analysed (Table 3; too few biopsies were methylated for CDKN2A for this analysis).

There was a significant association between the length of Barrett's esophagus and the number of methylated genes in each columnar biopsy from the reflux abnormal subjects (Figure 3; *P* = .0004). Methylation of ID4, RBP1, RUNX3, TIMP3 and TMEFF2, but not APC, MGMT or SFRP1, was significantly associated with the length of Barrett's esophagus (Table 4; too few biopsies were methylated for CDKN2A for this analysis).

DISCUSSION

We studied subjects who had an endoscopic and histologic diagnosis of Barrett's esophagus and underwent a fundoplication at least five years earlier, and objectively measured reflux by a 24-hour pH study to determine if the wrap remained functional. We measured DNA methylation in biopsies from the columnar and the squamous mucosa. We found that in subjects with persisting reflux DNA methylation remained at levels measured in those who had never had surgery, while in subjects whose reflux was in the normal range the DNA methylation was significantly less.

Of the 41 subjects enrolled in the study, the fundoplication was intact and pH studies were in the normal range for 31 subjects (pH normal), and abnormal for 10 subjects (pH abnormal). We found a significant reduction in the length of the Barrett's esophagus since the operation in the pH normal group, but not the pH abnormal group. Seven pH normal and one pH abnormal subjects had complete regression of their Barrett's esophagus in the time since their fundoplication. These results are consistent with other reports of endoscopic and histological regression of Barrett's esophagus following fundoplication.[18]

Methylation of DNA is a genomic change which is important in the development and progression of many cancers. In humans this involves the methylation of cytosines, and occurs at CpG dinucleotides. Regions of relatively high CpG content, termed CpG islands, are found in the promoter region of up to 70% of genes, and are generally unmethylated. Aberrant methylation of these normally unmethylated CpG islands is a common cause of altered gene expression in premalignant and malignant tissues. Its importance in the pathogenesis of esophageal adenocarcinoma is indicated by reports that the frequency of methylation in non-dysplastic Barrett's esophagus is similar to adenocarcinoma and

significantly higher than in normal squamous epithelium[16], and that methylation of specific genes in Barrett's esophagus predicts progression to high-grade dysplasia and adenocarcinoma.[19-22] In this study we show that DNA methylation correlates with known risk factors for the development of adenocarcinoma in subjects with Barrett's esophagus: length of the columnar lined epithelium and the presence of abnormal reflux.[2] There was no increase in methylation in the squamous epithelium, only the columnar lined epithelium which is where the cancers arise. As well, there was no significant difference in the number of methylated genes between squamous epithelium from subjects with normal compared to abnormal reflux.

We measured DNA methylation in the promoter region of nine genes by a technique which is sensitive and detects any pattern of methylation along the target sequence.[17] Methylation in the regions we analysed results in transcriptional silencing, is uncommon in squamous epithelium, and is common in Barrett's esophagus and adenocarcinoma, further supporting a role in the progression to cancer.[16]

We found no difference in the number of genes methylated between the pH abnormal and the no surgery subjects, but there were significantly fewer genes methylated in the pH normal group. Thus abnormal reflux correlated with increased methylation. There was no indication of selection bias in our groups. There was no difference in the proportion of males to females, age, pre-operative Barrett's esophagus length, and length of time after surgery between pH normal and pH abnormal groups. The subgroups from whom biopsies were available did not differ in any of these parameters either. There was also no difference in the demographic or clinical parameters between the Barrett's esophagus subjects who had never had surgery and pH abnormal subjects. In particular, the length of Barrett's esophagus in the no surgery group did not differ significantly from the pre-operative length of either the pH normal or abnormal subjects

The reason for this difference in methylation might be that a reduction in reflux since the fundoplication resulted in a decreased methylation in the pH normal subjects, or it might be that continuing reflux increased the methylation in the pH abnormal subjects (compared to the levels before the fundoplication). To distinguish between these two possibilities we compared the methylation in the fundoplication groups to that in no surgery subjects, which we would expect to be similar to the pre-operative methylation in our fundoplication groups. We found that the amount of methylation in the no surgery subjects was significantly more

than in the pH normal subjects, but did not differ from the pH abnormal subjects. We conclude from our results that long-term reflux control by fundoplication reduced aberrant DNA methylation, rather than preventing an increase over time. A prospective study, in which methylation is analysed prior to and after antireflux therapy, is required to confirm this.

There are two basic forms of fundoplication, total and partial. Our own data from 24-hour pH studies at six months showed no difference in reflux between total and partial fundoplications.[14] In this study we have not distinguished between the types of fundoplication, and have only been concerned whether the operation restricts reflux to within the normal range.

Fundoplication is effective in controlling clinical symptoms in the majority of patients[13], but measuring a reduction in cancer risk is more difficult. It has been estimated that this would require an extremely large sample size (4,000 for average risk patients), and they would need to be followed for more than five years, to detect a difference between groups.[23] Progression from intestinal metaplasia to dysplasia and adenocarcinoma following antireflux surgery has been reported, but it predominantly occurred in patients with long-segment Barrett's esophagus and a documented recurrent increased 24-hour pH study.[24, 25] In a recent meta-analysis Chang concluded that while antireflux surgery was associated with regression of Barrett's esophagus and/or dysplasia, it was less clear if it differed from medical treatment in preventing the development of adenocarcinoma.[18] The conclusion from combining all studies was that antireflux surgery was associated with a reduced incidence rate of cancer. However, many of the published studies were not controlled. Analysis of just controlled trials and cohort studies failed to show a difference between surgical and medical therapy. Interpretation of these trials is difficult because surgical patients generally suffer more severe reflux and may be considered at higher risk of progression to cancer, and, more importantly, in these studies it is not common to determine objectively if the antireflux surgery remains functional. The significance of this is evident from our finding that the fundoplication had failed in 10 (24.4%) of our 41 patients.

Fundoplication minimises the reflux of both gastric and duodenal contents, unlike proton pump inhibitors which reduce the acidity and volume of gastric secretions but do not prevent reflux of bile or enzymes. We measured reflux as the length of time that acidic contents were present in the esophagus, but this does not mean that it is acid that is responsible for DNA

methylation, as opposed to a combination of acid and some other component in the refluxate such as bile, or another component alone. Our findings are consistent with reports that fundoplication reverses molecular changes in Barrett's esophagus that promote carcinogenesis or reflect damage to the epithelium, such as oxidative stress altered expression of the cytokines IL-8, IL-1alpha, IL-1beta, or activated NF-kappaB, COX-2 and Cdx2.[26, 27]

We have shown that in patients whose fundoplication was proven to be intact by a pH study five or more years after surgery there was a reduction in the length of Barrett's esophagus over the time following fundoplication. We demonstrate a further benefit of fundoplication: there was significantly less methylation in the columnar mucosa of subjects with an intact fundoplication compared to subjects whose fundoplication had failed and have abnormal reflux, or those who have not had surgery. Thus successful surgery may reduce a genomic change which is thought to play a role in the development of cancer. Additionally, to our knowledge, we are the first to show an association between abnormal gastro-esophageal reflux and aberrant DNA methylation in Barrett's esophagus.

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Table 1. Primer sequences and annealing temperatures for methylation analysis

Gene	Primer sequences 5'-3'	Tm (^{o}C)	Location[a]	Size[b]	CpGs[b]
APC	Forward-GAAGYGGAGAGAGAAGTAGTTG Reverse-ACRAACTACACCAATACAACCACATA	55	chr5:112073441-112073572	132	10
CDKN2A	Forward-TYGGYGGYGGGGAGTAGTATGGAGTTT Reverse-RTTAAACAACRCCCCCRCCTCCAACAA	60	chr9:21974710 - 21974820	111	8
ID4	Forward-GGGGYGTAYGGTTTTATAAATATAGTTG Reverse-TAATCACTCCCTTCRAAACTCCGACTAAA	55	chr6:19945537-19945694	158	16
MGMT	Forward-IGIGTTTIGGATATGTTGGGATAGTT Reverse-ACIAAACIACCCAAACACTCACCAAA	55	chr10:131155458-131155572	115	12
RBP1	Forward-TGTGYGYGTTGGGAATTTAGTTG Reverse-CRAAAAATAACTAAAACCAATTAACCACAAA	55	chr3:140741067-140741230	164	15
RUNX3	Forward-YGTYGTTTTTTGYGTTTTGAGGTT Reverse-ACTTAAATCTACRAAAATACRCATAACAA	55	chr1:25128923-25129094	172	23
SFRP1	Forward-ATTTTYGGGAGTYGGGGYGTATT Reverse-RACCAATAACRACCCTCRACCTA	57	chr8:41285991-41286078	88	9
TIMP3	Forward-TTTGAGGGGGYGGGTTTTAATAGTT Reverse-AACRACCTCCCRACGAAAAAACAAA	55	chr22:31527757-31527632	174	13
TMEFF2	Forward-TTGTTTTTTYGTYGGGTGTTATTGTTAT Reverse-AACAAACRACTTCCRAAAAACACAAA	55	chr2:192767520-192767637	118	11

Y – C/T. R – A/G. I – inosine. Tm – annealing temperature. [a]Genome location, as determined by UCSC Genome Browser Database (GBD, http://genome.ucsc.edu) Human March 2006 (hg18) assembly. [b]Size of the PCR product. [c]Number of CpGs in the PCR product between the primer binding sites.

Table 2. Clinical characteristics of subjects

	All fundoplication subjects		Biopsied subjects		
	pH normal	pH abnormal	pH normal	pH abnormal	No surgery
Patient numbers	n = 31	n = 10	n = 21	n = 9	n = 17
Males, n (%)	23 (74)	6 (60)	17 (81)	5 (56)	13 (76)
Age (*y*)[a]	54.5	58.6	55.1	58.4	56.8
95% CI	50.2-58.9	47.0-70.2	49.6-60.7	45.2-71.6	51.8-61.7
range	35-78	34-88	37-78	34-88	38-71
Time interval (mo)[b]	97.2	104.5	95.0	106.0	
95% CI	90.2-104.2	88.3-120.7	86.7-103.2	88.0-124.1	
range	65-143	68-151	65-124	68-151	
Pre-op BE length (cm)[c]	4.0[2]	4.0	5.0[2]	5.0	
range	1.0-13.0	2.0-10.0	1.0-13.0	2.0-10.0	
BE length at study (cm)[d]	0.5[1,2]	2.5[1]	1.0[2,3]	3.0	4.0[3]
range	0-8.0	0-12.0	0.5-8.0	1.0-12.0	3.0-9.0

[a]Mean age at the time of the study. [b]Mean time interval between surgery and this study. [c]Median pre-operative (pre-op) Barrett's esophagus (BE) length. [d]Median BE length at the time of the study. [1]$P = .0404$. [2]$P < .0001$. [3]$P = .0026$.

Table 3. Relationship between methylation of individual genes and reflux group

Gene	Odds ratio	95% CI	*P* value
APC	0.7800	0.0825-7.3692	.8284
ID4	9.0580	0.9132-90.0900	.0598
MGMT	4.0000	0.8326-19.2308	.0834
RBP1	4.1911	0.6612-26.5252	.1283
RUNX3	3.1666	0.5811-17.2414	.1827
SFRP1	1.6801	0.1508-18.7266	.6732
TIMP3	5.2493	1.2598-21.8818	.0228
TMEFF2	2.3832	0.5200-11.1982	.2714

To test whether reflux group (normal or abnormal) had an influence on the odds of an individual gene being methylated, univariate logistic GEE regression models were fitted to the data. There were too few observations where CDKN2A was methylated for analysis.

Table 4. Relationship between methylation of individual genes and Barrett's esophagus length

Gene	Odds ratio	95% CI	*P* value
APC	1.0628	0.8592-1.3148	.5745
ID4	1.5957	1.0727-2.3736	.0211
MGMT	1.0829	0.8467-1.3847	.5259
RBP1	1.7023	1.1427-2.5358	.0089
RUNX3	1.3259	1.0139-1.7339	.0393
SFRP1	1.2798	0.9581-1.7095	.0949
TIMP3	1.2844	1.0994-1.5007	.0016
TMEFF2	1.6901	1.1935-2.3934	.0031

To test whether the length of Barrett's esophagus had an influence on the odds of an individual gene being methylated, univariate logistic GEE regression models were fitted to the data. There were too few observations where CDKN2A was methylated for analysis.

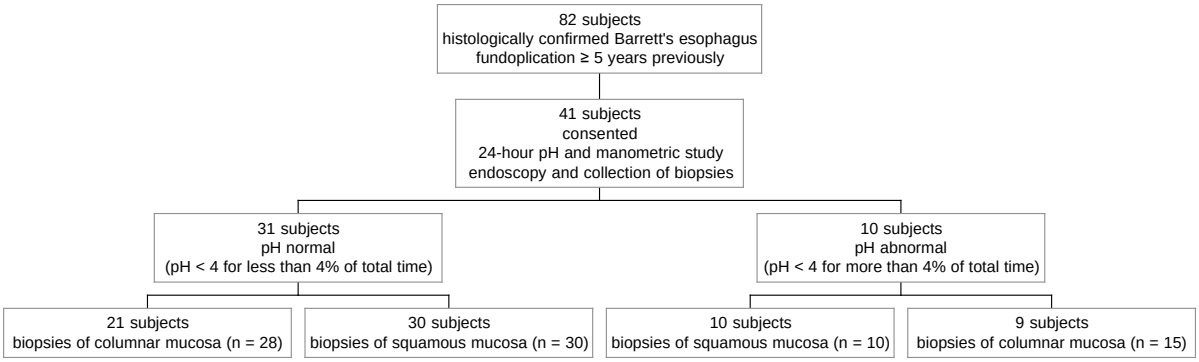

Figure 1. Subject recruitment for the study. Of the 41 subjects with Barrett's esophagus who had a fundoplication more than five years earlier, 31 currently had a normal and 10 an abnormal pH study. A squamous biopsy was collected from each of these subjects except from one pH normal subject who became distressed during the endoscopy. Biopsies of columnar mucosa were collected from 21 of the pH normal and nine of the pH abnormal subjects. Columnar biopsies could not be collected from seven pH normal and one of the pH positive subjects because the columnar lined esophagus had completely regressed, and were not collected from three pH normal subjects because the amount of columnar lined esophagus was too short to biopsy confidently. A total of 28 and 15 columnar biopsies were collected from the pH normal and pH abnormal subjects respectively.

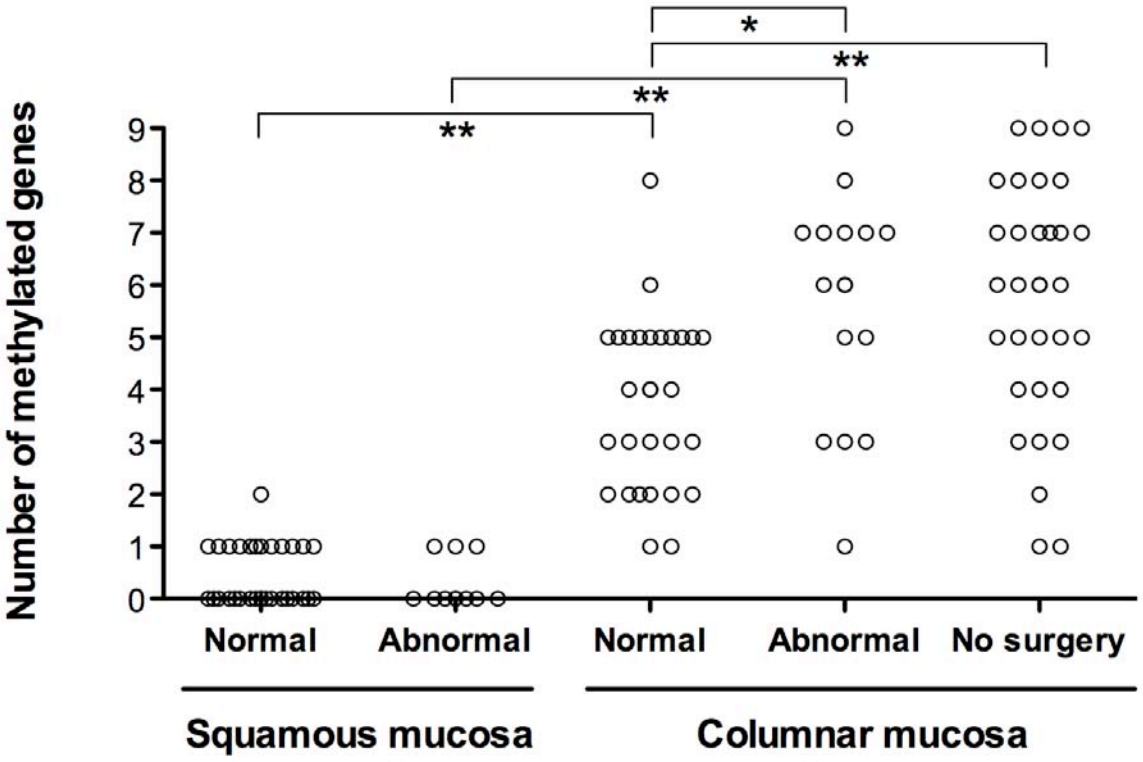

Figure 2. The number of methylated genes in biopsies of squamous and columnar mucosa from pH normal and pH abnormal fundoplication subjects and no surgery subjects. Methylation of each of the nine genes was analysed in each biopsy. The adjusted mean number of methylated genes, as estimated by a linear mixed effects model, was .49, .30, 3.65, 5.32 and 6.04 (left to right respectively). **P* = .0067. ***P* < .0001.

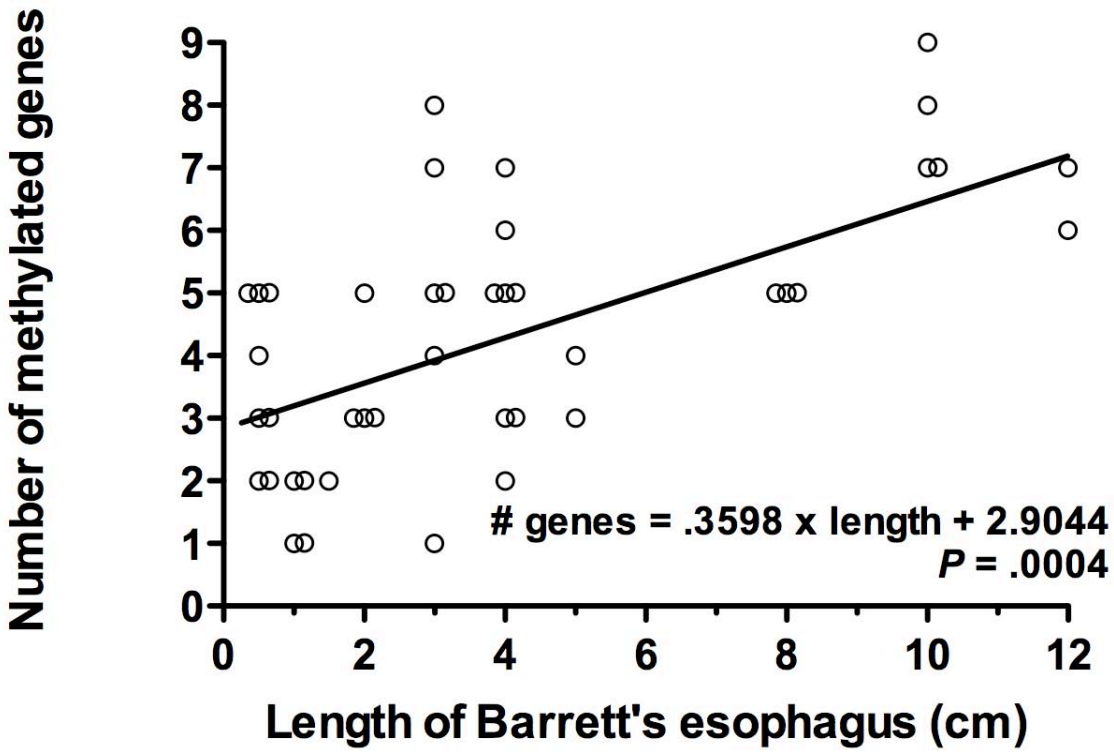

Figure 3. The relationship between the number of methylated genes in columnar mucosa from pH normal and pH abnormal fundoplication subjects (P = .0004). Methylation of each of the nine genes was analysed in each biopsy.

CHAPTER 9: EFFECT OF HIGH-DOSE ESOMEPRAZOLE ON GASTRIC AND ESOPHAGEAL ACID EXPOSURE AND MOLECULAR MARKERS IN BARRETT'S ESOPHAGUS

Awni Abu-sneineh[1], William Tam[1], Mark Schoeman[1,2], Robert Fraser[2,3], Andrew R. Ruszkiewicz[4], Eric Smith[5], Paul A. Drew[6], John Dent[1,2], Richard H. Holloway[1,2]

[1]Department of Gastroenterology, Hepatology and General Medicine, Royal Adelaide Hospital, Adelaide, 5000, South Australia;
[2]Discipline of Medicine, The University of Adelaide, Adelaide, 5005, South Australia;
[3]Gastroenterology Unit, Repatriation Hospital, Daw Park, 5041, South Australia;
[4]Surgical Pathology, SA Pathology, Adelaide, 5000, Australia;
[5]Discipline of Surgery, The University of Adelaide, Adelaide, 5005, Australia;
[6]School of Nursing and Midwifery, Flinders University, Bedford Park, 5042, Australia.

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ABSTRACT

Background & Aims:

We determined the effect of 40 mg twice daily esomeprazole (high-dose) on gastric and esophageal pH and symptoms, and biomarkers relevant to adenocarcinoma progression, in patients with Barrett's esophagus (BE).

Methods:

Eighteen patients with BE, treated with proton pump inhibitors (PPIs) as prescribed by their treating doctor, had their therapy increased to high-dose esomeprazole for 6 months.

Results:

At entry into the study 9/18 patients had excessive 24-hour esophageal acid exposure, and gastric pH remained <4 for >16 hours in 8/18. With high-dose esomeprazole excessive acid exposure occurred in 2/18 patients, and gastric pH <4 was reduced from 38% of the overall recording time and 53% of the nocturnal period to 15% and 17% respectively ($P < .001$). There was a reduction in self-assessed symptoms of heartburn ($P = .0005$) and regurgitation ($P < .0001$), and decreased inflammation and proliferation in the Barrett's mucosa. There was no significant change in p53, MGMT or COX-2 protein expression, nor in aberrant DNA methylation of 9 genes tested.

Conclusion:

High-dose esomeprazole achieved high levels of gastric acid suppression and effective control of esophageal acid reflux and symptoms in almost all BE patients, and was associated with significant decreases in inflammation and epithelial proliferation. There was no reversal of aberrant DNA methylation.

Keywords:

Barrett's esophagus; esomeprazole; biomarkers.

INTRODUCTION

Barrett's esophagus (BE) is a complication of gastro-esophageal reflux disease in which the normal esophageal squamous epithelium is replaced by a columnar epithelium which contains goblet cells. Approximately 0.5 to 1% of patients with BE will develop esophageal adenocarcinoma each year, and BE patients have a 30- to 125-fold increase in the risk of esophageal adenocarcinoma compared to the general population.[1-3] Exposure to reflux contributes to symptoms and is an independent risk factor for BE and adenocarcinoma.[4]

Proton pump inhibitors (PPIs) are the mainstay of treatment of reflux disease. Esomeprazole at a dose of 40 mg once-daily provides more effective control of gastric pH at steady state than once-daily omeprazole 20 mg, lansoprazole 30 mg, pantoprazole 40 mg or rabeprazole 20 mg.[5] However, in patients with BE esophageal acid exposure is often difficult to control with commonly used dosages of PPI therapy even though symptom control may be adequate.[6, 7] In particular, control of esophageal acid exposure in the second 12 hour period after morning dosing is frequently insufficient in BE patients, and better pH control over the full 24 hours may require a second dose.

Whilst one goal of treatment is the control of symptoms, another should be the prevention of progression to esophageal adenocarcinoma. Non-randomised studies suggest that acid suppression by PPIs may reduce the risk of developing dysplasia and therefore potentially reduce the risk of developing cancer.[8-10] However, data on the protective benefit of medical therapy is not clear. Previous studies did not assess the effectiveness of pH control, and very large trials with long follow-up would be required to assess whether PPI therapy reduces the risk of adenocarcinoma.[11] In this study we used 40 mg twice-daily esomeprazole to control acid reflux, and measured molecular markers of inflammation and proliferation, and aberrant DNA methylation as surrogates for the effect of acid suppressant therapy on the potential for development of adenocarcinoma in patients with BE.

METHODS

Patients and study design

Nineteen patients with histologically confirmed BE ≥3 cm in length, being treated with PPI therapy as prescribed by their treating doctor, agreed to enter the study. The PPIs and the doses at which they were prescribed are detailed in the Results, and for the purposes of this study are collectively described as standard PPI therapy. At entry into the study, whilst on their standard PPI therapy, patients had an endoscopy with collection of biopsies, esophageal manometry, ambulatory 24-hour gastric and esophageal pH monitoring, and completed a symptom score questionnaire. Then PPI therapy was increased to 40 mg twice-daily (high-dose) esomeprazole for 6 months. One patient withdrew within 3 days of starting high-dose esomeprazole because of nausea and was not included in the analysis. Whilst on high-dose esomeprazole, gastric and esophageal pH monitoring and a symptom score questionnaire were repeated at 2 months, and endoscopy repeated and esophageal biopsies taken at 6 months. The study was approved by the Research Ethics Committee of the Royal Adelaide Hospital and all patients gave their written informed consent.

Esophageal manometry

Esophageal manometry was performed at entry into the study. After an overnight fast, a 3.5 mm multi-lumen assembly that incorporated a sleeve sensor (Dentsleeve Pty, Ltd, Wayville, South Australia) was used to measure lower esophageal sphincter (LES) pressure, gastric pressure, esophageal body motility and swallowing. After intubation the proximal margin of the LES was determined by performing a stepwise pull-through of the distal 3 esophageal side-hole sensors. Following a 10 - 15 minute period of accommodation, esophageal responses to 10 water swallows were assessed. Basal LES pressure was measured at end-expiration relative to intragastric pressure as a 1 minute visual mean over the last minute of the accommodation period. Esophageal peristalsis was assessed for wave amplitude and peristaltic progression as described previously.[12]

Ambulatory 24-hour pH monitoring

Ambulatory esophageal and gastric pH monitoring were performed on PPI therapy at entry into the study and after 2 months of treatment with high-dose esomeprazole. Esophageal and gastric pH were measured using a dual antimony catheter with pH electrodes spaced 15 cm apart (Medtronic Functional Diagnostics Zinetics Inc, Utah, USA). The proximal electrode

was positioned 5 cm above the upper border of the LES; accordingly, the distal electrode was positioned in the upper stomach. The electrodes were connected to a portable data-logger and ambulatory pH recordings were made for 24 hours.

Ambulatory 24-hour pH data were analysed using the EsopHagram software (Medtronic Functional Diagnostics Zinetics Inc). The time that esophageal pH was <4 was determined for the total, upright and recumbent periods as well as for the nocturnal period, defined as 22.00 - 08.00 hours.[13] Median and mean gastric pH, as well as the duration of gastric pH <4, were determined for each hour as well as the nocturnal period.

Assessment of symptoms

Heartburn and regurgitation that had occurred over the previous 4 weeks were assessed by the Reflux Disease Questionnaire[16] which was completed by the patient without assistance, at entry into the study and after 2 months of high-dose esomeprazole. The frequency and severity of heartburn and regurgitation were assessed separately. The maximum score for heartburn or regurgitation was 20, with a maximum combined score of 40.

Biopsies

Biopsies were taken at endoscopy at entry into the study and after 6 months of high-dose esomeprazole according to a standard protocol: 4 quadrantic biopsies were taken at the gastro-esophageal junction (GEJ), defined as at the tops of the gastric folds, and 4 each at 2 cm intervals in the columnar lined esophagus, beginning in the esophagus 1 cm above the GEJ. Biopsies were placed in neutral buffered formalin for histological assessment. An additional biopsy was taken from each level in the columnar lined esophagus, and from macroscopically normal squamous mucosa 5 cm proximal to the maximal extent of the squamo-columnar junction, and preserved either in RNAlater (Ambion, Austin, TX, USA), or snap frozen in liquid nitrogen, and then stored at -80^{o} C until measurement of DNA methylation.

DNA methylation analysis

Biopsies from the esophageal columnar and squamous mucosae, taken at entry into the study and after 6 months of high-dose esomeprazole, were used for DNA methylation analysis. The DNA was isolated and bisulfite modified as described previously.[17] Bisulfite modified DNA was polymerase chain reaction amplified using primers and conditions described in Supplementary Table 1. Methylation was quantified by calculating the T50, the temperature

at which half the amplicons are melted, as described previously.[18] The average T50 was calculated when multiple columnar biopsies were available.

Histological assessment

Biopsies from the columnar lined esophagus taken at entry into the study and after 6 months of high-dose esomeprazole were used for histological assessment. Formalin-fixed paraffin-embedded sections were stained with haematoxylin and eosin (H&E) for routine histology, or incubated with antibodies for Ki67, COX-2, MGMT or p53 for immunohistochemistry. Staining for Ki67 was assessed separately for the crypts and glands, and for the luminal surface epithelium, and scored as 0 (none), 1 (low) or 2 (high). Staining for COX-2 was assessed separately for the crypts and glands, and for the luminal surface epithelium, and scored as 0 (none), 1 (low) or 2 (high). Staining for MGMT was scored as 0 (normal), 1 (borderline reduced expression) or 2 (reduced expression with complete or clonal loss). Staining for p53 was scored as 0 (negative), 1 (borderline positive staining) or 2 (>10% cells with strong nuclear staining). Inflammation was assessed on the H&E sections and scored as 0 (none), 1 (mild chronic), 2 (more intense chronic) or 3 (substantial admixture of neutrophils). The aggregate score of the 4 biopsies immediately proximal to the GEJ was used in the analysis.

Statistical analysis

Normally distributed data were summarised using means and ranges and were compared between reflux groups using unpaired t-tests. Non-normally distributed continuous data were summarised using the median and lower and upper quartiles and were compared using either a Wilcoxon signed-rank test for paired data or a Mann Whitney test for independent samples. The P-values were adjusted for multiple comparisons using the Sidak procedure. All statistics were considered significant when the two tailed $P \leq .05$.

RESULTS

Eighteen patients (14 males) with a median age of 56 years (range 38 - 71) and BE ≥3 cm in length (median circumferential length 4 cm, range 3 - 8 cm) completed the study. At entry into the study all patients had BE with histologically confirmed intestinal metaplasia, and none had erosive or ulcerative esophagitis. Patients were taking a range of PPI therapies at

entry into the study. Ten were on a once-daily morning dose (omeprazole 20 mg (n = 5), pantoprazole 40 mg (n = 3), lansoprazole 30 mg (n = 1), or esomeprazole 40 mg (n = 1)), and eight on a twice-daily dose (omeprazole 20 mg (n = 6), lansoprazole 30 mg (n = 2).

Esophageal acid exposure

At entry into the study, whilst on their standard PPI therapy, the median 24-hour esophageal acid exposure was 4.5% (.9 - 15.5%) (Figure 1). Nine of the 18 (50%) patients had excessive total 24-hour acid exposure (pH <4 for >5% of the total time): one because of excessive upright exposure, 5 because of excessive recumbent exposure and 4 because of excessive upright and recumbent acid exposure. There were no significant differences in total, upright or recumbent acid exposure between patients on once- or twice-daily therapy, although the four highest acid exposure patients were each on once-daily therapy (Figure 1).

Whilst on high-dose esomeprazole the median 24-hour esophageal acid exposure was only .2% (0 - .6%). Only 2 patients had excessive acid exposure, 1 as a result of excessive upright reflux and the other due to excessive upright and recumbent reflux. These 2 patients had the highest esophageal acid exposure at entry into the study (Figure 1).

Gastric acidity

The gastric pH profiles in Figure 2 show the median pH each hour of the entire 24-hour period. At entry into the study gastric pH remained below 4 for a median of 38% (29 - 57%) of the 24-hour period (Figure 3). The percentage of the time that gastric pH was below 4 did not differ between the upright (33% (26 - 48%)) and recumbent periods (45% (35 - 60%). There were no significant differences in any of these variables between patients on once- or twice-daily therapy.

On high-dose esomeprazole the overall time that gastric pH remained below pH 4 fell to 15% (5 - 22%; P < .001). However, the percentage of the time that gastric pH was below 4 was significantly greater during the recumbent period (17.2% (9 - 33%)) than during the upright period (9% (2 - 17%). At entry into the study, gastric pH remained above pH 4 for more than 16 hours in only 8 of 18 patients, compared with 17 of 18 patients on high-dose esomeprazole (Fisher's Exact Test: P = .0027).

Nocturnal gastric and esophageal acidity

At entry into the study the median duration that gastric pH was below 4 was 320 minutes (208 - 440 minutes) or 53% (35 - 73%) of the nocturnal recording period (Figure 4). There were no differences between patients on once- and twice-daily therapy. On high-dose esomeprazole the median time that gastric pH was below 4 (100 minutes (40 - 171 minutes) or 17% (7 - 27%)) was significantly less than at entry into the study ($P < .001$).

At entry into the study, nocturnal esophageal acid exposure was 5.5% (0.4 - 36.3%). Patients on once-daily therapy had similar levels to those on twice-daily therapy. Nocturnal esophageal acid exposure was almost completely eliminated on high-dose esomeprazole (0%; 0 - 0.2%) ($P < .004$) (Figure 5).

Esophageal motility

At entry into the study basal LES pressure was uniformly low (3.5 ± 0.8 mm Hg). The rate of peristalsis was high and successful peristaltic responses occurred with 100% (90 - 100%) of water swallows. Maximum peristaltic pressure wave amplitudes were 54 ± 6.3 mm Hg in the distal esophagus.

Symptoms

At entry into the study the median combined heartburn and regurgitation score was 12 (7.25 - 18) over the previous 4 weeks, with 17 of the 18 patients reporting symptoms. The median heartburn score was 5 (0 - 7.8), with 12 patients reporting symptoms. The regurgitation score was 8 (4.5 - 10), with 16 patients reporting symptoms. Following 2 months of treatment with high-dose esomeprazole, the median combined heartburn and regurgitation score was 2 (0 - 4.75) ($P < .0001$), with 11 of the 18 patients reporting symptoms. The median heartburn score was 0 (0 - 0) ($P = .0005$), with 3 patients reporting symptoms. The regurgitation score was 1.5 (0 - 4) ($P < .0001$), with 10 patients reporting symptoms.

Histology

Over the 6 months of high-dose treatment there was a significant decrease in the expression of Ki67 in the crypts and glands (median 6 (5 - 7) vs 5 (4 - 5.25); $P = .0442$) and in the surface epithelium (median 3 (2 - 4) vs 1 (0 - 2); $P = .0029$). There was also a significant decrease in inflammation score (median 8 (5.75 - 9.25) vs 4 (3.75 - 5.25); $P = .0016$). Detectable p53 or

COX-2 or altered MGMT expression, when present, was low and did not change with high-dose treatment.

Methylation

The T50, a measure of the amount of DNA methylation in a sample, was significantly greater in the biopsies from the columnar epithelium than in those from the squamous epithelium for all 9 genes (Supplementary Table 2). There was no significant change in methylation in the columnar lining following 6 months of high-dose treatment (Supplementary Table 3). Significantly increased methylation of MGMT (median 75.69 (75.66 - 77.27) vs 77.53 (77.12 - 77.76); $P < .0001$) was associated with reduced MGMT protein expression in columnar biopsies at the equivalent esophageal level.

DISCUSSION

Patients with BE have increased esophageal acid reflux that is often difficult to control with standard dose PPIs. If acid reflux plays a role in the progression from BE to cancer, then medical treatment must not simply result in symptomatic relief, but ensure effective control of esophageal acid exposure as well. The aim of this study was to assess prospectively the effect of high-dose (40 mg twice daily) esomeprazole on acid suppression, symptoms and molecular markers in a heterogeneous group of 18 patients selected on the basis of having at least 3 cm of BE with confirmed intestinal metaplasia. Treatment with high-dose esomeprazole achieved high levels of gastric acid suppression and effective control of esophageal acid reflux in 16 (89%) patients, with a significant improvement in symptoms. In the BE segment, while there was a significant reduction in inflammation and epithelial cell proliferation, there was no change in p53, COX-2 or MGMT protein expression, and, importantly, no reduction in aberrant DNA methylation of the 9 genes assessed.

The management of BE is sometimes difficult because control of esophageal acid exposure by PPIs is often less effective in patients with BE compared to patients with reflux esophagitis.[6, 13] Persistent acid reflux, particularly at night, is common [7] even when reflux symptoms are reported to be controlled.[19, 20] Persistent esophageal acid exposure would most likely undermine any potential benefit of PPI therapy in reducing the risk of dysplasia and adenocarcinoma. The reasons for the difficulty in suppressing acid reflux and secretion in

patients with BE are unclear. The underlying high levels of acid reflux may require greater levels of acid suppression. However, whether acid secretion is increased in BE is controversial.[21, 22] We found that, on PPI therapy as used in routine clinical practice, whether once-daily or twice-daily dosing, high levels of gastric acidity and excessive levels of esophageal acid exposure were common among our patients.

Twice-daily 40 mg esomeprazole, however, was highly effective in controlling gastric acidity and esophageal acid exposure. Gastric pH remained above pH 4 for 85% of the time overall, and 17/18 (94%) patients maintained a gastric pH above 4 for more than 16 hours. Esophageal acid exposure, which had been excessive in half of the patients on their initial doses of PPI, was reduced to normal levels in all but 2 patients.

Our findings following treatment with high-dose esomeprazole contrast with earlier studies which reported a significant minority of patients having persistent symptoms when treated with what was considered a maximal dose of PPIs (but lower than used in our study).[7, 23] High-dose esomeprazole was also very effective in controlling heartburn, but had less impact on regurgitation. These findings are consistent with other recent reports and underline the need for twice daily PPI therapy to control acid secretion and acid reflux reliably in BE.[24]

While symptom relief is one major aim of treatment of reflux disease, in patients with BE it would also be desirable that treatment should reduce the significant risk of progression to esophageal adenocarcinoma. There are reports that high-dose PPIs may decrease the length of BE[25-28], but there is no evidence that they can completely reverse the condition.[25, 29-32] Whilst there are claims that acid suppression with a PPI alone reduces the risk for development of dysplasia in patients with BE[8-10], these studies were uncontrolled and retrospective, and information on the effectiveness of the control of esophageal acid exposure was not available because pH monitoring was not included. In the current study the high levels of acid inhibition that were achieved by high-dose esomeprazole were documented by detailed gastric and esophageal pH monitoring.

Because of the large cohort size and long follow up period required to measure cancer as an end-point of treatment, we selected a range of molecular measures as surrogate markers for cancer risk. Treatment with high-dose esomeprazole was associated with a reduction in epithelial cell proliferation, as measured by proliferating cell nuclear antigen Ki67, in both the

crypt and glands and the luminal surface cells. In normal tissues, cell proliferation staining is absent from the luminal cells, and it increases there as the tissues progress along the metaplasia-dysplasia-carcinoma sequence of esophageal adenocarcinoma.[33] Our findings are similar to other studies which have shown that the increased proliferation and expression of key cell cycle regulatory genes, which occurs early in the neoplastic progression associated with BE, is reduced by treatment with PPIs.[34-36]

Chronic inflammation is thought to be causally associated with the progression from premalignant disease to cancer, including BE and esophageal adenocarcinoma.[37] We noted a significant decrease in the inflammation score over the 6 months of high-dose treatment. One measure of inflammation which has been associated with malignancy, COX-2 expression, remained unchanged at low or undetectable levels over the course of the study. In other studies of the effects of PPI treatment, COX-2 has been variably reported to increase[34] or not change[38] following acid suppression. It is possible that the reduction in the inflammation score might have resulted from anti-inflammatory effects which may be exerted by PPIs independent of acid inhibition.[39] Our results suggest a dose response to PPIs, as inflammation was reduced with the high-dose esomeprazole compared to the doses of PPIs that patients were on before entering the study. We also found absent or low levels of p53 expression and reduced MGMT expression in several patients at the start of the study. No significant changes were measured following treatment, reflecting the fact that most of the participants had normal expression of these markers on entry into the study.

The progression to cancer in a tissue requires the up- or down-regulation of critical genes. Aberrant methylation of CpG islands in the promoter regions of genes, particularly tumor suppressor genes, can lead to gene silencing and is common in cancer, including esophageal adenocarcinoma.[40, 41] We have previously reported that methylation of 7 of the genes analysed in this study is as frequent in BE as in EAC.[17] Despite the strong link between this and the development of cancer, little is known about changes in aberrant methylation in BE as a result of treatment of reflux. We did not find any significant change after 6 months treatment with high-dose esomeprazole. Methylation and demethylation most commonly occur at cell division. As columnar epithelial cells have a short cell cycle time, even in treated patients, we would expect there to have been more than enough cell divisions to have seen a change in DNA methylation profile over the 6 month period if control of acid would result in its reversion back to normal.

In this study we have shown that high-dose esomeprazole significantly reduces gastric and esophageal acid levels, and reflux-associated symptoms, in patients with ≥3 cm of BE. An important finding is that despite this convincing benefit in the control of acid and symptoms, as well as reduced inflammation and epithelial cell proliferation, there was no reversal of aberrant DNA methylation, a biomarker believed to be causally associated with development of cancer. These findings suggest that while high-dose esomeprazole offers an appropriate treatment for difficult to control reflux symptoms, it may not prevent the development of Barrett's-associated esophageal adenocarcinoma in patients with BE.

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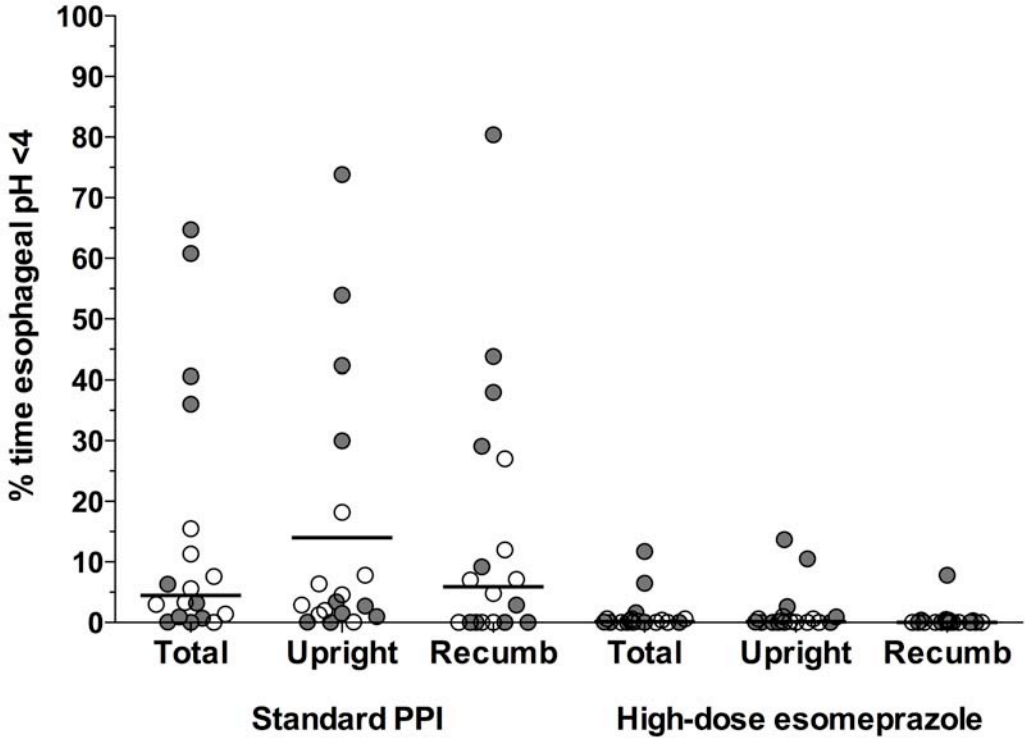

Figure 1. Esophageal acid exposure in patients with Barrett's esophagus on proton pump inhibitor therapy at entry into the study (standard PPI) and after 2 months treatment with 40 mg twice-daily (high-dose) esomeprazole. Data are presented for the total 24-hours, upright and recumbent periods. Each data point represents an individual patient, either on once-daily dose of PPI at entry into the study (grey), or twice-daily dose (white). The horizontal lines represent the medians of the group.

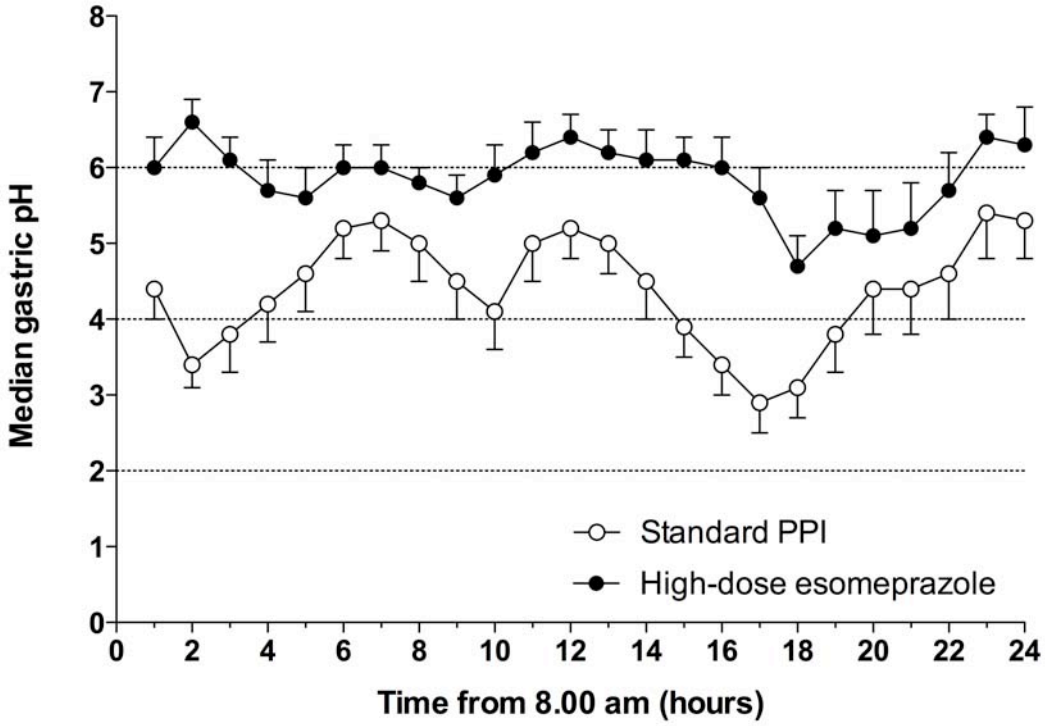

Figure 2. 24-hour gastric pH in patients with Barrett's esophagus on proton pump inhibitor therapy at entry into the study (standard PPI) and after 2 month's treatment with 40 mg twice-daily (high-dose) esomeprazole. Data are presented as the median and lower and upper quartiles of the median pH for each hour over the 24-hour period in patients on standard-dose proton pump inhibitor (white) and esomeprazole 40 mg bid for 2 months (black).

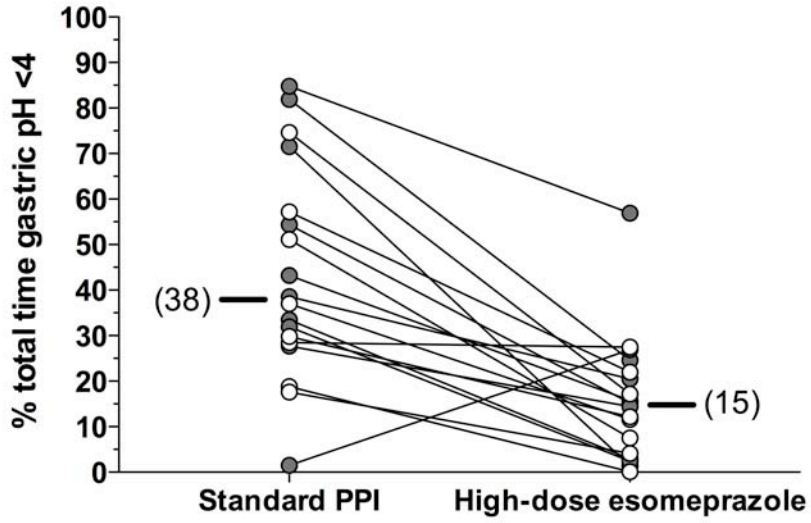

Figure 3. Gastric acid exposure in patients with Barrett's esophagus on proton pump inhibitor therapy at entry into the study (standard PPI) and after 2 months treatment with 40 mg twice-daily (high-dose) esomeprazole. Each data point represents an individual patient, either on once-daily dose of PPI at entry into the study (grey), or twice-daily dose (white). The horizontal lines represent the medians of the group.

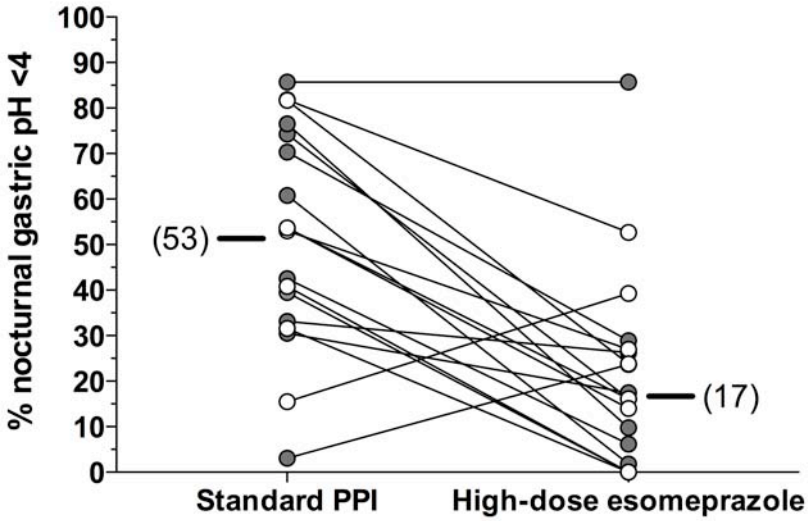

Figure 4. Nocturnal gastric acid exposure in patients with Barrett's esophagus on proton pump inhibitor therapy at entry into the study (standard PPI) and after 2 months treatment with 40 mg twice-daily (high-dose) esomeprazole. Each data point represents an individual patient, either on once-daily dose of PPI at entry into the study (grey), or twice-daily dose (white). The horizontal lines represent the medians of the group.

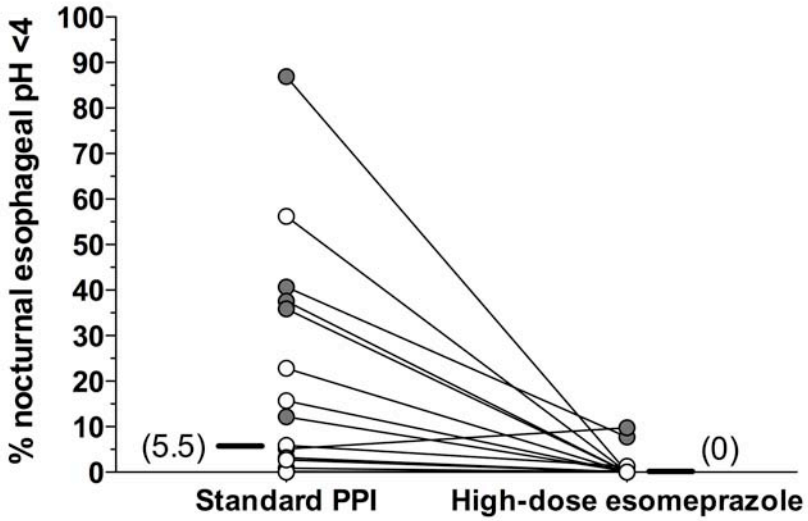

Figure 5. Nocturnal esophageal acid exposure in patients with Barrett's esophagus on proton pump inhibitor therapy at entry into the study (standard PPI) and after 2 months treatment with 40 mg twice-daily (high-dose) esomeprazole. Each data point represents an individual patient, either on once-daily dose of PPI at entry into the study (grey), or twice-daily dose (white). The horizontal lines represent the medians of the group.

Supplementary Table 1. Primer sequences and annealing temperatures for DNA methylation analysis

Gene	Primer sequences 5'-3'	Tm (^{o}C)	Location[a]	Size[b]	CpGs[b]
APC	Forward-GAAGYGGAGAGAGAAGTAGTTG Reverse-ACRAACTACACCAATACAACCACATA	55	chr5:112073441-112073572	132	10
CDKN2A	Forward-TYGGYGGYGGGGAGTAGTATGGAGTTT Reverse-RTTAAACAACRCCCCCRCCTCCAACAA	60	chr9:21974710 - 21974820	111	8
ID4	Forward-GGGGYGTAYGGTTTTATAAATATAGTTG Reverse-TAATCACTCCCTTCRAAACTCCGACTAAA	55	chr6:19945537-19945694	158	16
MGMT	Forward-IGIGTTTIGGATATGTTGGGATAGTT Reverse-ACIAAACIACCCAAACACTCACCAAA	55	chr10:131155458-131155572	115	12
RBP1	Forward-TGTGYGYGTTGGGAATTTAGTTG Reverse-CRAAAAATAACTAAAACCAATTAACCACAAA	55	chr3:140741067-140741230	164	15
RUNX3	Forward-YGTYGTTTTTTGYGTTTTGAGGTT Reverse-ACTTAAATCTACRAAAATACRCATAACAA	55	chr1:25128923-25129094	172	23
SFRP1	Forward-ATTTTYGGGAGTYGGGGYGTATT Reverse-RACCAATAACRACCCTCRACCTA	57	chr8:41285991-41286078	88	9
TIMP3	Forward-TTTGAGGGGGYGGGTTTTAATAGTT Reverse-AACRACCTCCCRACGAAAAAACAAA	55	chr22:31527757-31527632	174	13
TMEFF2	Forward-TTGTTTTTTYGTYGGGTGTTATTGTTAT Reverse-AACAAACRACTTCCRAAAAACACAAA	55	chr2:192767520-192767637	118	11

Y – C/T. R – A/G. I – inosine. Tm – annealing temperature. [a]Genome location, as determined by UCSC Genome Browser Database (GBD, http://genome.ucsc.edu) Human March 2006 (hg18) assembly. [b]Size of the PCR product. [c]Number of CpGs in the PCR product between the primer binding sites.

Supplementary Table 2. The median (interquartile range) amount of methylation (T50) in all squamous and columnar biopsies

	Squamous	Columnar	P-value[a]
APC	77.95 (77.70 - 78.41)	79.45 (79.02 - 79.97)	< .0001
CDKN2A	81.77 (81.75 - 81.79)	81.81 (81.76 - 82.01)	.0223
ID4	81.70 (81.11 - 81.93)	82.80 (82.31 - 83.72)	< .0001
MGMT	75.68 (75.66 - 75.74)	76.70 (75.70 - 77.52)	< .0001
RBP1	75.78 (75.68 - 76.29)	76.18 (75.88 - 76.88)	.0004
RUNX3	78.76 (78.69 - 78.86)	79.08 (78.81 - 80.12)	< .0001
SFRP1	75.82 (75.22 - 75.90)	77.62 (76.86 - 78.35)	< .0001
TIMP3	76.10 (76.03 - 76.54)	77.48 (76.69 - 78.07)	< .0001
TMEFF2	75.85 (75.84 - 75.88)	77.39 (76.31 - 78.08)	< .0001

[a]Mann Whitney test

Supplementary Table 3. The median (interquartile range) amount of methylation (T50) in columnar biopsies from patients on standard PPI and following 40 mg twice-daily (high-dose) esomeprazole for 6 months

	Standard PPI	High-dose esomeprazole	Adjusted P-value[a]
APC	79.66 (79.16 - 80.29)	79.38 (78.83 - 79.89)	.6733
CDKN2A	81.79 (81.76 - 81.96)	81.81 (81.76 - 82.05)	.9975
ID4	83.06 (82.47 - 83.98)	82.59 (81.98 - 83.62)	.2850
MGMT	76.70 (75.73 - 77.53)	76.70 (75.66 - 77.51)	1.000
RBP1	76.10 (75.84 - 76.85)	76.36 (75.94 - 77.07)	.8522
RUNX3	79.20 (78.93 - 80.09)	79.04 (78.78 - 80.17)	.9995
SFRP1	77.44 (76.89 - 78.33)	77.78 (76.64 - 78.37)	1.000
TIMP3	77.33 (76.49 - 78.04)	77.61 (77.08 - 78.10)	.9953
TMEFF2	77.14 (76.33 - 78.41)	77.43 (76.23 - 77.97)	1.000

[a]Wilcoxon signed-rank test adjusted for multiple comparisons by Sidak procedure

CHAPTER 10: CONCLUSIONS

In this thesis I describe (1) the development of a simple method to quantitate DNA methylation, (2) the discovery of novel aberrantly methylated genes that are associated with transcriptional silencing in BE, EAC and ESCC, and (3) the effect of treating gastro-oesophageal reflux surgically by fundoplication or medically with a high-dose of the PPI esomeprazole on the aberrant DNA methylation present in patients with BE.

Development of a simple quantitative assay to measure methylation

There are a number of available methods to measure DNA methylation, each with their advantages and disadvantages. When I commenced my studies methylation-specific PCR (MSP) was one of the more commonly used assays for detection of DNA methylation. Despite it's continuing widespread use, MSP is technically difficult, being prone to false positives (due to, for example, mispriming) and false negatives (if insufficient template). In Chapter 2 I proposed an approach to determine the optimal annealing temperature for MSP primers using a mixture of unmethylated and methylated reference DNA. The sensitivity of an MSP could be improved by using a higher annealing temperature. Another disadvantage of MSP is that it interrogates only a limited number (at most 2-4) of CpGs, and only those located at the 3'-end of the primers. In Chapter 4 I utilized combined bisulfite restriction analysis (COBRA) to analyse methylation of the MT3 promoter. The COBRA method overcomes some of the false positive and false negative issues observed with MSP, as a region of interest is amplified independent of the methylation status, and interrogates CpGs across the length of the amplicon. Some of the disadvantages of COBRA are that it requires multiple steps, and only those CpGs recognised by restriction enzymes can be analysed. To overcome these disadvantages I developed and validated a method utilising melt curve analysis that quantifies the overall methylation of all CpGs within the amplicon in a single step (Chapter 3). Melt curve analysis is a rapid, simple, in-tube method that is robust and reproducible, utilizing easily designed primers and not requiring proprietary algorithms or software. Melt curve analysis has many advantages over other common PCR based techniques, whether for screening for or quantitating methylation. In the remainder of my studies DNA methylation was analysed by melt curve analysis.

Discovery of novel aberrantly methylated genes in oesophageal disease

At the commencement of my project only a few genes were reported as methylated in BE, EAC or ESCC. Techniques for genome-wide analysis of methylation were in their infancy, and were technically demanding and prohibitive because of cost. I attempted a systematic

approach to discover novel methylated genes present in oesophageal diseases. The EAC cell line OE33 and the ESCC cell line TE7 were treated with the demethylating drug 5-aza-2'-deoxycytidine or vehicle, and genes whose expression was increased by treatment were identified using the QIMR 4.5K cDNA microarray (Appendix B4). This approach lead to the discovery that MT3 was frequently methylated in ESCC primary tumours (Chapter 4; Appendix B3), and ID4 was frequently methylated in primary EAC and BE (Chapter 7; Appendix B7). However, this study was disappointing as very few of the genes which were up-regulated on the expression array could be independently validated as methylated. Subsequently the demethylation experiment was repeated for OE33 and the ESCC cell line OE21, and up-regulation of genes was measured on the Affymetrix GeneChip HG-U133 Plus 2.0 expression microarray. This array proved much more reliable, and a significantly higher number of candidate genes were validated as methylated in EAC or ESCC (Chapter 6; Appendix A10). The validation of methylation, prognostic significance, and functional studies on genes identified by this approach are continuing (Appendices B10, B11, and B12).

In Chapter 4 I reported that MT3 was frequently down-regulated in ESCC by DNA methylation. The methylation of the individual CpGs about the transcription start site of MT3 was region-specific in both cell lines and tissues. The biological and functional significance of such region-specific methylation is currently unknown. The density of CpG methylation correlated with the level transcriptional silencing of MT3. These findings emphasized the value of measuring multiple CpGs when investigating control of transcription by DNA methylation, highlighting a potential weakness of MSP as an analytical technique. No correlations were observed between methylation of MT3 and survival time, patient age, gender, smoking or drinking history, tumour stage, volume, or lymph node involvement.

I then examined methylation of TIMP3 in cell lines and in ESCC tissues from Chinese patients (Chapter 5). Methylation of the CpGs about the transcription start site of TIMP3 in cell lines was region-specific, with some regions of methylation correlating with silencing of expression, and others not. Similar findings that methylation of only certain CpGs and not others is associated with gene silencing, has been reported in a few other genes. I observed that both DNA methylation and the loss of expression of TIMP3 were rare in ESCC from Chinese patients. This contrasted with an earlier study of ESCC in Japanese patients which found that decreased TIMP3 protein expression was common in the tumour cells and

correlated with invasive activity and metastasis and decreased patient survival. No measurement of DNA methylation was reported in the Japanese study.

One of the original aims of my thesis was to find prognostic methylation biomarkers which would predict progression from BE to EAC, and could be used to stratify patients according to risk. This would assist in developing a targeted and more cost-effective surveillance strategy. I hypothesized that there would be an increase in the number of aberrantly methylated genes, and the extent of methylation, in the progression from BE through dysplasia to EAC. To achieve this aim I firstly needed to know what methylation changes took place between the different stages of disease. I wanted to identify a panel of genes which were methylated in EAC but not BE or squamous oesophageal epithelium, which would have lead to a search for genes which were methylated only in those BE patients who progressed to high-grade dysplasia or EAC. I investigated candidate genes previously reported as methylated in EAC (APC, CDKN2A, MGMT, RBP1, RUNX3, SFRP1, TIMP3, TMEFF2) and ID4 which I had discovered to be methylated in the first set of demethylation experiments which I had undertaken. I was the first to identify ID4 as methylated in EAC and BE (Chapter 7). I observed that for most of these genes (APC, ID4, MGMT, RBP1, SFRP1, TIMP3 and TMEFF2, but not CDKN2A or RUNX3) aberrant DNA methylation was as frequent in BE as EAC. I reported that BE is a highly abnormal tissue, at least in terms of DNA methylation, more similar to cancer than to squamous oesophageal epithelium. As discussed in the manuscript, there is accumulating evidence that the genome in BE has widespread genomic abnormalities. Although there are several reports of methylation profiles which indicate an increased risk of progression from non-dysplastic to dysplastic BE, these have not been validated prospectively. Based on my results such a profile may lack specificity and sensitivity simply because of the frequency and extent of DNA methylation of genes in BE.

As part of searching for methylated genes which might be useful biomarkers, I discovered other genes which are methylated in BE and EAC, but not in squamous epithelium (Appendices B10 and B11). I identified eight genes (BNIP3, FBN2, ID4, MLF1, PRDM2, RBP4, RARRES1, TFAP2C) that had been reported methylated in other cancers, but not before in BE or EAC, and four genes (CLDN6, DCBLD2, FNBP1 and MGC16824) that had not previously been reported as methylated in any cancer. These findings suggest that the high frequency of methylation in BE compared to squamous epithelium is widespread,

affecting a large number of gene promoters. The recent development of genome-wide methylation analysis platforms, such as the Illumina Infinium Methylation Assay (HumanMethylation27BeadChip) and next generation sequencing of bisulphite modified DNA, will inevitably answer this question. My findings raise a significant question for future studies: what are the factors and mechanisms responsible for the extensive differences in methylation between squamous and non-dysplastic Barrett's epithelium?

Effect of treatment on methylation in Barrett's oesophagus

Given that reflux is an accepted risk factor for BE, I hypothesized that preventing reflux would reduce aberrant DNA methylation in the columnar mucosa of patients with BE. In Chapter 8 I compared methylation of the panel of nine genes (as used in Chapter 7) between subjects who, at least five years following fundoplication, had normal reflux and those with abnormal reflux. There was a significant regression of BE length in those patients with normal levels of reflux, but no regression in those with abnormal reflux. The number of methylated genes correlated with BE length, which is a known risk factor for EAC. There were significantly fewer genes methylated in the pH normal than the pH abnormal group. The reason for this difference in methylation might be that a reduction in reflux resulted in a decreased methylation in the pH normal subjects, or it might be that continuing reflux increased the methylation in the pH abnormal subjects (compared to the levels before the fundoplication). To distinguish between these two possibilities I compared the methylation in the fundoplication groups to that in no surgery subjects, which I would expect to be similar to the pre-operative methylation in our fundoplication groups. I found that the amount of methylation in the no surgery subjects was significantly more than in the pH normal subjects, but did not differ from the pH abnormal subjects. I concluded that long-term reflux control by fundoplication reduced aberrant DNA methylation, rather than preventing an increase over time. A significant implication of this study is that it provides the first published evidence that reflux is directly implicated in the aberrant DNA methylation found in BE. A longitudinal prospective study in which methylation is compared in biopsies obtained from the same patient before and after anti-reflux therapy is required to confirm this.

Finally, in Chapter 9 I examined the effect of treatment for six months with high-dose esomeprazole on methylation of the panel of nine genes (as used in Chapters 7 and 8) in BE patients whose reflux symptoms were uncontrolled by normal doses of PPIs. Their symptoms were well controlled by the high-dose of esomeprazole, and there was a reduction in tissue

inflammation and cell proliferation. However, there was no significant reduction in the extent of DNA methylation of any of the nine genes. These findings suggest that non-acid constituents of the refluxate (eg., bile and enzymes) are likely to be important in causing aberrant methylation, as reducing acid alone did not lead to its reversal. Given the central role of DNA methylation in the genomic changes which occur in the development and progression of cancer, the results suggest that long-term fundoplication has greater potential than medical treatment to reduce the risk of the development of cancer in patients with reflux and BE. A long-term longitudinal study of a large cohort of patients with objective measurement of reflux (oesophageal pH, and impedance or bile testing) comparing surgical with medical anti-reflux therapy is required.

Overview

In the studies described in this thesis I have developed a rapid and robust method to assess DNA methylation, and discovered a number of novel genes which are aberrantly methylated in oesophageal disease including cancer. I have also shown that in non-dysplastic (metaplastic) BE methylation in a number of genes is similar to that found in cancer. I have used DNA methylation to measure the efficacy of the most common surgical and medical treatments for BE, and shown that long-term fundoplication but not short-term high-dose PPI treatment, reverses methylation, which may lead to a reduction in the risk of development of dysplasia and adenocarcinoma. Finally, I have suggested extensions to the work published in this thesis. Further understanding of which genes are methylated in BE, EAC and ESCC, the mechanisms responsible for this aberrant methylation, and the function of the genes, would improve our insight into the underlying biology of oesophageal diseases, and potentially lead to new biomarkers or treatment options.

APPENDIX A: STATEMENT OF AUTHORSHIPS

APPENDIX B: ABSTRACTS PUBLISHED

B1: Methylation of the APC promoter in oesophageal adenocarcinoma

Presented: Australian Gastroenterology Week, Gastroenterological Society of Australia, Adelaide, 15-18 October, 2002.
Published: Journal of Gastroenterology and Hepatology, 17 (Suppl), A186 (2002).

Eric Smith, Paul A. Drew, David I. Watson, Glyn G. Jamieson, Alex Dobrovic

PURPOSE: Oesophageal adenocarcinoma is associated with many genetic changes. Structural changes to the DNA, such as deletions, amplifications or base changes, have been well studied. Hypermethylation of the promoter regions of genes (particularly tumour suppressor genes), which can lead to gene inactivation or silencing by preventing initiation of transcription, is another method by which the phenotype of cells can be altered. We report hypermethylation of the APC gene in this cancer in short and long term survivors.
METHODOLOGY: Regions of oesophageal adenocarcinoma were dissected from paraffin sections prepared from resections from 10 patients. DNA was isolated by standard techniques. Hypermethylation in the APC promoter region was detected by methylation specific PCR, as well as methylation sensitive single strand conformation analysis.
RESULTS: In patients who survived less than 1 year after resection, hypermethylation was detected in tumour in 2 of 4 patients. In patients who survived more than 1 year, hypermethylation was detected in 5 of 6 patients.
CONCLUSIONS: Hypermethylation of the APC promoter region was common in oesophageal adenocarcinoma, but did not correlate with duration of post-operative survival.

B2: Methylation of the APC promoter in adenocarcinoma of the oesophagus

Smith, E., Bianco-Miotto, T., Drew, P.A., Watson, D.I., Jamieson, G.G. & Dobrovic, A. (2002) Methylation of the APC promoter in adenocarcinoma of the oesophagus (abstract). *Proceedings of the Australian Health and Medical Research Congress, Melbourne,* 25 -29 November 2002.

B3: Methylation of MT-3 promoter in oesophageal squamous cell carcinoma

Smith, E., Zi-Qing Tian, Bianco-Miotto, T., De Young, N.J., Drew, P.A., Watson, D.I. & Jamieson, G.G. (2003) Methylation of MT-3 promoter in oesophageal squamous cell carcinoma (abstract 4.2).
Proceedings of The Surgical Research Society of Australasia, Melbourne, 2003.

B4: Microarray transcriptional profiling reveals novel methylated genes in Barrett's associated adenocarcinoma cell lines

Smith, E., Pavey, S., De Young, N.J., Bianco-Miotto, T., Drew, P.A., Hayward, N., Watson, D.I. & Jamieson, G.G. (2003) Microarray transcriptional profiling reveals novel methylated genes in Barrett's associated adenocarcinoma cell lines (abstract P19). *Proceedings of The Surgical Research Society of Australasia, Melbourne, 2003.*

B5: Is methylation a good prognostic marker in oesophageal adenocarcinoma?

Bianco-Miotto, T., Smith, E., Drew, P.A. & Watson, D.I. (2003) Is methylation a good prognostic marker in oesophageal adenocarcinoma? (abstract P15).
Proceedings of The Surgical Research Society of Australasia, Melbourne, 2003.

B6: No Methylation Or Reduction In The Expression Of TIMP3 In Squamous Cell Carcinoma Of The Esophagus From A Region Of High Incidence In China

Presented: Digestive Diseases Week, Los Angeles, 2006.
Published: Gastroenterology 130 (Supplement 2), A415, 2006.

Eric Smith, Maria Caruso, Zi-Qing Tian; Jun-Feng Liu, John J. Kelly, Andrew R. Ruszkiewicz, Paul A. Drew

ABSTRACT BODY: TIMP3 is an inhibitor of the proteolytic activity of matrix metalloproteinases and therefore may have the potential to reduce tumour invasion and metastasis. It has also been reported to induce apoptosis in cancer cells and suppress tumour growth and angiogenesis. It is downregulated in a number of cancers, and linked to a poor outcome. Loss of expression has been associated with methylation of the TIMP3 gene promoter in tumors, including esophageal adenocarcinoma. In a study of patients from Gunma, Japan, the only report of TIMP3 expression in esophageal squamous cell carcinoma, a reduction in its expression was seen in 67% of 90 tumours, which correlated with invasive activity and metastasis. Although methylation was suggested as a possible mechanism for the observed downregulation, it was not assessed in the study. The aim of this study was to measure methylation in the promoter of the TIMP3 gene in squamous cell carcinoma and relate this to any change of gene expression. Tumours and proximal resection margins were obtained from 51 patients operated on for esophageal squamous cell cancer at the 4th Hospital of Hebei Medical University, China. During the follow up period (mean 569 days) 9 patients had died (mean survival of 476 days after the operation), 27 were alive, and 15 were lost to follow up. There were 3 T1 tumours, 11 T2, 30 T3 and 4 T4, with node involvement recorded for 30%, and for 3 no staging information was available. No methylation was detected in the TIMP3 gene promoter in any of the tumour or proximal margin tissues. Expression of TIMP3 was determined by immunohistochemistry. After microwave induced antigen retrieval the sections were stained using a monoclonal antibody to TIMP3 (Chemicon) and the MACH4™ universal probe HRP-polymer kit (Biocare Medical). All cases showed strong heterogeneous cytoplasmic staining in cancer cells. Strong heterogeneous cytoplasmic expression of TIMP-

3 was also observed in the normal proximal resection margin mucosa in 5 cases, but was limited to the basal layers of squamous epithelium. There was no evidence of reduced or partial staining. In this cohort of patients with esophageal squamous cell carcinoma of the esophagus there was no methylation of the TIMP3 gene promoter nor alteration in TIMP3 protein expression, regardless of the stage of or outcome from the disease. Further studies are required to determine if the conflicting results on TIMP3 expression in this cancer are a result of methodological differences, or reflect a difference in aspects of the molecular biology of the disease between regions.

B7: Gene Promoter Methylation Of ID4 And ARL4D In Barrett's Esophagus And Esophageal Adenocarcinoma

Presented: Digestive Diseases Week, Los Angeles, 2006.
Published: Gastroenterology 130 (Supplement 2), A565, 2006.

Eric Smith, Derek J. Nancarrow, Sandra Pavey, Nick K. Hayward, Neville J. De Young, John J. Kelly, David C. Whiteman, B. Mark Smithers, David C. Gotley, Paul A. Drew

INTRODUCTION: DNA methylation has been reported in the promoter region of a number of genes in oesophageal adenocarcinoma and its precursor lesion Barrett's esophagus, and may contribute to the progression to cancer. Analysis of gene methylation is important because there is a need for biomarkers in non-dysplastic Barrett's which might predict risk of progression to cancer, and it may provide insights into the pathogenesis of the disease.
AIM: To determine the frequency of methylation in the promoter region of 10 genes in esophageal tissues and cell lines.
RESULTS: Methylation in the promoter region of 10 genes was measured in 3 oesophageal adenocarcinoma cell lines, single biopsies of Barrett's associated adenocarcinoma from 16 patients (AdCa), and multiple biopsies of metaplastic (BE) and single biopsies of proximal squamous epithelium from 18 patients with Barrett's esophagus. The percentage of patients or cell lines with methylation in each of the genes tested is shown in the table. In patients with Barrett's esophagus from whom multiple biopsies of BE were available, methylation often differed between the biopsies.
DISCUSSION: For the gene promoters examined there was less methylation in the squamous epithelium of the esophagus compared to the metaplastic or adenocarcinoma tissues. There was no consistent relationship between the methylation in the Barrett's and adenocarcinoma tissue. Two genes are of particular interest. ID4 is a member of the inhibitor of DNA-binding (ID) family, which inhibits DNA binding of basic helix-loop-helix transcription factors, and has been suggested to suppress tumour progression and metastasis. ARL4D is a member of the ARF family, and may play a role in membrane-associated intracellular trafficking. This is the first report of methylation of ID4 in Barrett's esophagus and esophageal adenocarcinoma, and of ARL4D in any cancer.

This work is part of the Study of Digestive Health (Australia).

Percentage of patients or cell lines with methylation for each of the genes tested.

	APC	MGMT	RBP1	CDKN2A	TMEFF2	RUNX3	SFRP1	TIMP3	ID4	ARL4D
Squamous	47	13	33	20	0	0	0	0	13	0
BE	100	67	72	50	94	72	100	71	94	10
AdCa	81	50	56	62	69	50	81	56	63	25
Cell lines	33	33	100	0	100	67	100	33	67	33

B8: DNA Methylation In The Esophageal Mucosa Of Patients 5 Or More Years After A Fundoplication For Barrett's Esophagus

Presented: Digestive Diseases Week, Los Angeles, 2006.
Published: Gastroenterology 130 (Supplement 2), A8, 2006.

John J. Kelly, **Eric Smith**, Paul A. Drew, Stuart R. Phillis, Andrew R. Ruszkiewicz, Glyn G. Jamieson

INTRODUCTION: Barrett's esophagus results from reflux and is a major risk factor for adenocarcinoma of the distal esophagus. Aberrant DNA methylation is frequent in Barrett's esophagus and adenocarcinoma and is thought to contribute to the disease progression. Fundoplication protects the oesophagus from reflux and may result in regression of the Barrett's mucosa, but it is not known what effect it has on methylation in the esophagus.
AIM: To test the hypothesis that reflux control by fundoplication in patients with Barrett's esophagus will reduce DNA methylation in the esophageal mucosa.
METHOD: We studied 23 patients with histologically proven Barrett's esophagus preoperatively who had a laparoscopic fundoplication more than 5 years ago. Reflux was measured by 24 hour pH monitoring. Biopsies at 2 cm intervals from any columnar epithelium and squamous epithelium were obtained at endoscopy. Methylation was measured in the promoter region of 9 genes - APC, CDKN2A, ID4, TEMMF2, MGMT, RBP1, RUNX3, SFRP1 and TIMP3.
RESULTS: The median follow up was 8 years (60 - 127 months). Of the 18 patients with no reflux, 17 had an apparent regression of the columnar mucosa. This regression was complete in 9 patients (5 long segment and 4 short segment) and partial in 8 patients. Five patients had reflux and persistent Barrett's esophagus. There was significantly less methylation in the columnar epithelium of patients without reflux compared to those with reflux ($p<0.0001$). The squamous biopsies from patients without reflux but continuing Barrett's had more methylation than either squamous epithelium proximal to the Barrett's in patients with reflux ($p=0.005$), or from patients with complete regression ($p<0.001$). This may reflect the squamous biospies coming from areas in which metaplastic changes are reversing.

CONCLUSIONS: We observed complete regression to squamous mucosa in 50% of patients with a fundoplication and no acid reflux. In patients with complete regression the regenerated squamous had minimal methylation which did not differ from the squamous proximal to Barrett's. In patients with partial regression there was significantly less methylation in the columnar lining compared to the patients with continuing reflux, but more methylation in the squamous regenerating region than in the normal esophagus. The significance of the methylation in the partially regressed esophagus warrants further investigation.

B9: Suppression of Acid Reflux with Double-Dose Esomeprazole for 6 Months does not Alter DNA Methylation in Patients with Barrett's Esophagus

Presented: Digestive Diseases Week, Los Angeles, 2006.
Published: Gastroenterology 130 (Supplement 2), A265, 2006.

Eric Smith, Paul A. Drew, Awni Abu-Sneineh, William C. Tam, Andrew R. Ruszkiewicz, Mark N. Schoeman, John Dent, Richard H. Holloway

INTRODUCTION: Barrett's esophagus is a sequela of longstanding gastroesophageal reflux and is a major risk factor for adenocarcinoma. Aberrant DNA methylation is frequent in Barrett's esophagus and adenocarcinoma and may contribute to the disease progression. AIM: To test the hypothesis that short term high-level acid reflux control by esomeprazole 40 mg bd in patients with Barrett's esophagus will reduce DNA methylation in the esophageal mucosa.

METHOD: Eighteen patients with histologically proven Barrett's esophagus on standard doses of PPI (omeprazole 20 mg, lanseprazole 30 mg or pantoprazole 40 mg) were studied before and after 6 months treatment with esomeprazole 40 mg bd. Esophageal acid exposure was measured by 24 hour pH monitoring before and 2 months after starting esomeprazole. Biopsies were obtained at 2 cm intervals from the columnar epithelium and from the adjacent squamous epithelium of the esophagus before and after 6 months of treatment. In each biopsy methylation was measured in the promoter region of 9 genes - APC, CDKN2A, ID4, HPP1, MGMT, RBP1, RUNX3, SFRP1 and TIMP3.

RESULTS: Nine patients of the 18 patients had excessive (pH>4 of >5% of the time) acid reflux on standard PPI therapy. Esomeprazole 40 mg bd effectively eliminated acid reflux in 16 of 18 patients (pH<4, <1%) and substantially reduced it in the other 2 patients although it remained increased (6.5% and 11.7%). There was significantly more methylation in the columnar epithelium of all patients compared to the squamous epithelium ($p<0.0001$) both before and after 6 months of treatment. However, there was no statistical difference in the methylation in the columnar epithelium of patients who had reflux compared to those that did not have reflux, either before commencement or after 6 months of treatment.

CONCLUSIONS: Effective control of acid reflux with esomeprazole 40 mg bd neither increased nor decreased the amount of methylation in the panel of relevant gene promoters tested, either in patients whose reflux was controlled at the start of the trial, or those with reflux at the start of the trial which was reversed by the treatment. These findings suggest the need for further study to determine the benefit of the control of acid exposure in preventing or reversing abnormal DNA methylation in Barrett's esophagus, and the possible development of adenocarcinoma. (Supported by Grant-in Aid from AstraZeneca)

B10: Novel genes methylated in the oesophageal adenocarcinoma cell line OE33

Presented: 3rd Australian Medical and Health Research Congress, Melbourne, November 26 - December 1 2006.

Paul A. Drew, **Eric Smith**, Neville J. De Young, Stuart J. Phillis, and for the Study of Digestive Health

INTRODUCTION: DNA methylation has been reported in the CpG islands associated with the promoter region of a number of genes, and, by silencing critical genes, may contribute to the progression to cancer. Methylation may be of value as a diagnostic or prognostic biomarker, and may provide insights into the pathogenesis of the disease. To screen for genes methylated in oesophageal adenocarcinoma, we searched for genes in the oesophageal cell line OE33 whose expression was increased when the cells were cultured with the demethylating drug 5-aza-dC. Expression was measured using the Affymetrix HGU133Plus2.0 chip. Methylation was measured by melt curve analysis of bisulphite modified DNA.

RESULTS: A number of genes whose expression was upregulated in drug treated cultures did not have GpG islands associated with their promoters and were not investigated further. Five genes which were upregulated and had promoter CpG islands were investigated further. Each had been described as methylated in other cancers, but not in oesophageal adenocarcinoma. Each was methylated in OE33, and variably in other cancer cell lines. The genes were (1) TFAP2C, a transcription factor required for the development of tissues of ectodermal origin; (2) MLF1, myeloid leukemia factor 1; (3) BNIP3, a pro-apoptotic gene involved in hypoxia-induced cell death; (4) FBN2, a large modular extracellular matrix glycoprotein; and RBP4, retinol binding protein. None of these genes was methylated in the oesophageal squamous cell carcinoma cell line OE21.

CONCLUSION: This is the first report suggesting the methylation of these 5 genes in oesophageal adenocarcinoma.

This project was funded by the NIH as part of the Study of Digestive Health.

B11: Novel aberrantly methylated genes in esophageal adenocarcinoma

Presented: Digestive Diseases Week, Washington DC, 2007.
Published: Gastroenterology 132 (Supplement 2), 322, 2007.

Eric Smith, Neville J. De Young, Andrew R. Ruszkiewicz, Peter G. Devitt, Glyn G. Jamieson, Paul A. Drew

BACKGROUND: The incidence of esophageal adenocarcinoma (EAC) in the Western world has risen dramatically over the last three decades, such that it has the greatest increase of any malignancy and is the most common cancer of the esophagus. EAC is one of the most lethal cancers, with a five year survival of less than twenty percent. The only known risk factor is the premalignant Barrett's metaplasia (BE). Aberrant DNA methylation is frequently observed in BE and EAC, and is thought to contribute to the disease progression and to influence outcome. Knowledge of gene methylation may assist in diagnosis, treatment, and understanding the biology of the disease.
AIM: To discover genes which have expression altered by methylation in EAC.
MATERIALS AND METHODS: The EAC cell line OE33 was demethylated with 5-aza-2'-deoxycytidine (aza-dC). Gene expression was compared between cells grown with and without aza-dC using Affymetrix Human Genome U133 Plus 2.0 Array. The relationship between DNA methylation and gene expression was determined in four esophageal cancer cell lines (TE7, OE19, OE21 and OE33) by melt curve analysis and quantitative real time RT-PCR respectively. Methylation was measured in tumors, Barrett's and squamous mucosa from the resection margin of 25 patients with EAC.
RESULTS: Eleven genes, selected from the array screen, were methylated in one or more esophageal cancer cell lines, but unmethylated in lymphocytes from patients with no known disease. All of these genes were frequently methylated in EAC. Seven of these genes (BNIP3, FBN2, MLF1, PRDM2, RBP4, RARRES1, TFAP2C) have been reported methylated in other cancers, but not BE or EAC. Four genes (CLDN6, DCBLD2, FNBP1 and MGC16824) have not previously been reported as methylated in any cancer. CLDN6 encodes a calcium-independent cell-cell adhesion molecule. DCBLD2, an intracellular cell adhesion molecule, is involved in receptor-mediated signaling, and negative regulation of cell growth.

FNBP1 is involved in protein biosynthesis and MGC16824 is an esophageal cancer associated protein.

CONCLUSION: This study reports significant DNA methylation of four novel genes in EAC and BE. Methylation of these genes has not been reported before in any cancer. Their role in tumorogeneis is unknown and warrants further investigation. (Supported by the NIH as part of the Study of Digestive Health)

Frequency of DNA methylation in tumor, Barrett's and squamous mucosa from patients with esophageal adenocarcinoma

	BNIP3	CLDN6	DCBLD2	FBN2	FNBP1	MGC1624	MLF1	PRDM2	RBP4	RARRES1	TFAP2C
Tumor (n = 25)	15 (60%)	5 (20%)	17 (68%)	22 (88%)	16 (64%)	6 (24%)	22 (88%)	22 (88%)	12 (48%)	9 (36%)	11 (44%)
Barrett's (n = 7)	6 (86%)	0 (0%)	5 (71%)	7 (100%)	5 (71%)	2 (29%)	5 (71%)	7 (100%)	5 (71%)	6 (86%)	2 (29%)
Squamous (n = 22)	10 (45%)	1 (5%)	0 (0%)	7 (32%)	0 (0%)	1 (5%)	1 (5%)	11 (50%)	9 (41%)	6 (27%)	1 (5%)

B12: Aberrant methylation of follistatin-like 1 (FSTL1) in esophageal squamous cell carcinoma.

Presented: Digestive Diseases Week, Washington DC, 2007.
Published: Gastroenterology 132 (Supplement 2), 636, 2007.

Shigeru Tsunoda, **Eric Smith**, Neville J. De Young, Zi-Qing Tian, Jun-Feng Liu, Glyn G. Jamieson and Paul A. Drew

BACKGROUND: Esophageal squamous cell carcinoma (ESCC) is one of the most lethal cancers of the gastrointestinal tract, with a dismal survival rate despite recent advances in multidisciplinary therapy. Aberrant DNA methylation is frequent in ESCC as well, and is thought to contribute to the disease progression and to influence outcome. Knowledge of gene methylation may assist in diagnosis, treatment, and understanding the biology of the disease.
AIM: To discover genes which have expression altered by methylation in ESCC.
MATERIALS AND METHODS: The ESCC cell line OE21 was demethylated with 5-aza-2'-deoxycytidine (aza-dC). Gene expression was compared between cells grown with and without aza-dC, using Affymetrix GeneChip HG-U133 Plus 2.0. Methylation was measured by melt curve analysis, and gene expression by quantitative real time RT-PCR. Six tumor cell lines (PC3, DU145, TE7, OE19, OE21 and OE33) were used to explore the relationship between methylation status and gene expression. Tumors and proximal resection margins were obtained from 65 patients operated on for esophageal ESCC at the 4th Hospital of Hebei Medical University, China.
RESULTS: Twelve genes (CLDN1, NMES1, PHLDA1, FSTL1, DTR, CDH13, DUSP6, TFPI2, DUSP4, TNFRSF10D, THBS1 and CTGF) were selected for further study from the array screen. Among them, 4 genes (FSTL1, CDH13, TFPI2 and TNFRSF10D) showed methylation in melt curve analysis and up-regulation of mRNA expression after aza-dC treatment was confirmed by RT-PCR. Of these follistatin-like 1 (FSTL1) had not previously been reported as methylated in cancer. FSTL1, which is also called follistatin-related protein (FRP) or TSC-36, is the smallest member of the SPARC family, which can down-regulate cancer cell proliferation and migration. Methylation status and mRNA expression correlated in the six cell lines, and aza-dC treatment induced demethylation in the promoter of FSTL1

and increased its mRNA expression in methylated cell lines (DU145, TE7, OE19, OE21 and OE33), but not in an unmethylated cell line (PC3). Moreover, 19/65 (29.2%) of the tumors had methylation in the FSTL1promoter, significantly higher (p=0.0001) than in the matched normal epithelium (4/65)(6.2%).

CONCLUSION: This is the first report of aberrant methylation of FSTL1 in any cancer. This is consistent with FSTL1 being a tumor suppressor in ESCC.

B13: Methylation of Wnt associated genes in carcinoma cell lines.

Presented: ASMR Medical Research Week, Adelaide, June, 2007.

Karissa Phillis, **Eric Smith**, Neville J. De Young, Paul A. Drew

INTRODUCTION: Disruptions to the Wnt signalling pathway are important in the development and progression of many cancers. Hypermethylation of CpG islands associated with gene promoters can repress gene transcription. The measurement of this methylation could provide diagnostic and prognostic biomarkers for these cancers.

METHOD: Eighteen genes associated with the Wnt signalling pathway were selected for analysis. Methylation was measured by melt curve analysis of bisulphite modified DNA that was prepared from ten carcinoma cell lines. The effect of methylation on gene expression was determined by comparing mRNA levels in cells cultured with or without the demethylating agent 5-aza-2'-deoxycytidine.

RESULTS: Methylation was measured in eighteen genes involved with the Wnt signalling pathway. Some have not been reported as methylated in any cancers, including: Bone Morphogenetic Protein 2 (BMP2), a component of the BMP signalling pathway which acts in opposition to the Wnt pathway; NOGGIN, a direct inhibitor of the BMP signalling pathway; ICAT, a β-Catenin interacting protein; PYGO1, a novel gene which interacts with the Wnt signalling pathway within the nucleus; FZD7, a receptor for Wnt ligands and a protein phosphatase, PP2A. Several genes whose methylation has been reported in other cancer cell lines were also found to be methylated in a number of oesophageal cancer cell lines, including AXIN2, a protein involved in the phosphorylation of β-Catenin and DKK3, DACT1 and WIF1 which are all secreted Wnt antagonists.

CONCLUSION: This is the first report of methylation in a number of genes in the Wnt signalling pathway in carcinoma cell lines. This methylation could provide insight into the development of these cancers or aid in the identification of biomarkers.

B14: Antireflux surgery reduces aberrant DNA methylation in Barrett oesophagus.

Presented: 4th Australian Medical and Health Research Congress, Brisbane, November 16 - December 21 2008.

Eric Smith, John J. Kelly, Glyn G. Jamieson and Paul A. Drew

INTRODUCTION: Barrett's oesophagus is a metaplastic change from squamous to columnar epithelium resulting from reflux, and is a major risk factor for oesophageal adenocarcinoma. Aberrant DNA methylation is frequent in Barrett's oesophagus and adenocarcinoma and is thought to contribute to the disease progression. Fundoplication is an operation which protects the oesophagus from reflux, but it is not known what effect it has on DNA methylation in the oesophagus.

AIM: To determine if reflux suppression by fundoplication in patients with Barrett's oesophagus reduces DNA methylation in the oesophageal mucosa.

METHOD: We studied 41 patients with histologically proven Barrett's oesophagus who had a fundoplication more than 5 years ago. Reflux was measured by 24 hour pH monitoring. Biopsies were taken from the squamous and at 2 cm intervals from any columnar epithelium. Methylation was measured in the promoter region of 9 genes.

RESULTS: The median age was 55 years (range 34 - 88), and follow up was 8 years (5.4 - 12.6). Of the 41 patients, 31 had no reflux or reflux in the normal range, and 10 had abnormally high reflux. There was no difference in methylation of squamous mucosa between patients with or without reflux. There was more methylation in the columnar tissues compared to the squamous. More genes were methylated in the columnar mucosa from patients with reflux compared to those without ($P = 0.0061$). There was a significant correlation between the number of methylated genes and the percent time $pH < 4$ ($P = 0.0231$). There was a significant correlation between the number of methylated genes in each biopsy and the length of columnar mucosa ($P < 0.0001$).

CONCLUSION: DNA methylation is frequent in the columnar mucosa of Barrett's patients with reflux. Abnormal reflux is associated with maintenance of methylation in the columnar mucosa.

B15: Changes in oesophageal mucosa in patients 5 or more years after a fundoplication for Barrett's oesophagus.

Presented: International Society for Diseases of the Esophagus, 11th World Congress, Budapest, September 10-13, 2008.

John J. Kelly, Glyn G. Jamieson, Jennifer C. Myers, **Eric Smith**, Paul A. Drew and Andrew R. Ruszkiewicz

INTRODUCTION: Barretts oesophagus results from reflux and is a major risk factor for adenocarcinoma of the distal oesophagus. Aberrant DNA methylation is observed in Barretts oesophagus and adenocarcinoma and may contribute to the disease progression. Fundoplication protects the oesophagus from reflux and may result in regression of the Barretts mucosa but it is not known what effect it has on methylation.

METHOD: Forty five patients with preoperative histologically proven Barrett's oesophagus 5 or more years following laparoscopic fundoplication underwent 24 hour pH monitoring followed by endoscopy. Biopsies were taken at 2 cm intervals in columnar epithelium and the adjacent squamous epithelium. Methylation was measured in the promoter region of 9 genes - APC, CDKN2A, ID4, HPP1, MGMT, RBP1, RUNX3, SFRP1 and TIMP3.

RESULTS: The median follow up was 8 years (60 - 127 months). An oesophageal pH of < 4 for < 4% of the time was seen in 33 (73%) patients. Median length of Barretts fell from 4 (1-13)cm preoperatively to 1 (0-12)cm at follow up ($p = 0.0001$). Intestinal metaplasia was less frequent when acid reflux was controlled 33%vs75% but chronic inflammation was consistent regardless of acid control. DNA methylation was significantly less in the columnar epithelium of patients without reflux compared to those with reflux ($p<0.0001$).

CONCLUSIONS: The elimination of abnormal reflux correlates with regression of Barretts oesophagus. Changes in aberrant DNA methylation may reflect reversing metaplastic change but chronic inflammation persists.

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