Aberrant DNA Methylation In Oesophageal Cancer And Barrett's Oesophagus

by

Eric Smith

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

A thesis by publication submitted for the degree of

Doctor of Philosophy

Discipline of Surgery, School of Medicine

Faculty of Health Science

The University of Adelaide

April 2010

TABLE OF CONTENTS

TABLE OF CONTENTS ..... **i**

ABSTRACT ..... **iv**

DECLARATION ..... **vi**

ACKNOWLEDGMENTS ..... **viii**

ABBREVIATIONS ..... **ix**

CHAPTER 1: INTRODUCTION **1**

1.1 Thesis overview 2

1.2 Cancer of the oesophagus 2

1.2.1 Introduction 2

1.2.2 Oesophageal squamous cell carcinoma 2

1.2.3 Oesophageal adenocarcinoma 3

1.2.4 Treatment of oesophageal cancer 4

1.2.5 Barrett's oesophagus 5

1.2.5.1 Incidence and risk factors for Barrett's oesophagus 6

1.2.5.2 Barrett's oesophagus – a precursor to oesophageal adenocarcinoma 7

1.2.5.3 Treatment of Barrett's oesophagus 8

1.2.5.3.1 Medical treatment of Barrett's oesophagus 8

1.2.5.3.2 Surgical treatment of Barrett's oesophagus 9

1.2.5.3.3 Ablation and mucosal resection for Barrett's oesophagus 11

1.2.5.4 Risk stratification in Barrett's oesophagus 11

1.3 DNA Methylation 13

1.3.1 DNA methylation is an epigenetic change 13

1.3.2 Mechanisms of DNA methylation-mediated transcriptional repression 14

1.3.3 DNA methyltransferases catalyse DNA methylation 15

1.3.4 Altered DNA methylation in cancer 15

1.3.5 Measurement of DNA methylation 17

1.3.6 Identifying genes with aberrant DNA methylation 19

1.3.7 DNA methylation in oesophageal cancer and its precursors 21
1.3.7.1 DNA methylation in oesophageal adenocarcinoma and Barrett's oesophagus. 21
1.3.7.2 DNA methylation in oesophageal squamous cell carcinoma 22
1.3.8 Clinical applications of DNA methylation..... 23
1.3.8.1 DNA Methylation and Predicting Progression to Cancer 23
1.3.8.2 Methylated DNA in serum or plasma from oesophageal cancer patients..... 24
1.3.8.3 DNA methylation as a predictor of survival in oesophageal cancer..... 25
1.3.8.4 Demethylation as a therapeutic intervention 26
1.4 Aims of the Study..... 26

CHAPTER 2: METHOD FOR OPTIMIZING METHYLATION-SPECIFIC PCR 29

CHAPTER 3: QUANTITATION OF DNA METHYLATION BY MELT CURVE ANALYSIS 33

CHAPTER 4: METALLOTHIONIEN 3 EXPRESSION IS FREQUENTLY DOWN-REGULATED IN OESOPHAGEAL SQUAMOUS CELL CARCINOMA BY DNA METHYLATION 47

CHAPTER 5: METHYLATION OF TIMP3 IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA ... 59

CHAPTER 6: METHYLATION OF CLDN6, FBN2, RBP1, RBP4, TFPI2, AND TMEFF2 IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA 69

CHAPTER 7: SIMILARITY OF ABERRANT DNA METHYLATION IN BARRETT'S ESOPHAGUS AND ESOPHAGEAL ADENOCARCINOMA 79

CHAPTER 8: THE EFFECT OF LONG TERM CONTROL OF REFLUX BY FUNDOPLICATION ON ABERRANT DNA METHYLATION IN PATIENTS WITH BARRETT'S ESOPHAGUS 93

CHAPTER 9: EFFECT OF HIGH-DOSE ESOMEPRAZOLE ON GASTRIC AND ESOPHAGEAL ACID EXPOSURE AND MOLECULAR MARKERS IN BARRETT'S ESOPHAGUS 115

CHAPTER 10: CONCLUSIONS 139

APPENDIX A: STATEMENT OF AUTHORSHIPS 145

APPENDIX B: ABSTRACTS PUBLISHED 205

B1: Methylation of the APC promoter in oesophageal adenocarcinoma..... 206
B2: Methylation of the APC promoter in adenocarcinoma of the oesophagus 207

B3: Methylation of MT-3 promoter in oesophageal squamous cell carcinoma 208
B4: Microarray transcriptional profiling reveals novel methylated genes in Barrett's associated adenocarcinoma cell lines..... 209
B5: Is methylation a good prognostic marker in oesophageal adenocarcinoma?..... 210
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..... 211
B7: Gene Promoter Methylation Of ID4 And ARL4D In Barrett's Esophagus And Esophageal Adenocarcinoma 213
B8: DNA Methylation In The Esophageal Mucosa Of Patients 5 Or More Years After A Fundoplication For Barrett's Esophagus..... 215
B9: Suppression of Acid Reflux with Double-Dose Esomeprazole for 6 Months does not Alter DNA Methylation in Patients with Barrett's Esophagus 217
B10: Novel genes methylated in the oesophageal adenocarcinoma cell line OE33..... 219
B11: Novel aberrantly methylated genes in esophageal adenocarcinoma 220
B12: Aberrant methylation of follistatin-like 1 (FSTL1) in esophageal squamous cell carcinoma. 222
B13: Methylation of Wnt associated genes in carcinoma cell lines. 224
B14: Antireflux surgery reduces aberrant DNA methylation in Barrett oesophagus. 225
B15: Changes in oesophageal mucosa in patients 5 or more years after a fundoplication for Barrett's oesophagus. 226

BIBLIOGRAPHY 227

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

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

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

The author acknowledges that copyright of published works contained within this thesis (as listed below) resides with the copyright holder(s) of those works.

1. **Eric Smith**, Tina Bianco-Miotto, Paul A. Drew, David I Watson. Method for optimizing methylation-specific PCR. *Biotechniques. 2003; 35: 32-33.* Copyright © 2003 Smith et al.
2. **Eric Smith**, Michael E. Jones, Paul A. Drew. Quantitation of DNA methylation by melt curve analysis. *BMC Cancer 2009; 9: 123.* Copyright © 2009 Smith et al; licensee BioMed Central Ltd.
3. **Eric Smith**, Paul A. Drew, Zi-Qing Tian, Neville J. De Young, Jun-Feng Liu, George C. Mayne, Andrew R. Ruszkiewicz, David I. Watson, Glyn G. Jamieson. Metallothionien 3 expression is frequently down-regulated in oesophageal squamous cell carcinoma by DNA methylation. *Molecular Cancer 2005; 4: 42.* Copyright © 2005 Smith et al; licensee BioMed Central Ltd.
4. **Eric Smith**, Neville J. De Young, Zi-Qiang Tian, Maria Caruso, Andrew R. Ruszkiewicz, Jun-Feng Liu, Glyn G. Jamieson, Paul A. Drew. Methylation of TIMP3 in esophageal squamous cell carcinoma. *World Journal of Gastroenterology 2008; 14: 203-210.* Copyright © 2008 WJG; all rights reserved.
5. Shigeru Tsunoda, **Eric Smith**, Neville J. De Young, Xian Wang, Zi-Qing Tian, Jun-Feng Liu, Glyn G. Jamieson, Paul A. Drew. Methylation of CLDN6, FBN2, RBP1, RBP4, TFPI2, and TMEFF2 in esophageal squamous cell carcinoma. *Oncology*

Reports 2009; 21: 1067-1073.

6. **Eric Smith**, Neville J. De Young, Sandra J. Pavey, Nicholas K. Hayward, Derek J. Nancarrow, David C. Whiteman, B. Mark Smithers, Andrew R Ruszkiewicz, Andrew D. Clouston, David C. Gotley, Peter G. Devitt, Glyn G. Jamieson, Paul A. Drew. Similarity of aberrant DNA methylation in Barrett's esophagus and esophageal adenocarcinoma. *Molecular Cancer 2008; 7: 75*.

7. **Eric Smith**, John J. Kelly, Andrew R. Ruszkiewicz, Thomas Sullivan, Paul A. Drew, Glyn G. Jamieson. The effect of long term control of reflux by fundoplication on aberrant DNA methylation in patients with Barrett's esophagus. *Accepted in Annals of Surgery, 2009*.

8. Awni Abu-sneineh, William Tam, Mark Schoeman, Robert Fraser, Andrew R. Ruszkiewicz, **Eric Smith**, Paul A. Drew, John Dent, Richard H. Holloway. Effect of high-dose esomeprazole on gastric and esophageal acid exposure and molecular markers in Barrett's esophagus. *Submitted to Gastroenterology, 2010*.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Eric Smith

8 April 2010

ACKNOWLEDGMENTS

I am grateful for this opportunity to acknowledge all those who have made this thesis possible.

With much gratitude I thank my supervisors Dr Paul Drew and Professor Glyn Jamieson for their encouragement, support, invaluable guidance and patience.

I thank my numerous collaborators, co-authors and all those who were members of the Discipline of Surgery during my journey. During the period that these studies were carried out I was employed by the University of Adelaide. Without this position I would not have pursued this project.

In particular I acknowledge my friends and family, especially my wife Catherine and my daughter Madeleine, for their constant support and encouragement.

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