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CLINICAL SIGNIFICANCE AND MOLECULAR FEATURES OF THE SERRATED COLORECTAL CANCER PATHWAY

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A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Surgery University of Auckland

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Dr James Church
Associate Professor Matthew Kalady

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DEDICATION

My ever loving and ever supportive parents:

My mother, Mary Liang, who gave me the freedom to dream and be who I am, she inspires me with her passion for higher education.

My father, Joe Liang, a natural-born teacher who taught me to think beyond the boundaries. He inspires me with his strength and perseverance, and has over the years shown me his earnest heart that always strives to improve the lives of others.

My siblings and sisters-in-law:

Michael and Mary Liang and James and Tina Liang, whose support, resoluteness and grace continually embrace me.

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Thank you Lord, for your words which are my cornerstone and thank you for your people, brothers and sisters, whom you have sent onto my path especially when things are getting tough.

"With men this is impossible; but with God all things are possible"

- Matthew 19:26
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Summary

Colorectal cancer (CRC) affects approximately 150,000 Americans and 2716 New Zealanders annually, and approximately one third of Americans and 45 percent of New Zealanders will eventually succumb to the condition. In the past, CRC has been one of the best understood malignancies in terms of its molecular alterations. The first step of carcinogenesis involves the development of pre-malignant lesions (polyps) in the colonic mucosa. Certain histological features of the polyp determine its malignant potential, and the risk of cancer will be significantly reduced if all the precursor lesions are removed endoscopically. However, despite close surveillance, many studies have shown that a significant number of CRCs will still develop. This could be because some cancers are missed on prior endoscopy, their precursor lesions are not easily recognised at the time of colonoscopy, or they may be a result of rapid development of cancer. There are two major polyp subtypes which serve as precursors to colorectal cancers (adenoma and serrated polyps). Colorectal cancers mostly arise from adenomas but a lesser proportion can arise from serrated polyps. Once thought to be harmless, the spectrum of serrated colorectal polyps includes some potentially dangerous lesions, such as the
sessile serrated adenoma/polyp (SSA/P) and the traditional serrated adenoma (TSA). SSA/Ps develop through a distinct molecular pathway featuring proto-oncogene B-Raf or v-Raf murine sarcoma viral oncogene homolog B (BRAF) mutations, hypermethylation of CpG sites in gene promoters, and loss of expression of mismatch repair gene MLH1. The first part of this thesis focuses on determining the natural history of serrated neoplasms, and the endoscopic detection and treatment of these lesions in Cleveland Clinic, Ohio. The main body of the thesis is based on the hypothesis that hypermethylation of CpG islands results in epigenetic silencing of specific microRNAs (miRNAs) that function as tumour suppressors, and this silencing results in increased cell growth and development of serrated neoplasia.

**Methods and Results**

In the first part of thesis I have determined the natural history of the serrated polyps, compared their detection rate with the adenoma detection rate, and have demonstrated that endoscopic removal of these precursor lesions is feasible. microRNAs are post-transcriptional regulators that may function as tumor suppressors. Previously, with global DNA methylation screening, miR-1247 is found to be hypermethylated in three methylated tumors when compared with three non-methylated tumors and normal colonic mucosa. By TaqMan® quantitative polymerase chain reaction (PCR), this was further confirmed in 20 tumours of each group. Treatment with the DNA
de-methylation reagent, 5-Aza-2′-deoxycytidine (5′-Aza), has resulted in upregulation of miR-1247 expression in CRC cell lines. Transiently transfected miR-1247 cells have shown reduced cell growth and less cell migration \textit{in vitro}. Moreover, stable transfected miR-1247 cells have significantly impaired tumour development when implanted into immunocompromised mice. These results strongly suggest that miR-1247 is a tumor suppressor. Demethylation with 5′-Aza has significantly decreased tumor growth in nude mice. The predicted gene target of miR-1247 is E3 ubiquitin-protein ligase MYCBP2 also known as myc-binding protein 2 or protein associates with myc (PAM), a significant decrease in MYCBP2 protein levels has been seen in cells transfected with miR-1247. Moreover, inhibition of endogenous miR-1247 by a miR inhibitor significantly has increased MYCBP2 expression. Direct targeting of MYCBP2 3′UTR by miR-1247 is confirmed with luciferase assays. Most importantly, with immunohistochemistry staining, a reciprocal relationship between MYCBP2 expression and miR-1247 level in seven methylated versus non-methylated cancers has been demonstrated.

\textbf{Conclusion}

This thesis has demonstrated that epigenetic regulation plays an important part in the tumorigenesis of serrated CRC. DNA hypermethylation is a key feature in this subtype of colorectal CRC, which in turn suppresses miR-1247 expression and causes an increase in MYCBP2 and its downstream MYC.
protein. The findings of this thesis open up a whole new area for therapeutic approaches in the treatment of serrated colorectal cancer.
# TABLE OF CONTENTS

CHAPTER I – INTRODUCTION 22

CHAPTER II – SERRATED POLYP PATHWAY 25

2.1 Definition and classification of serrated lesions 25

2.2 Hyperplastic polyps 26
   2.2.1 Risk factors and epidemiology 27
   2.2.2 Endoscopic appearance 28
   2.2.3 Histological appearance 29
   2.2.4 Clinical approach 31

2.3 Sessile serrated adenoma/polyp without dysplasia 33
   2.3.1 Endoscopic appearance 34
   2.3.2 Histological appearance 35

2.4 Sessile serrated adenoma/polyp with dysplasia 37

2.5 Traditional serrated adenoma 38
   2.5.1 Endoscopic appearance 38
   2.5.2 Histological appearance 38

2.6 Rationale for clinical research 39

CHAPTER III – ADVANCED SERRATED LESIONS OF THE COLON AND RECTUM 41

3.1 Introduction 41

3.2 Methods 43

3.3 Results 44

3.4 Discussion 48

3.5 Summary 53
CHAPTER IV – SERRATED POLYP DETECTION RATE VERSUS ADENOMA DETECTION RATE DURING COLONOSCOPY 55

4.1 Introduction 55
4.2 Methods 57
4.3 Results 58
4.4 Discussion 59
4.5 Summary 64

CHAPTER V – SNARING LARGE SERRATED POLYPS 65

5.1 Introduction 65
5.2 Methods 66
5.3 Results 68
5.4 Discussion 71
5.5 Summary 76

CHAPTER VI – SERRATED PATHWAY COLORECTAL CANCERS 77

6.1 Historical evidence 77
6.2 Recent evidence 79
6.3 Unpublished date from the Cleveland Clinic laboratory 81
6.4 Guidelines for post-polypectomy surveillance 82
6.5 Summary 83

CHAPTER VII – MOLECULAR MECHANISMS UNDERLYING COLORECTAL CANCERS 85

7.1 Key molecular pathways 85
   7.1.1 Classical "suppressor" pathway 85
   7.1.2 Microsatellite instability-high pathway 86
   7.1.3 Mild "mutator" microsatellite instability-low pathway 86
7.2 Molecular alterations in serrated pathway lesions 87
   7.2.1 MAPK-ERK pathway 87
   7.2.2 Alterations in regulation of apoptosis 89
   7.2.3 Abnormal methylation 90
   7.2.4 Microsatellite instability 92
   7.2.5 Key molecular changes in colorectal polyps 94
7.3 miRNAs and colorectal cancer 96
7.4 Epigenetic changes in serrated pathway 101
   7.4.1 miRNA and methylation 101
   7.4.2 DNA methylation 101
   7.4.3 Epigenetics and regulation of methylation 103
7.5 Rationale for laboratory research 106

CHAPTER VIII – IDENTIFICATION OF miRNAs RELEVANT IN THE SERRATED COLORECTAL CANCER PATHWAY 107

CHAPTER IX – TUMOUR-SUPPRESSIVE FUNCTIONS OF miR-1247 AND ITS TARGET PATHWAY 119
9.1 Introduction 119
9.2 Methods and materials 120
   9.2.1 Patient and sample collection 120
   9.2.2 Antibodies and reagents 121
   9.2.3 Cell lines and miR transfection 122
   9.2.4 Taqman quantitative PCR 122
   9.2.5 Genomic DNA isolation, bisulphite conversion and methylation specific PCR 122
   9.2.6 Cell proliferation, migration and anoikis assays 124
   9.2.7 3’UTR luciferase reporter assays 124
   9.2.8 Immunohistochemistry 125
   9.2.9 Immunofluorescence 126
   9.2.10 Lentivirus packaging and infection 126
9.2.11 Xenografts and treatment with 5’-Aza 127

9.3 Statistical analysis 127

9.4 Results 128

9.4.1 miR-1247 expression is suppressed in patients methylated colon cancer as well as in the corresponding immortal colon cancer cell lines 128

9.4.2 miR-1247 inhibits colon cancer cell proliferation via cell apoptosis 130

9.4.3 miR-1247 regulates colon cancer cell motility 134

9.4.4 DNA methylation regulates miR-1247 expression and in turn proliferation and migration of colorectal cancer cells 138

9.4.5 miR-1247 regulates colorectal cancer cell xenograft progression in vivo 141

9.4.6 miR-1247 targets MYCBP2 in colon cancer 146

9.5 Discussion 149

CHAPTER X – SUMMARY AND DISCUSSION 158

10.1 Summary of clinical research 158

10.2 Summary of experimental findings 160

10.3 Clinical implications 163

10.4 Future perspective and conclusion 168

CHAPTER XI– APPENDIX- LABORATORY MATERIALS AND METHODS 169

11.1 Human tissue samples 169

11.2 Colorectal cancer cell lines 169

11.2.1 Selection 169

11.2.2 Origin 170

11.2.3 Maintenance 171
11.3 DNA and RNA purification 172
   11.3.1 Purification of DNA from cell lines and human tissues 172
   11.3.2 RNA extraction 175
   11.3.3 Estimation of DNA and RNA concentration 178

11.4. Techniques for studying DNA methylation 179
   11.4.1 5-Aza-2'-deoxycytidine treatment 179
   11.4.2 Bisulfite conversion 180
   11.4.3 MethyLite primer design and sequence 182
   11.4.4 Protocol: MethyLite 184
   11.4.5 Methylation-specific PCR primer design and sequence 187
   11.4.6 Protocol: Methylation-specific PCR 189

11.5 DNA polyacrylamide gel electrophoresis 191
   11.5.1 Buffers and solution 191
   11.5.2 DNA polyacrylamide gel electrophoresis protocol 192

11.6 Real-time quantitative PCR 194
   11.6.1 Instrument 194
   11.6.2 Reverse transcription of microRNA 198
   11.6.3 Quantitative real-time PCR: Taqman analysis 199
   11.6.4 Data analysis (ΔΔCT) 200

11.7 Transient transfection of cell lines 202

11.8 Cell proliferation assays 203

11.9 Annexin V apoptosis assay 204
   11.9.1 Principles 204
   11.9.2 Apoptosis assay protocols 205

11.10 Cell migration assay 207

11.11 Western blot 208

11.12 Immunohistochemistry 211

11.13 Immunofluorescence 213

11.14 Lentiviral infection 214

11.15 Subcutaneous xenografts 215
TABLES

Table 2.1 World Health Organisation classification of serrated lesions 26

Table 3.1 Patient demographics and indications for colonoscopy 45

Table 3.2 Polyp location, size and shape 46

Table 3.3 Treatment and follow-up of advanced serrated polyp 47

Table 4.1 Serrated polyp detection rate and adenoma detection rate for all examinations and for average risk screening examinations only 59

Table 4.2 Pearson correlation coefficient for relationship between SDR and ADR in colonoscopies done for average risk screening 59

Table 5.1 Polyp location, size and outcomes of polypectomy according to polyp histology 70

Table 5.2 Subtypes of serrated polyps of the large bowel 71

Table 5.3 Recent series describing resection of large adenomas 72

Table 6.1 Colonoscopy surveillance 83

Table 7.1 Summary of key molecular changes in colorectal polyps 95

Table 7.2 Comparison of the molecular profiles of serrated polyps, traditional colonic adenomas and colorectal cancers 96

Table 11.1 Profiling of colorectal cancer cell lines 170

Table 11.2 MethyLite primers for miR-1247 183

Table 11.3 PCR conditions 186
Table 11.4 Methylation-specific PCR primers of miR-1247 and miR-1258 promoter regions 189

Table 11.5 Components of methylation-specific PCR system 190

Table 11.6 Methylation-specific PCR cycle settings 191

Table 11.7 Recipe for sodium dodecyl sulphate polyacrylamide gel electrophoresis gel 192

Table 11.8 Terminology of TaqMan quantitative PCR 197

Table 11.9 Components of the TaqMan relative quantitation of microRNAs 198

Table 11.10 Components of microRNA reverse transcription 199

Table 11.11 Components of the TaqMan relative quantitation of microRNAs 200
FIGURES

Figure 2.1 Features of hyperplastic polyps 30

Figure 2.2 Histological appearances of serrated neoplasia 36

Figure 2.3 Sessile serrated polps/adenoma with dysplasia 38

Figure 7.1 Pathways of microRNA (miRNA) biogenesis and mechanism of action 98

Figure 7.2 Model of the colorectal cancer network showing the role of miRNAs and mutations in colorectal cancer pathogenesis 101

Figure 7.3 Regulation of gene transcription by DNA methylation 104

Figure 7.4 Suppression of DNMT by 5’-Aza 105

Figure 8.1 Mapping and comparisons of DNA methylomes in normal colon, non-CIMP, CIMP colon cancers 110

Figure 8.2 Top predicted pathway for miR-1247 114

Figure 8.3 Predicted gene target for miR-1247 in the Wnt pathways 115

Figure 8.4 Top predicted pathway for miR-1258 116

Figure 8.5 Predicted gene target for miR-1258 in the Wnt pathways 117

Figure 9.1 Expression of miR-1247 the colorectal cancer cell lines 129

Figure 9.2 miR-1247 regulates methylated CRC cells’ proliferation via promoting cell apoptosis 132

Figure 9.3 miR-1247 regulates methylated CRC cell proliferation via
promoting cell apoptosis 133

**Figure 9.4** miR-1247 regulates colorectal cancer cell motility 136

**Figure 9.5** Colorectal cancer cell motility is regulated by miR-1247 137

**Figure 9.6** miR-1247 expression in colon cancer cells is regulated by DNA methylation 139

**Figure 9.7** DNA methylation regulates viability and motility of methylated colon cancer cells 140

**Figure 9.8** Overexpression of miR-1247 expression suppresses growth of the colorectal cancer xenograft 142

**Figure 9.9** miR-1247 or control virus, along with luciferase, was introduced into LS411N cells by lentiviral infection 143

**Figure 9.10** 5′-Aza treatment suppresses growth of colorectal cancer xenografts 145

**Figure 9.11** TargetScan was used to predict putative coding gene target of miR-1247 146

**Figure 9.12** MYCBP2 is the direct target of miR-1247 in colorectal cancer 148

**Figure 9.13** MYCBP2 mRNA level correlates with poorer survival in colorectal cancer 152

**Figure 10.1** Changes in expression of miRNA in colorectal adenomas, colorectal carcinomas, and their correlation with Union for International Cancer Control stage or survival 165
Figure 11.1 CpG island in the promoter region of the has-mir-1247 gene 182

Figure 11.2 CpG island in the promoter region of the has-mir-1258 gene 188

Figure 11.3 Real-time quantitative PCR instruments and workflow 194

Figure 11.4 Representative amplification plot 196

Figure 11.5 Instruments and principles for Transwell® migration assays 207
ABBREVIATIONS

ACF-H, aberrant crypt foci
5'Aza 5-Aza-2'-deoxycytidine
AZA, azacitidine
APC, Adenomatous polyposis coli
ADR, adenoma detection rate
BMBC, brain metastatic breast cancer
BRAF, proto-oncogene B-Raf or v-Raf murine sarcoma viral oncogene homolog B
CIMP, CpG island methylator phenotype
CIMP-L, low-level CIMP
CIMP-H, high-level CIMP
CRC, Colorectal cancer
Ct, cycle threshold
DNMT, DNA methyltransferase
FITC, fluorescein isothiocyanate
GFP, green fluorescent protein
HP, hyperplastic polyp
miRNAs, microRNAs
MSI, microsatellite instability
**MSI-L**, microsatellite instability-low

**MSI-H**, microsatellite instability-high

**MSP**, methylation specific PCR

**MSS**, microsatellite stable

**MYCBP2**, E3 ubiquitin-protein ligase MYCBP2 or myc-binding protein 2 or protein associated with myc (PAM)

**PI**, propidium iodide

**SDR**, serrated polyp detection rate

**SSA**, sessile serrated adenoma

**SA**, serrated adenoma

**SSA/P**, sessile serrated adenoma/polyp

**PCR**, polymerase chain reaction

**TEMED**, tetramethylethylenediamine

**TBE**, Tris/Borate/EDTA

**TSA**, traditional serrated adenoma

**UTR**, untranslated region
 CHAPTER I
INTRODUCTION

Colorectal cancer (CRC) affects approximately 150,000 Americans and 2716 New Zealanders annually. Approximately one third of Americans and 45% of New Zealanders with the disease will eventually succumb to it (1, 2). The high prevalence and natural history of colorectal cancer lends itself well to population screening. Endoscopy has evolved to the extent that small precursor lesions can now be clearly identified and readily removed by polypectomy, thereby reducing subsequent morbidity and mortality (3-6).

There are 2 major types of colorectal epithelial polyps (adenomas and serrated polyps). Until recently, serrated polyps were known as hyperplastic polyps and were considered innocuous. In 1990, Longacre and Fenoglio-Preiser (7) coined the term "serrated adenoma" to describe serrated-appearing polyps with dysplastic cytology. Later, Torlakovic et al subdivided these polyps further into traditional serrated adenomas (TSAs) and sessile serrated adenomas (SSAs), with the latter lacking uniform cytologic dysplasia (8). These lesions are now referred to as "sessile serrated adenoma/polyps" (SSA/Ps) (9).

CRC is one of the best understood malignancies in terms of its stepwise
molecular alterations (10). The first step of carcinogenesis involves development of a pre-malignant neoplasm (polyp) in the colonic mucosa. Certain histological features of the polyp determine its malignant potential such as findings of adenoma with villous histology or high grade dysplasia or ≥ 10 mm in size, or ≥ 3 adenomas; serrated polyps ≥ 10 mm or with dysplasia (11-14) and the incidence and mortality from cancer is significantly reduced if the precursor lesion is removed endoscopically (15).

However, many studies have shown that a significant number of CRCs will still develop despite close surveillance (6, 13). This can be attributed to some cancers being missed on previous endoscopy or their precursor lesions not being easily recognised at the time of colonoscopy, or they may be a result of rapid development of cancer (13, 16) Once thought to be harmless, the spectrum of serrated colorectal polyps includes some potentially dangerous lesions, including SSA/Ps and TSAs. SSA/Ps develop via a distinct molecular pathway featuring BRAF mutations and promoter hypermethylation of tumour suppressor genes and DNA repair genes. Large SSA/Ps are usually right-sided, and it has been suggested that missed serrated polyps are at least partially responsible for the apparent failure of screening colonoscopy to protect against development of right-sided cancer (17, 18). The recognition of serrated polyps and their contribution to CRC underscores the importance of their detection and management during colonoscopy. This field is rapidly
evolving secondary to recent breakthroughs in our understanding of the molecular basis of SSA/Ps and associated cancers.

This PhD research dissertation contains both clinical studies and laboratory work. In the clinical section, the focus is on understanding the natural history of serrated polyps. It also compares detection rates of serrated polyps and classical adenomas on endoscopy. Lastly, outcomes of endoscopic management of large sessile serrated polyps and classical adenoma are reviewed. The second part of the dissertation describes our laboratory work identifying key miRNAs as potential tumour suppressors in the serrated neoplasia pathway. Specifically, I have investigated miRNA expression levels in human CRCs and in CRC cell lines, and attempted to identify the anti-tumour function of miRNAs in CRC cell lines. This work confirms hypermethylation of DNA as a key regulatory mechanism in the expression of miRNAs in serrated CRC cell lines. Finally, the anti-tumour effects of these miRNAs are explored in vivo, and miRNA targets and their associated pathways are identified and validated.

With increased understanding of the biological nature of different subtypes of CRC, we will be better equipped when designing effective preventative, diagnostic and therapeutic tools to help reduce the burden of this disease.
CHAPTER II
SERRATED POLYP PATHWAY

2.1 Definition and classification of serrated lesions

CRC ranks among the three most common cancers in terms of incidence and cancer-related deaths in Western countries. The estimated worldwide incidence is 570,000 new cases per year, and the lifetime risk of CRC may reach up to 6% in developed nations (1, 19).

The term "serrated polyp" is used to describe a range of colorectal polyps with a sawtooth-like appearance on histological section due to infolding of the surface and crypt epithelium. This infolding has been hypothesised to be the result of decreased apoptosis and delayed migration of the cell from the crypt to the surface (20, 21). Serrated polyps is divided into subtypes by the World Health Organisation classification published in 2010 (9) as shown in Table 2.1.
WHO Classification of Serrated Lesions of the Large Intestine

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<td>Hyperplastic Polyp</td>
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<td>Microvesicular type (MVHP)</td>
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<td>Goblet cell-rich type (GCHP)</td>
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<td>Mucin poor type (MPHP)</td>
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<td>Sessile serrated adenoma/polyp</td>
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Table 2.1 World Health Organisation classification of serrated lesions (9).

The earliest lesions identified in the serrated pathway are aberrant crypt foci (22-26). Aberrant crypt foci are minute non-polypoid lesions, which can be visualised by low-magnification stereomicroscopy stained with methylene blue (27) and in vivo by high-magnification chromoendoscopy techniques (28). These are further classified into two subtypes, i.e., hyperplastic/heteroplastic and dysplastic, resembling miniature versions of hyperplastic polyps or adenomas.

2.2 Hyperplastic polyps

The first description of hyperplastic polyps dates back to the late 19th century,
but it was not until 1962 that Morson made a clear distinction between hyperplastic polyps and adenomas (29). Until recently, hyperplastic polyps have conventionally been regarded as non-neoplastic lesions.

2.2.1 Risk factors and epidemiology

Hyperplastic polyps are fairly common, with large cohort studies reporting a prevalence of 10%–30% in asymptomatic populations (30-32). They account for 80%–90% of serrated polyps, and are typically located in the rectosigmoid region (7, 8, 33, 34).

Specific lifestyle and dietary factors that appear to be associated with an increased risk of hyperplastic polyps are also those linked with the development of CRC. (35, 36). These include cigarette smoking, alcohol consumption, obesity and low folate intake (37-39). On the other hand, several factors have been reported to protect against development of hyperplastic polyps, including high calcium intake, use of non-steroidal anti-inflammatory drugs, and hormone replacement therapy (39).

Hyperplastic polyps seem to develop earlier than adenomas; however, the incidence of hyperplastic polyps does not correlate with increasing age beyond 50 years (38, 40, 41). An autopsy series from three European centres found that 23% of cases aged 20–54 years had hyperplastic polyps whereas only
11% of cases in this age group had adenomas (40). Furthermore, an endoscopy study of an asymptomatic and average risk population demonstrated a 2.5-fold increase in the prevalence of adenoma from the sixth to eighth decades, but no statistically significant change in the prevalence of hyperplastic polyps over the same time frame (42).

2.2.2 Endoscopic appearance

Typically, hyperplastic polyps are diminutive (80%–90% measuring <5 mm) and located in the distal colon (75%–80% occurring in the rectosigmoid region) (43). Brady et al showed that 57% of distal polyps are hyperplastic whereas only 25% of proximal polyps are hyperplastic in asymptomatic, average-risk patients undergoing colonoscopy (44).

The classical description of a hyperplastic polyp is "a pearl-coloured dew drop on the colonic mucosa", with hyperplastic polyps tending to be flat and more difficult to see when the lumen is fully distended (45). It is often difficult to distinguish hyperplastic polyps from small adenomas by endoscopy, but hyperplastic polyps tend to be smaller in size, lighter in colour, and closer to the anal verge than adenomas (46, 47).

Newer high-definition endoscopes with narrow band imaging as well as chromoendoscopy have been used to help identify hyperplastic polyps. The
surfaces of hyperplastic polyps have crypt openings that are larger than the surrounding normal crypts, and the lumens are either round or stellate (asteroid crypt pattern) in shape (48-54). The stellate-appearing pattern has also been identified in association with microvesicular-type hyperplastic polyps, and the rounded pattern has been associated with goblet cell-type hyperplastic polyps (55). These endoscopic advances, as well as newer techniques such as optical coherence tomography and autofluorescence, should make the diagnosis of hyperplastic polyps more reproducible, but the current standard method for diagnosis is polypectomy and histology.

2.2.3 Histological appearance

Figure 2.1 shows the histological appearance of hyperplastic polyps. Under light microscopy, hyperplastic polyps are small (usually less than 5 mm in diameter), symmetrical, and uniform in appearance. Hyperplastic polyps typically have non-branched but convoluted crypts and an epithelium with a sawtooth pattern of infoldings (serrations) in the upper half of the crypt (56). This serrated pattern gives a stellate-like appearance to hyperplastic polyps cut in cross-section.

The serrated pattern is thought to be due to a relatively normal rate and regulation of cell proliferation in the crypt base alongside failure of normal apoptosis and shedding of epithelial cells at the upper crypt and surface
mucosa. This unbalanced rate of cell proliferation versus cell loss results in accumulation of non-proliferating cells in the upper crypt, with infolding to allow more surface area for the retained cells (57).

Figure 2.1 Hyperplastic polyps contain serrated crypts that show surface maturation (A). Bizarre multinucleated epithelial cells may be detected in rare cases, but likely represent a degenerative phenomenon (B). Hyperplastic polyps of the distal colorectum may contain misplaced epithelium within the submucosa (C) that is often associated with haemorrhage and hemosiderin deposits (D). Reproduced with the permission of Vakiani et al. (56) and the publisher.

There are five histological features that are generic to hyperplastic polyps:

1. The serrated pattern is confined to the surface and upper portion of the crypt
2. They have a simple crypt architecture characterised by elongated, enlarged mucosal crypts separated by lamina propria that is similar to that of normal mucosa

3. The crypt epithelium is composed of goblet cells, columnar cells and cells with abundant foamy cytoplasm in varying proportions, showing maturation towards the upper crypt and surface epithelium

4. The proliferative activity, measured by mitotic activity or immunohistochemical staining for Ki67 (58), is largely confined to the round or oval slightly enlarged vesicular nuclei of the lower and mid crypt

5. The hallmark feature distinguishing a hyperplastic polyp from an adenoma is the enlarged pseudo-stratified "cigar-shaped" nuclei

It has been proposed that hyperplastic polyps can be further classified according to their cellular composition as microvesicular, goblet cell and mucin-poor variants. It is not known whether these subgroups differ in terms of their clinical behavior (59-61).

2.2.4 Clinical approach

In the early research, Ansher et al. has found that there is a greater than three-fold increase in the likelihood of patients with hyperplastic polyps in the colon harbouring an adenoma compared with those without hyperplastic
polyps (62). At around the same time, Provenzale et al has demonstrated that patients with distal hyperplastic polyps are 4.5 times more likely to have a proximal adenoma than those without distal hyperplastic polyps (63). However, a subsequent prospective study by the same investigators could not identify a true difference in risk, hence they have concluded that colonoscopy is not indicated if hyperplastic polyps are the only findings on sigmoidoscopy (64).

A recent meta-analysis that has calculated the risk for proximal adenomas in asymptomatic patients with distal hyperplastic polyps has shown an incidence of 21%–25%; this risk is intermediate between that for patients without distal polyps (16%) and those with distal adenomas (36%). Based on four screening colonoscopy studies (44, 65-67), the relative risk of any proximal neoplasia in patients with distal hyperplastic polyps is 1.3 (95% confidence interval 0.9–1.8) compared with those without distal hyperplastic polyps (68). The absolute risk of advanced proximal neoplasia (villous features, high-grade dysplasia, cancer) with distal hyperplastic polyps alone is 2.8%–4%, which is up to 2.6 times (95% confidence interval 1.1–5.9) greater than in those without distal polyps (68, 69).

The current practice is to remove all large right-sided polyps encountered on colonoscopy. When several small hyperplastic polyps are present in the rectosigmoid region, biopsies are usually taken of representative lesions rather
than attempting complete polypectomy of each lesion (70). Currently, endoscopic surveillance for detection of metachronous hyperplastic polyps or adenomas following removal of hyperplastic polyps alone is not recommended in the guidelines published by the American College of Gastroenterology, American Gastroenterological Association, or American Society for Gastrointestinal Endoscopy (70-74). When the large lesion is removed in piecemeal fashion technique, another colonoscopy at 3 to 6 months is recommended to check for completeness of excision (75).

2.3 Sessile serrated adenoma/polyps without dysplasia (SSA/P)

The original description of SSA/Ps as an entity by Longacre and Fenoglio-Preiser identified an incidence of <0.6% among over 18,000 polyps reviewed (7). Based on several studies involving selected patient populations, SSA/Ps are estimated to occur in 1%–7% of cases (76-80). Early studies have reported that SSA/Ps are more common in men than in women (2:1), with a mean age at diagnosis of approximately 60–65 years (7, 76, 79-81).

The morphological features of sessile serrated polyps were originally described in 1996 by Torlakovic et al and Snover (82), and the difference between SSA/Ps and TSAs is that the former lack intracellular dysplasia. This concept was re-introduced in 2003 by Torlakovic (8) and Goldstein (83), who both reported associations between abnormal proliferation and metachronous
CRC with microsatellite instability-high (MSI-H). In their studies, these represented 18%–22% of all types of serrated polyps (8, 83), but subsequent demographic cohorts have yielded lower numbers (34).

2.3.1 Endoscopic appearance

SSA/Ps usually appear flat with a soft, smooth surface. They are often covered with mucus, giving them an initial yellow appearance (60, 84, 85). When the mucus is washed off, the underlying polyp may be similar in colour to the adjacent mucosa or may be slightly more reddish. Iwabuchi et al. has reported that 60% of serrated adenomas have a “reddish” appearance (86). A distinctive feature of a SSA/P is the mucus cap, comprising a layer of mucus adherent to the surface of the lesion, giving the lesion a yellow or rust-colored appearance in contrast with the surrounding mucosa (87).

SSA/Ps are commonly found in the proximal colon, with 75% being located proximal to the splenic flexure. In one of the earlier endoscopic studies of SSA/Ps (78), 42.5% appeared to be sessile, 4% were pedunculated, and 53.5% were flat. This contrasts with a subsequent study showing that 63% of 52 SSA/Ps were pedunculated, 29% were sessile, and 8% were flat (79, 80). When viewed by chromoendoscopy or narrow-band imaging, the surface characteristics of SSA/Ps are similar to those of microvesicular hyperplastic polyps, that is, with an enlarged stellate pit pattern (88). However, this pattern
is clearly different from that of goblet cell hyperplastic polyps and TSAs (84, 88, 89).

2.3.2 Histological appearance

Figure 2.2C and 2.2D demonstrates the histological features of SSA/Ps. SSA/Ps differ from hyperplastic polyps by having a more disorganised architectural structure, an extended proliferative zone, and subtle cellular atypia. SSA/P crypts are often dilated and extend laterally to grow parallel to the muscularis mucosae, forming a crypt shaped like an inverted "T" or an "L" (8, 57, 90, 91). The proliferation zone extends from the crypt bases, and cells often have vesicular nuclei with slight chromatin irregularities (8, 82, 83). SSA/Ps do not have classic dysplasia; they may have mild cytological atypia, but even this is not required for the diagnosis. Bariol et al. has found that the presence of serration in >20% of the crypts and adenomatous change or nuclear dysplasia involving the surface epithelium are jointly associated with high diagnostic accuracy (76). Other histological features supporting the diagnosis of SSA/P are maintenance of basal goblet cells (92) and the presence of mitotic figures in the middle and upper third of the crypts (93). The polyp is classified as SSA/P with low-grade dysplasia when nuclear hyperchromasia and elongation, pseudostratification, apoptosis, increased mitotic activity, and loss of mucin are present, as is seen in conventional adenomas. On the other hand, high-grade dysplasia is characterised by greater nuclear enlargement, with enlarged nucleoli, loss of polarity, and
accompanying architectural complexity. SSA/Ps can be distinguished from both hyperplastic polyps and adenomas using immunohistochemical markers of proliferation, apoptosis and cell cycle control (Ki67, Bcl-2, p21^{WAF1/CIP1}). Generally, SSA/Ps have indices for these markers that are intermediate between those for hyperplastic polyps and adenomas (94-96).

**Figure 2.2** (A) Hyperplastic polyp showing mild glandular serration that is limited to the upper crypt and surface epithelium. Differentiation deviates minimally from normal, and goblet cells are easily distinguished from absorptive cells. (B) Hyperplastic polyp showing more serration and goblet cells do not stand out from columnar cells containing mucin droplets in their apical cytoplasm. (C) In sessile serrated adenoma/polyps, there is abundant mucin in the columnar cells and in the distended lumina. Crypts show exaggerated serration and branching, and the epithelium to stroma ratio is increased such that the overall architecture approximates that of serrated adenoma. (D) In serrated adenoma, the columnar cells are tall and eosinophilic, and mucin droplets are inconspicuous. Nuclei are elongated and pseudostratified but the nucleus to cytoplasm ratio is low and there is little nuclear
pleomorphism (low-grade dysplasia). Reproduced with the permission of the publisher (97).

2.4 Sessile serrated adenoma/polyp with dysplasia

Before re-classification by WHO (9), sessile serrated adenoma/polyp with dysplasia is known as ‘mixed polyps’ representing collision of tumours or that the adenomatous foci represent a non-serrated adenomatous component (7). Figure 2.3 shows the histology of sessile serrated adenoma/polyp with dysplasia.

In one colonoscopic series, sessile serrated adenoma/polyps with dysplasia accounted for about 2% of all colonic polyps and 4% of all serrated neoplasias of the colon (43). Sessile serrated adenoma/polyps with dysplasia are now commonly thought to be SSA/PSs that have developed focal dysplasia similar to that seen in classical adenomas; the dysplastic foci are thought to be immediate precursors of a unique subset of colorectal adenocarcinomas.
Figure 2.3 (A) Sessile serrated polyp/adenoma with dysplasia showing dysplastic tubular or serrated crypts (B) Admixed with serrated crypts that lack dysplastic epithelium. Reproduced with the permission of Vakiani et al.(56) and the publisher.

2.5 Traditional serrated adenoma (TSA)

“Traditional serrated adenoma” has replaced the term “serrated adenoma” coined in 1990 by Longacre and Fenoglio-Preiser (7). TSAs are thought to be uncommon lesions, accounting for less than 1% of all colonic polyps and about 2% of all serrated neoplasias of the colon (43). Traditional serrated adenomas have been shown to have a high proliferative capacity and a tendency to develop into higher-grade neoplasms (96, 98, 99).

2.5.1 Endoscopic appearance

Traditional serrated adenomas have a predilection for the distal colon and rectum, and usually have a more pedunculated or broad-based polypoid growth pattern than hyperplastic polyps. They are said to be more commonly reddish and show a villiform protuberant configuration (34, 79, 80, 100). The endoscopic surface appearance of TSAs is not well characterised, but is
thought to be similar to that of colonic adenomas, with tubular, cerebriform or flower-petal crypt openings (84, 85).

2.5.2 Histological appearance

The crypt architecture of TSAs gives them a villiform appearance when viewed at low-power magnification. Traditional serrated adenomas share the same serrated crypt architecture as hyperplastic polyps and SSA/Ps, but contain unequivocal cytological dysplasia throughout the polyp. They are described as polyps with columnar configuration of the glandular epithelium, eosinophilic cytoplasm, and uniform non-stratified glandular dysplasia (101). Loss of orientation is seen in the basal crypts, as well as detachment of the crypt from the muscularis mucosae.

2.6 Rationale for clinical research

Once considered to be innocent, SSA/Ps and TSAs are now believed to be a cause of missed CRCs. Interobserver reproducibility among pathologists (including gastrointestinal pathologists) when differentiating SSA/Ps from other polyps has been assessed by several groups (102-106). These studies only showed poor to moderate reproducibility with kappa values of 0.14–0.55. It is anticipated that increased awareness in the pathology community and release of the 2010 World Health Organisation criteria (9) will result in improved reproducibility of the diagnosis of serrated polyps (107). In the past five years
there has been an increasing body of evidence addressing this topic, but given the vast variation in reporting of serrated polyps using various histological classifications and reporting by endoscopists with different degrees of experience, these issues need to be addressed: 1. The natural history of serrated colorectal lesions. 2. Serrated polyp detection rate versus adenoma detection rate during colonoscopy. 3. Snaring large serrated polyps.
CHAPTER III
ADVANCED SERRATED LESIONS OF THE COLON
AND RECTUM

3.1 Introduction

Serrated colorectal polyps are defined histologically by a sawtooth-like appearance of the epithelium, and comprise a spectrum of lesions, including hyperplastic polyps, SSA/Ps with or without dysplasia and TSAs. Opinion regarding the clinical significance of serrated polyps has changed from considering them safe and irrelevant to cancer to seeing them as having a role as important precursors of right-sided colon cancer with a methylator phenotype (108). SSA/Ps without dysplasia seem to be particularly dangerous lesions because they are more common than TSAs and have been more often incriminated as immediate precursors of cancer (75). However, TSAs are different from SSA/Ps in that, by definition, they all contain adenomatous epithelium. SSA/Ps with dysplasia also contain adenomatous epithelium, so these three subtypes of serrated polyps can be regarded as “advanced”.

Little is known about the natural history of the different subtypes of serrated colorectal lesions. Hyperplastic polyps are the most numerous, and are generally small and left-sided. Development of carcinoma directly from hyperplastic polyps is uncommon, and the studies describing their natural
history are confounded by past difficulties in separating serrated lesions histologically. SSA/Ps without dysplasia are more direct precursors of CpG island methylator phenotype (CIMP) cancer (109). These are found in 2%–6% of patients undergoing screening colonoscopy (76, 110) and have a characteristic sessile or flat mucus-covered appearance. TSAs are less common, accounting for less than 1% of polyps in these same series. Because TSAs are much less common than SSA/Ps, there are fewer data on their molecular profile. TSAs may have KRAS or BRAF mutations, and either low or high levels of CIMP (22, 111). They do not show hypermethylation of MLH1 or develop microsatellite instability, but they do commonly have hypermethylation of the DNA repair gene MGMT (O6-methylguanine-DNA methyltransferase) (111). MGMT promoter methylation has been associated with both CIMP-low and CIMP-high colorectal cancer (112). TSAs are not usually diagnosed endoscopically, but rather are thought to be adenomas. It is only when the pathology report is read that the diagnosis is appreciated. A third category of advanced serrated colorectal polyp is the SSA/Ps with dysplasia. Here, adenomatous epithelium coexists with serrated epithelium in a way that is different to that observed with TSA. Current opinion is that SSA/Ps with dysplasia represent adenomatous dysplasia arising in an SSA/P (75), but I have made them a separate group here to identify whether there are any qualities that make them unique lesions. In the absence of systematic data describing the natural history of serrated lesions, the SSA/Ps with or without
dysplasia and TSAs in Cleveland Clinic, Ohio of one surgeon were investigated.

3.2 Methods

This was a retrospective review of data collected prospectively from 2004 (the year when pathology reporting of serrated polyps became acceptably reproducible at Cleveland Clinic, Ohio) to 2010, and stored in a single-surgeon colonoscopy database. The institutional review board at the Cleveland Clinic approved this database and a waiver of consent was granted for this study.

All clinically significant lesions seen during colonoscopy were removed or biopsied, and tissue was routinely submitted for pathology. When there were multiple, small, pale polyps in the rectum and sigmoid, not all were removed or biopsied, but a random representative sample of one or two lesions was excised or biopsied.

All patients with a histological diagnosis of SSA/P with or without dysplasia or TSA were identified. Medical records, histological slides and endoscopic reports were reviewed. Demographic data, indication for colonoscopy, polyp location, and treatment were extracted.
Continuous data were described as the mean and standard deviation (for normally distributed data) and the median and range (for non-parametric data). Categorical data were given as numbers and percentages. The significance of differences between groups was assessed using the Student's t-test for parametric continuous data and the Chi-squared or Fisher's Exact test for categorical data.

3.3 Results

From 2004 to 2010, 154 patients were identified as having 211 histologically advanced serrated polyps. There were 157 SSA/Ps without dysplasia in 111 patients (72.1%), 33 TSAs in 24 patients (15.6%), and 21 SSA/Ps with dysplasia in 19 patients (12.3%). The patient demographics are shown in Table 3.1. Patients with TSA were younger than the others (54.0±7.1 years versus 63.8±10.6 years for SSA/P and 66.5±7.8 years for SSA/Ps with dysplasia; p<0.05). Women comprised 55.9%, 50.0% and 63.2% of patients with SSA/Ps, TSAs and SSA/Ps with dysplasia, respectively (p=0.682). A family history of CRC was significantly more common in the SSA/P group (35.1% versus 8.4% in the TSA group versus 5.3% in the SSA/Ps with dysplasia group; p=0.001).
### Table 3.1 Patient demographics and indications for colonoscopy.

**Abbreviations:** CRC, colorectal cancer; MP, mixed polyp; SSA/P, sessile serrated adenoma/polyp; TSA, traditional serrated adenoma.

Indications for colonoscopy are also shown in Table 3.1. The most common indication for colonoscopy for patients in whom SSA/Ps without dysplasia group and TSAs were found was a past history of polyps (48.6% and 33.3%, respectively), while SSA/Ps with dysplasia group were more commonly detected on screening colonoscopies (31.6%). There was no statistically significant difference in indication for colonoscopy.
### Table 3.2 Polyp location, size and shape.

<table>
<thead>
<tr>
<th>Polyp Location</th>
<th>Without Dysplasia</th>
<th>With Dysplasia</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caecum</td>
<td>35 (22.3%)</td>
<td>10 (28.6%)</td>
<td>3 (15.0%)</td>
</tr>
<tr>
<td>Ascending</td>
<td>52 (33.1%)</td>
<td>5 (14.3%)</td>
<td>5 (33.3%)</td>
</tr>
<tr>
<td>Hepatic flexure</td>
<td>14 (8.9%)</td>
<td>2 (5.7%)</td>
<td>1 (5.0%)</td>
</tr>
<tr>
<td>Transverse</td>
<td>34 (21.7%)</td>
<td>2 (5.7%)</td>
<td>4 (20.0%)</td>
</tr>
<tr>
<td>Splenic flexure</td>
<td>3 (1.9%)</td>
<td>1 (2.9%)</td>
<td>1 (5.0%)</td>
</tr>
<tr>
<td>Descending colon</td>
<td>5 (3.2%)</td>
<td>1 (2.9%)</td>
<td>2 (10.0%)</td>
</tr>
<tr>
<td>Sigmoid</td>
<td>8 (5.1%)</td>
<td>9 (22.9%)</td>
<td>3 (15.0%)</td>
</tr>
<tr>
<td>Rectum</td>
<td>6 (3.8%)</td>
<td>3 (5.7%)</td>
<td>2 (10.0%)</td>
</tr>
</tbody>
</table>

**Shape**

<table>
<thead>
<tr>
<th>Shape</th>
<th>SSA/P without</th>
<th>SSA/P with</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat</td>
<td>7</td>
<td>1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sessile</td>
<td>102</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>pedunculated</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

**Mean diameter**

<table>
<thead>
<tr>
<th>Diameter</th>
<th>SSA/P without</th>
<th>SSA/P with</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flat</td>
<td>14.0±10.1mm</td>
<td>5.5±3.5mm</td>
<td></td>
</tr>
<tr>
<td>Sessile</td>
<td>15.0±7.1mm</td>
<td>15.0±7.1mm</td>
<td></td>
</tr>
<tr>
<td>pedunculated</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Adenomatous dysplasia**

<table>
<thead>
<tr>
<th>Polyp Characteristics</th>
<th>SSA/P without</th>
<th>SSA/P with</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSA/P</td>
<td>10 (6.4%)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Abbreviations:** HP, hyperplastic polyp; SSA/P, sessile serrated adenoma/polyp; TSA, traditional serrated adenoma; MP, mixed polyp; TA, tubular adenoma; NA, not applicable.

Table 3.2 shows details of the polyps. SSA/Ps without dysplasia were more often located in the right colon (proximal to and including the splenic flexure) than were TSAs and SSA/Ps with dysplasia (87.9% versus 60.6% versus 66.7%, respectively; p<0.05). Both SSA/Ps with or without dysplasia were significantly larger than TSAs (14.0±10.1 mm and 15.0±7.1 mm versus 5.5±3.5 mm, respectively; p<0.001). Most (60.0%–85.4%) of the polyps were snared.
### Table 3.3 Treatment and follow-up of advanced serrated polyps.

**Abbreviations:** SSA/P, sessile serrated adenoma/polyp; TSA, traditional serrated adenoma; MP, mixed polyp.

<table>
<thead>
<tr>
<th></th>
<th>Cold biopsied</th>
<th>Hot biopsied</th>
<th>Snared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistence of index polyp</td>
<td>0 patients (0.0%)</td>
<td>0 patients (0.0%)</td>
<td>4 patients (3.6%)</td>
</tr>
<tr>
<td>Any metachronous polyp</td>
<td>10 (91%)</td>
<td>11 (100%)</td>
<td>79 (91%)</td>
</tr>
<tr>
<td>Metachronous adenoma</td>
<td>7 (64%)</td>
<td>8 (73%)</td>
<td>31 (36%)</td>
</tr>
<tr>
<td>Metachronous SSA/P</td>
<td>2 (18%)</td>
<td>1 (9%)</td>
<td>48 (55%)</td>
</tr>
<tr>
<td>Metachronous hyperplastic polyp</td>
<td>10 (91%)</td>
<td>8 (73%)</td>
<td>38 (44%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mean follow-up, years</th>
<th>5.4±3.2</th>
<th>3.6±3.3</th>
<th>4.0±3.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients followed up (n)</td>
<td>87</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

There were 310 follow-up colonoscopies in 87 patients with an index SSA/P without dysplasia (3.6 per patient), 26 follow-up colonoscopies in 11 patients with an index TSA (2.4 per patient), and 22 follow-up colonoscopies in 11 patients with an index SSA/Ps with dysplasia (2.0 per patient). Results of surveillance examinations are shown in Table 3.3. Persistence of the index polyp was noted in four patients with an index SSA/P without dysplasia, but in no patient with an index TSA or index SSA/Ps with dysplasia. However,
metachronous lesions were common. While only one patient developed cancer (with an index TSA), most patients had at least one type of polyp found on follow-up. Although there was some overlap, adenomas were the most common metachronous polyp after TSA, SSA/P without dysplasia were most common after an index SSA/P without dysplasia, and hyperplastic polyps were most common after an initial SSA/Ps with dysplasia. The mean follow-up times were 5.4±3.2 years, 3.6±3.3 years and 4.0±3.2 years for SSA/Ps without dysplasia, TSAs and SSA/Ps with dysplasia, respectively.

3.4 Discussion

The diagnosis of SSA/P without dysplasia is based solely on histological criteria, which were uniformly applied in Cleveland Clinic from 2004 onwards. Therefore, it is important to define the histological characteristics of these advanced serrated polyps. In SSA/Ps without dysplasia, the basal half of the crypts are often dilated and extend laterally to grow parallel to the muscularis mucosae, forming a crypt shaped like an inverted "T" or an "L" (113). The proliferation zone extends from the crypt bases, and cells often have vesicular nuclei with slight chromatin irregularities (8, 82, 93). SSA/Ps without dysplasia do not usually have adenomatous dysplasia; they may have mild cytological atypia, but even this is not required for the diagnosis. In this current study, four SSA/Ps with low-grade adenomatous dysplasia, indicating progression along
the pathway to CIMP-high cancer.

In this series, TSAs comprised 1.6% of “advanced” serrated lesions. They tend to be left-sided, are endoscopically indistinguishable from adenomas, and are smaller than the more right-sided SSA/Ps. In addition, TSAs are much less often associated with a family history of CRC. Because of their small size, they are relatively easily controlled endoscopically, and are rarely multiple.

The genetic background of TSAs is unique. They show abnormality of the epithelial growth pathway, although sometimes this is by KRAS mutation and sometimes by BRAF mutation (22, 111). Hypermethylation is part of their phenotype, but unlike SSA/Ps without dysplasia this affects genes other than hMLH1 (112). Other characteristic genetic changes seen in TSAs, but not in SSA/Ps without dysplasia or hyperplastic polyps, include mutations in mitochondrial DNA (potentially affecting apoptosis) and overexpression of cyclo-oxygenase-2 (114-116).

TSAs share the same serrated crypt architecture as hyperplastic polyps and SSA/Ps without dysplasia. They feature budding of proliferative crypts that grow perpendicular to the long axis of the crypt (ectopic crypt formation) but also contain unequivocal cytological dysplasia throughout the polyp. SSA/Ps with
dysplasia also always contain adenomatous dysplasia, but have been distinguished histologically from TSA in that the adenomatous dysplasia is more localised and more locally dominant, representing either a collision tumour or a serrated polyp with a non-serrated adenomatous component (7).

SSA/Ps with dysplasia are the same size as SSA/Ps without dysplasia but are less often right-sided and significantly less associated with a positive family history. Although the numbers are small, there is a slight female predominance for SSA/Ps with dysplasia. These findings suggest that SSA/Ps with dysplasia may be a distinct entity.

Based on several studies involving selected populations, SSA/P without dysplasia incidence estimates range from 1% to 7% of colonoscopies (7, 8, 76, 110). TSAs are less common, accounting for less than 1% of all colonic polyps and about 2% of all serrated lesions in the large bowel. The incidence and natural history of SSA/Ps with dysplasia are even less well documented. In one colonoscopic series, mixed polyps accounted for about 2% of all colonic polyps and 4% of all serrated lesions in the colon (117).

We have shown that patients with TSAs are younger than those with other forms of advanced serrated lesions. Han and Zhou showed that these patients
were also younger than those with tubular adenomas (117). However, in this study, TSAs were smaller than SSA/Ps with or without dysplasia, implying that they were found at an earlier stage in their development or they have a different biology. They were also anatomically more left-sided than SSA/Ps without dysplasia. It is notable that the incidence of a positive family history for CRC was significantly lower for TSAs and SSA/Ps with dysplasia than for SSA/Ps without dysplasia, suggesting that TSAs are largely sporadic and not part of a hereditary syndrome.

In this series, most polyps were snared. At a mean follow-up of 5.4±3.2 years, 3.6±3.3 years and 4.0±3.2 years for SSA/Ps without dysplasia, TSAs and SSA/Ps with dysplasia, respectively, only four patients (3.6%) with SSA/Ps without dysplasia had persistent polyps. One cancer was reported in a patient with an index TSA during this period. In the literature, patients with serrated adenomas are reported to have a higher propensity for metachronous serrated adenomas (26%) and synchronous cancer (19.6%–24%) (118, 119). Furthermore, in a few case reports, high-grade serrated adenomas have been associated with rapid recurrence and, ultimately, the development of serrated adenocarcinoma (120, 121). Metachronous CRC was also observed in 5.3% of patients with serrated adenomas in contrast with 2.2% of patients with conventional adenomas (98). In all of these studies, dated around 2005–2006,
when the data were accumulated prior to publication, the tendency to group all advanced serrated polyps together and call them “serrated adenomas” makes it difficult to draw conclusions. Some significant differences between the three types of advanced serrated lesions on follow-up were noted. Almost all (>90%) patients who were followed up had some sort of polyp, and the pattern of metachronous lesions is interesting. Patients with an index SSA/P without dysplasia tended to have SSA/Ps without dysplasia on follow-up, while patients with an index TSA tended to have more adenomas. Patients with an index SSA/Ps with dysplasia mainly had hyperplastic polyps at follow-up. Taken together, synchronous and metachronous polyyps, this indicate the inclination of the colorectal mucosa giving rise to the polyps. For SSA/Ps without dysplasia, metachronous lesions suggest dominant methylation, whereas TSA chromosomal instability and SSA/Ps with dysplasia are likely to be associated with a KRAS mutation. Studies of normal colorectal mucosa are needed to confirm these hypotheses.

The relatively small numbers and restriction of data to the experience of a single endoscopist limit this study. The missing hyperplastic polyps may provide an extra dimension to this research but would make the study unwieldy without necessarily adding anything to the information about high-risk serrated lesions. The indications for colonoscopy reflect the surgical nature of the endoscopist’s
practice, which is dominated by surveillance examinations, with a moderate rate of average-risk and family history-based screening and a low number of symptomatic patients. The incidences of these serrated lesions are specific to this type of practice, but can be extrapolated to other practices by looking at the indications for examination. However, limiting the study to one endoscopist avoids interindividual differences in polyp detection and philosophy. In this study, all polyps seen were removed or biopsied.

3.5 Summary
Consistent with the data reported in the literature, SSA/Ps without dysplasia has a female predominance and tended to be proximal in location. TSAs are a rare type of serrated polyp that share some characteristics with SSA/Ps with and without dysplasia. However, they are smaller than both SSA/Ps with or without dysplasia, are more evenly split between the left and right colon, are usually sessile or pedunculated, and are almost always sporadic rather than inherited. In this series TSAs comprise almost 16% of advanced serrated lesions, and are found in 2.6% of colonoscopies. These incidence rates are higher than previously reported, and emphasize the need for colonoscopists to be aware of these potentially premalignant lesions. The following section examines the detection rate of serrated polyps as an indicator of quality in colonoscopy when compared with the detection rate of conventional adenoma.

The detection and recognition of serrated lesions is an important part of cancer
prevention hence in the next chapter this is analysed which will ultimately guide us to the management of these lesions.
Chapter IV

SERRATED POLYP DETECTION RATE VERSUS ADENOMA DETECTION RATE DURING COLONOSCOPY

4.1 Introduction

The primary aim of screening and surveillance colonoscopy is to prevent CRC by detecting and removing premalignant lesions, usually polyps. For this to be an effective strategy, premalignant lesions must be seen, recognised and effectively treated. In recent years, the adenoma detection rate (ADR) has become increasingly important as an index of the quality of screening and surveillance colonoscopy (122, 123). Although this use of the ADR has some disadvantages (124), there is no doubt that a missed adenoma is an opportunity for an interval cancer to develop. At the same time as the importance of the ADR has been increasingly emphasised, the role of serrated polyps as precursors to CRC has also been increasingly recognised. (92, 125).

Three distinct entities exist under the general rubric of serrated polyps (9). The TSA and SSA/P are considered as precursor lesions that follow the serrated pathway to CRC. As mentioned in Chapter 2 and 3, SSA/Ps develop through a distinct molecular pathway featuring mutation of the serine threonine kinase, BRAF, a downstream target in the epidermal growth factor receptor signalling pathway (43, 109, 126, 127) and methylation of the promoter regions of tumour
suppressor genes and DNA repair genes. The cancers that are the end products of this pathway possess a CIMP (97, 128). TSAS are manifestations of a different molecular pathway, not quite as well delineated, but one study has suggested that KRAS mutation is largely responsible (100). However, they are equally dangerous in the development of CRC. Because large SSA/Ps are usually right-sided, there have been suggestions that missed serrated polyps at least partially account for the apparent failure of screening colonoscopy to protect against right-sided cancer (4, 129). In the context of interval cancers, these are 2.5 times more likely to be CIMP-positive, 2.7 times more likely to demonstrate MSI-H, and nearly twice as likely to occur in the proximal colon (18). It is currently believed that many of these interval cancers originate from the serrated neoplastic pathway outlined above. The developing story of serrated polyps and their contribution to CRC highlights the importance of their detection during colonoscopy. In fact, the serrated polyp detection rate (SDR) is probably just as important as the ADR in reflecting the quality of a colonoscopy aimed at preventing CRC. Unfortunately, serrated lesions can be difficult to see and recognize because of their low profile, pale colour, and the mucus cap that often obscures them. Currently, there are few studies that describe the SDR, and its measurement is potentially more difficult than that of the ADR because of variability in histological diagnosis and a tendency for colonoscopists to ignore tiny hyperplastic polyps. However, detection and recognition of serrated lesions of the colon and rectum is an important part of
cancer prevention, the SDR is therefore analysed.

4.2 Methods

This is a retrospective review of data collected prospectively and stored in a single-institution colonoscopy database. The database is approved by the institutional review board of the Cleveland Clinic and a waiver of consent was granted for this database study. Only colorectal surgeons with more than 1000 colonoscopies in the database were included in this study and detection rates were stratified by surgeon. Some of these data (ADR and withdrawal times) have been reported previously in a study of ADR (123). None of the SDR data have been reported previously. The following data were extracted from the database: indication for colonoscopy, number of examinations complete to the caecum or ileocolic anastomosis, ADR defined as percentage of examinations during which one or more histologically proven adenomas were removed and SDR defined as serrated polyps percentage of examinations during which one or more histologically proven serrated polyps were removed. Withdrawal time is defined as the time taken for withdrawal of the colonoscope from the caecum to the anus in patients with intact colon and with no intervention (130). SDR was the primary end point of the study, and ADR and withdrawal time were secondary end points. Regression analysis was planned to explore possible relationships between ADR and SDR, and between SDR and withdrawal time. The data are described as the mean and standard deviation or median and
range, depending on their distribution.

4.3 Results

A total of 18,003 colonoscopies performed from 2004 to 2008 were included and were tabulated according to colonoscopist in Table 4.1. The average incomplete colonoscopy rate for the six endoscopists was 3.7%±1.2%. The patients comprised 10,100 men (56.1%) and 7903 (43.9%) women, of average age 61.4±13.3 years. The primary indications were screening in 3060 (17.0%), rectal bleeding in 3529 (19.6%), history of previous CRC in 3727 (20.7%), and a history of previous polyps in 7687 (42.7%). Table 4.1 shows the SDR and ADR for all examinations and for average-risk screening examinations. Withdrawal times were also tabulated. There was marked variation in SDR, ADR and withdrawal time between colonoscopists. The overall SDR ranged from 15.0% to 27.3%, and SDR in screening examinations ranged from 6.4% to 18.7%. Corresponding data for ADR were 22.0% to 42.2% and 13.4% to 25.7%. The range of mean withdrawal time was from 5.5 minutes to 14.1 minutes. Regression of overall ADR against overall SDR did not show a significant relationship ($R=0.571$, $p=0.237$), but regression of ADR against SDR in average-risk screening examinations did show a significant relationship ($R=0.854$, $p=0.031$). There was also a significant relationship between time of withdrawal and SDR, both in screening examinations ($R=0.908$; $p=0.012$) and overall ($R=0.956$; $p=0.003$), as shown in Tables 4.1.
Table 4.1 Serrated polyp detection rate and adenoma detection rate for all examinations and for average-risk screening examinations only.

<table>
<thead>
<tr>
<th>Study</th>
<th>ADR vs Proximal SDR</th>
<th>ADR vs HPDR</th>
<th>ADR vs SSA/PDR</th>
<th>ADR vs SDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kahi et al (131)</td>
<td>0.86</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hetzel et al (132)</td>
<td></td>
<td>0.84</td>
<td>0.64</td>
<td></td>
</tr>
<tr>
<td>Current study</td>
<td></td>
<td></td>
<td></td>
<td>0.85</td>
</tr>
</tbody>
</table>

Table 4.2 Pearson correlation coefficient for relationship between SDR and ADR in colonoscopies done for average-risk screening. **Abbreviations:** ADR, adenoma detection rate; SDR, serrated polyp detection rate; SSA/PDR, sessile serrated adenoma/polyp detection rate; HPDR, hyperplastic polyp detection rate; vs, versus.

4.4 Discussion

Serrated polyps of the large bowel are a spectrum of lesions, and effective prevention of CIMP-H cancers with colonoscopy depends on accurate recognition and removal of the more dangerous serrated lesions, in the same way that adenoma detection and removal of advanced adenomas can prevent chromosomally unstable and mutator cancers. This type of secondary prevention depends on detection and recognition of serrated lesions. While the ADR has become widely accepted as an important index of quality in colonoscopy, the SDR has not. This study is one of a small number of early
studies examining serrated polyp detection during routine screening colonoscopy.

In this study, the SDR varies between endoscopists in the same way as the ADR and that, like the ADR, the SDR is related strongly to the time of colonoscope withdrawal. Clearly, spending more time looking increases the yield of polyp identification, either adenomatous or serrated. However, unlike the ADR, there is no “standard” for the SDR. This is because of the paucity of data and also because of the difficulties that serrated lesions pose for endoscopists in terms of recognition and for pathologists in terms of classification. While small, pale, left-sided hyperplastic polyps are easy to identify, they are often ignored by colonoscopists who regard them (usually correctly) as benign. This would tend to produce an underestimation of the SDR. The clinically significant serrated lesions are SSA/Ps, which are larger and usually right-sided. They are much more difficult to see than small, left-sided hyperplastic polyps or adenomas. They are less discrete, often flat, and usually of a similar colour to that of the surrounding mucosa. The mucus cap that is a clue to their presence may be dismissed as poor preparation or may be obscured by poor preparation. In addition, as previously described, TSAs are a precursor to CRC. These tend to be left-sided, are smaller than the more right-sided SSA/Ps, and much less often associated with a family history of CRC (see Chapter 3).
There have been two large studies reporting detection rates for adenomas and serrated polyps in patients undergoing colonoscopy for average-risk screening. Kahi et al. have reported the results for 15 colonoscopists, only one of whom performed more than 1000 examinations, and three of whom performed less than 100 (131). The ADR ranged from 17% to 47% (average 32.4%) and SDR (proximal to splenic flexure) from 1% to 18% (average 8.9%). Hetzel et al. included 13 colonoscopists, all of whom performed more than 100 examinations (132). However, only two endoscopists had done more than 1000 examinations and nine performed less than 600. The ADR ranged from 13.5% to 33% (average 23.4%), the hyperplastic polyp detection rate ranged from 8.8% to 31% (average 13.7%), and the SSA/P detection rate ranged from 0.3% to 2.2% (average 0.8%). Although the data are not directly comparable (Kahi et al. (131) report proximal serrated lesions only and Hetzel et al. (132) subdivide serrated lesions according to histology), they are in broad agreement with the results. This current data show a range of SDRs for all indications of 15.0%–27.3% (average 20.6%), while the range for screening examinations was 6.4%–18.7% (average 13.9%). The corresponding ADR for all examinations ranged from 22.0% to 42.2% (average 31.5%) and for screening examinations ranged from 13.4% to 25.7% (average 20.7%). The ADR values are somewhat lower than those reported by Kahi et al. but in line with those reported by Hetzel et al. (131, 132). The SDR values are very
similar to those reported by Hetzel et al, but are likely to be an underestimation for two main reasons. The first is that hyperplastic polyps may not be removed or biopsied because they are assumed to be insignificant. The second is the difficulty in actually seeing SSA/Ps. The tendency to ignore hyperplastic polyps in patients already on a surveillance protocol may be the reason for the lack of a significant relationship between ADR and SDR when analyzing all colonoscopies, regardless of indication. The majority of cases were done for polyp or cancer surveillance. The significant relationship between ADR and SDR that emerged when only screening examinations were analysed implies that, in the context of screening, all polyps tend to be removed. Both Kahi et al. and Hetzel et al. noted significant relationships between SDR and ADR for screening examinations (see Table 4.2) (131, 132).

There are no studies of serrated polyp miss rates at present, but expert opinion is that these are potentially higher than those already published for adenomas. The extent of the potential underestimation of the number of serrated polyps is seen in as yet unpublished data from a study of one year’s worth of colonoscopies prepared by the senior investigator (JC). These data are the results of a careful analysis of one year of consecutive outpatient colonoscopies, during which all polyps seen were prospectively documented. In 654 colonoscopies, adenomas were found in 280 examinations (42.8%), advanced adenomas in 90 (13.8%), serrated polyps in 146 (22.3%), sessile
serrated polyps in 32 (4.9%), and cancers in 12 (1.8%). In the 121 patients colonoscoped for average-risk screening, the numbers were 46 adenomas (38%) including 12 advanced adenomas (10% of total screening examinations), 29 serrated polyps (24%) including seven sessile serrated polyps (6% of total screening examinations), and two cancers. While the overall SDRs are almost identical to those in the present study (endoscopist 1, JC), SDRs for screening examinations are significantly higher in the prospective study. In addition, the prospective study provides data for high-risk adenomas and SSA/Ps, allowing an estimation of the percentage of such lesions that might have been expected in the retrospective study. Unfortunately, the capacity to analyze the serrated lesions in this study according to pathology, size or location is limited. It is therefore impossible to know the proportions of hyperplastic polyps, SSA/Ps and TSAs in the serrated group. This is a limitation of the study. However, strengths of the study are the large number of examinations included and the range of end points among colonoscopists, which allows relationships to be identified.

While the concept of SDR is useful in underlining the importance of detection and treatment of these potentially dangerous lesions, it suffers from some of the same difficulties as the ADR (124). Endoscopists fixed on increasing their score may reject patients with poor bowel preparation because of the major inconvenience likely to ensue, may take significantly longer to examine the
colon, thus putting pressure on schedules, and may biopsy multiple excrescences that turn out to be normal, thereby unnecessarily inflating the cost of the examination.

4.5 Summary

Detection and removal of serrated polyps requires keen colonoscopic skills that differ from those required for recognition of adenomas. Endoscopists need to recognize the malignant potential of these lesions and be aware of their own SDR. The strong relationship between colonoscope withdrawal time and SDR, both for screening and all examinations, is an indication that careful examination of the colorectal mucosa is key to detection of serrated lesions. In addition, in colonoscopies performed for polyp or cancer surveillance, an ability to find adenomas does not necessarily mean an ability to find serrated polyps. In the following chapter, the feasibility and safety of endoscopic removal serrated polps vs adenoma will be examined in order to form recommendations on endoscopic management and follow up surveillance.
Chapter V
SNARING LARGE SERRATED POLYPS

5.1 Introduction

Prevention of CRC by colonoscopy involves detection and removal of pre-malignant lesions, and surveillance to prevent their recurrence. The most common pre-malignant lesion in the large bowel is the adenoma, a neoplasm of glandular origin that is the precursor of approximately 80% of colonic cancers and over 90% of rectal cancers. The other common pre-malignant lesions in the large intestine are serrated polyps. Chapter 2 has described various subtypes in details, including hyperplastic polyps, SSA/Ps with or without dysplasia, and TSAs (133). There is an increasing body of evidence pointing to the malignant potential of SSA/Ps and TSAs, leading to cancer with a unique genetic phenotype, i.e., the CIMP. Up to 33% of all colon cancers (<5% of all rectal cancers) are CIMP-positive cancers (134) while 57% of interval (likely missed) colon cancers are CIMP-positive (18). Detection and effective removal of the pre-malignant serrated polyp is an important part of overall prevention of CRC, particularly the larger, usually right-sided SSA/P. However, experience with endoscopic removal of large serrated polyps is minimal, partly because they were initially thought to be harmless and not worth the risks of snare excision, and also because they often look different to
adenomas, making endoscopists reluctant to attempt their removal. In this study, the safety and outcomes of serrated polypectomy is examined.

5.2 Methods

Outcomes of endoscopic polypectomy were prospectively recorded in a single-endoscopist, polyp database approved by the institutional review board in Ohio. Records were reviewed and updated to ensure current follow-up information. Polypectomy of large serrated polyps was compared with polypectomy of large adenomas. Large polyps are defined endoscopically as those with a maximum diameter of at least 2 cm. Right-sided polyps include those in the caecum, ascending colon, transverse colon and both flexures. Left-sided polyps are those in the descending and sigmoid colons and rectum. End points were complications of polypectomy and polyp persistence. Serrated polyps included those labeled as hyperplastic polyps, SSA/Ps with or without dysplasia and TSAs. Malignant polyps were excluded. Patients with polyposis syndromes including familial adenomatous polyposis, MYH-associated polyposis, and serrated (hyperplastic) polyposis, were also excluded.

Most polypectomies in this series were performed without injection of adrenaline or saline prior to polypectomy, and were not done with typical endoscopic mucosal resection technique (135). The basics of the polypectomy
technique included positioning the patient in the most favorable position to access the polyp, shortening the colon over the scope to achieve good control of the tip, and use of jumbo, standard or mini snares to resect the polyp. The snare was opened over the polyp, often opening it against a fold to encourage a wide loop. The snare and scope was depressed to encourage the polyp to enter the loop, and the loop is then closed. A pure coagulation current of 25 Hz was used, with the current interrupted frequently to allow heat to dissipate. The largest piece snared at one application is 2 cm. For very large polyps, the polypectomy was started at one edge and proceeds across the lesion. Retroflexion was used for polyps spreading on the underside of a fold, and the patient position was changed as necessary. Serrated polyps come up into the snare more easily than adenomas. They were softer and easier to entrap; however, their margins were less distinct. For any large polyp, suspected margins were treated with cautery using hot biopsy forceps. In a minority of cases, adrenaline (1:10,000) was used after polypectomy to control immediate bleeding. If a large polyp was removed, smaller polyps in other parts of the colon were generally not treated, to make localisation, diagnosis and treatment of postpolypectomy complications easier.

For the purpose of this study, complications included post-polypectomy syndrome and post-polypectomy haemorrhage. Post-polypectomy syndrome was defined as localised pain and tenderness after polypectomy, with or
without fever, but with no signs of general peritonitis and no free gas on abdominal radiology. Haemorrhage was defined as lower gastrointestinal bleeding requiring admission to hospital.

Patients with large sessile polyps removed piecemeal are re-scoped within six months to ensure complete removal of the index lesion. At that time, smaller lesions left at the index procedure were removed. Patients with high-grade dysplasia in the polyp were rescoped as early as three months to ensure complete removal of the index lesion. Polyps removed in one piece, without high-grade dysplasia, are followed up at one year. In follow-up, a persistent polyp was defined as a residual index lesion at the polypectomy site. Metachronous polyps were lesions at different sites.

Continuous data were described as the mean and standard deviation if normally distributed and as the median and range if not normally distributed. Categorical data were described as numbers and percentages.

5.3 Results

From Jan 2009 to Jan 2010, there were 132 large serrated polyps in 112 patients and 563 large adenomas in 428 patients. Table 5.1 shows the basic patient demographics and size and location of the polyps. Serrated polyps
were more commonly located in the right colon (120 of 132, 92%) compared with adenomas (379 of 563, 67%; \(p<0.0001\)). The large serrated polyps were smaller than the large adenomas (25.5±7.90 mm versus 36.8±16.9 mm; \(p<0.001\)). There were four complications of serrated polypectomy in four patients (3% of polypectomies, 3.6% of patients), including three post-polypectomy bleeds and one post-polypectomy syndrome (see Table 5.1). There were no perforations. During the time of the study, no serrated polyp was referred for surgical resection; all serrated polyps were removed endoscopically. Complication rates for polypectomy of adenomas were similar, and included 31 post-polypectomy bleeds and two cases of post-polypectomy syndrome (6.3% of polypectomies, \(p=0.376\), 7.7% of patients, \(p=0.371\)). There were no perforations.

Follow-up details are also given in Table 5.1. At the time of performing this study, 51 patients with serrated polyps had at least one follow-up examination and 36 (71%) had metachronous polyps, while only 133 of 298 (45%) of the adenoma patients had metachronous polyps on follow-up \(p<0.01\). Mean time to first follow-up was similar between the groups at 12.4±9.0 months for serrated polyps and 8.4±7.7 months for adenomas \(p=0.643\). However, at first follow-up, there were significantly fewer residual polyps in the serrated group (4/51 (8%) versus 64/298 (21%); \(p=0.016\)).
<table>
<thead>
<tr>
<th></th>
<th>Serrated polyps n=132</th>
<th>Adenomatous polyps n=563</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>52:60</td>
<td>261:167</td>
<td>0.0055</td>
</tr>
<tr>
<td>Mean age, years</td>
<td>63.2±10.8</td>
<td>67.8±3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Caecum/ileocecal valve</td>
<td>28 (22%)</td>
<td>138 (25%)</td>
<td></td>
</tr>
<tr>
<td>Ascending</td>
<td>47 (36%)</td>
<td>124 (23%)</td>
<td></td>
</tr>
<tr>
<td>Hepatic flexure</td>
<td>11 (8%)</td>
<td>37 (7%)</td>
<td></td>
</tr>
<tr>
<td>Transverse</td>
<td>28 (22%)</td>
<td>70 (13%)</td>
<td></td>
</tr>
<tr>
<td>Splenic flexure</td>
<td>6 (5%)</td>
<td>10 (2%)</td>
<td></td>
</tr>
<tr>
<td>Descending</td>
<td>1 (1%)</td>
<td>30 (6%)</td>
<td></td>
</tr>
<tr>
<td>Sigmoid</td>
<td>7 (5%)</td>
<td>88 (16%)</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>2 (2%)</td>
<td>48 (9%)</td>
<td></td>
</tr>
<tr>
<td>No data</td>
<td>2</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Mean size, mm</td>
<td>25.5±7.9</td>
<td>36.3±16.9</td>
<td>0.01</td>
</tr>
<tr>
<td>Median (range) size, mm</td>
<td>25 (20–80)</td>
<td>30 (20–130)</td>
<td></td>
</tr>
<tr>
<td>Shape*</td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>Pedunculated</td>
<td>4</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Sessile</td>
<td>100</td>
<td>388</td>
<td></td>
</tr>
<tr>
<td>Flat</td>
<td>10</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Total complications</td>
<td></td>
<td></td>
<td>0.613</td>
</tr>
<tr>
<td>Bleeding</td>
<td>3%</td>
<td>6%</td>
<td></td>
</tr>
<tr>
<td>Post-polypectomy syndrome</td>
<td>3</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Mean time to first follow-up, months</td>
<td>12.4±9.0</td>
<td>8.4±7.7</td>
<td>0.05</td>
</tr>
<tr>
<td>Mean time to second follow-up, months</td>
<td></td>
<td>22.9±15.1</td>
<td></td>
</tr>
<tr>
<td>Findings at first follow-up</td>
<td></td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>No polyps</td>
<td>11 (22%)</td>
<td>101 (34%)</td>
<td></td>
</tr>
<tr>
<td>Metachronous polyps</td>
<td>36 (71%)</td>
<td>133 (45%)</td>
<td></td>
</tr>
<tr>
<td>Residual polyp</td>
<td>4 (8%)</td>
<td>64 (21%)</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1 Polyp location, size and outcomes of polypectomy according to polyp histology. **Note:** Data are for 114/132 serrated polyps and 561/563 adenomas. **Abbreviation:** SD, standard deviation.

Table 5.2 separated serrated polyps by histology. The most common type was SSA/P without dysplasia followed by hyperplastic polyp, TSA, and SSA/P with

70
dysplasia. All subtypes shared features of right-sided predilection and sessile shape, and were of similar size. TSAs occurred mostly in men, unlike the gender distribution of the other polyps. The only polyps to recur were SSA/Ps.

<table>
<thead>
<tr>
<th></th>
<th>HP</th>
<th>SSA/P without dysplasia</th>
<th>TSA</th>
<th>SSA/P with dysplasia</th>
<th>p-value</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>30</td>
<td>87</td>
<td>11</td>
<td>4</td>
<td>0.25</td>
<td>132</td>
</tr>
<tr>
<td>Gender: M:F</td>
<td></td>
<td>14:16</td>
<td>38:49</td>
<td>8:3</td>
<td>1:3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Mean size (mm)</td>
<td>26.5±12.1</td>
<td>25.4±6.4</td>
<td>24.4±4.6</td>
<td>23.8±4.8</td>
<td>&gt;0.05</td>
<td>25.5±7.9</td>
</tr>
<tr>
<td>Distribution right:left</td>
<td>29:1</td>
<td>79:8</td>
<td>11:0</td>
<td>3:1</td>
<td>0.29</td>
<td>122:10</td>
</tr>
<tr>
<td>Shape</td>
<td>6:24:0</td>
<td>2:64:3</td>
<td>1:10:0</td>
<td>1:2:1</td>
<td>10:100:4</td>
<td></td>
</tr>
<tr>
<td>Flat:sessile: pedunculated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Piecemeal excision</td>
<td>28/30</td>
<td>50/69</td>
<td>10/11</td>
<td>1/4</td>
<td>89/114</td>
<td></td>
</tr>
<tr>
<td>Residual polyp at first follow-up</td>
<td>0/8</td>
<td>4/36</td>
<td>0/5</td>
<td>0/2</td>
<td>4/51</td>
<td></td>
</tr>
<tr>
<td>Mean (± SD) time to first follow-up (months)</td>
<td>13.0±6.1</td>
<td>9.8±3.7</td>
<td>10.0±3.5</td>
<td>5.0±2.8</td>
<td>0.05</td>
<td>12.4±9.0</td>
</tr>
</tbody>
</table>

Table 5.2 Subtypes of serrated polyps of the large bowel. Abbreviations: HP, hyperplastic polyp; SD, standard deviation; SSA/P, sessile serrated adenoma/polyp; TSA, traditional serrated adenoma; mixed, mixed adenomatous/hyperplastic polyp.

5.4 Discussion

Large serrated polyps are clinically significant. They are an intermediate step in the serrated pathway to CRC and must be effectively removed for secondary prevention of cancer. Although clinicians often avoid endoscopic resection of these lesions for fear of complications, this study demonstrates that they can be removed safely with an endoscopic snare.
<table>
<thead>
<tr>
<th>Study</th>
<th>Inclusion criteria</th>
<th>n</th>
<th>Rectosigmoid</th>
<th>Complications</th>
<th>Residual polyps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dell’Abate (136)</td>
<td>&gt;30 mm</td>
<td>104</td>
<td>72%</td>
<td>3.8%</td>
<td></td>
</tr>
<tr>
<td>Luigiano (137)</td>
<td>&gt;20 mm</td>
<td>168</td>
<td>66%</td>
<td>8.6%</td>
<td>14.3%</td>
</tr>
<tr>
<td>Jameel (138)</td>
<td>&gt;10 mm</td>
<td>33</td>
<td>57%</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stergiou (139)</td>
<td>&gt;30 mm</td>
<td>68</td>
<td>59%</td>
<td>4%</td>
<td>29%</td>
</tr>
<tr>
<td>Doniec (140)</td>
<td>&gt;30 mm</td>
<td>186</td>
<td>81%</td>
<td>2.5%</td>
<td>3%</td>
</tr>
<tr>
<td>Hochdorffer (141)</td>
<td>&gt;30 mm</td>
<td>167</td>
<td>73%</td>
<td>3.4%</td>
<td>16.2%</td>
</tr>
<tr>
<td>Binmoeller (142)</td>
<td>&gt;30 mm</td>
<td>176</td>
<td>73%</td>
<td>3.4%</td>
<td>16.2%</td>
</tr>
<tr>
<td>Kao (143)</td>
<td>1–9 cm</td>
<td>104</td>
<td>37% rectal</td>
<td>7%</td>
<td>12%</td>
</tr>
<tr>
<td>Ahlawat (144)</td>
<td>&gt;20 mm</td>
<td>183</td>
<td>32%</td>
<td>7%–13%</td>
<td>12%</td>
</tr>
<tr>
<td>Caputi (145)</td>
<td>&gt;20 mm</td>
<td>151</td>
<td>80%</td>
<td>3.8%</td>
<td>6.9%</td>
</tr>
<tr>
<td>Current series,</td>
<td>&gt;20 mm</td>
<td>563</td>
<td>25%</td>
<td>6%</td>
<td>21%</td>
</tr>
<tr>
<td>adenomas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current series,</td>
<td>&gt;20 mm</td>
<td>110</td>
<td>8%</td>
<td>3%</td>
<td>1%</td>
</tr>
<tr>
<td>serrated polyps</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.3 Recent series describing resection of large adenomas.

There are very few studies in the literature describing techniques and outcomes of polypectomy for large serrated lesions, and likewise few studies reporting surgical resection of sessile serrated polyps. Leonard et al. have
reviewed the literature and produced some recommendations (146), but these are not based on any direct experience with management. However, there are multiple studies addressing removal of large adenomas, and some of the more recent ones are summarised in Table 5.3 (136-145). The data reported here for adenomas are an extension of a previous study reported in 2003 (147) and are compared with those available in the literature.

A comparison of polypectomy for serrated lesions with polypectomy for adenomas is helpful, in that it provides a context for adenoma studies within which the outcomes for polypectomy of serrated lesions can be evaluated. However, adenomas are more often pedunculated than serrated polyps, and are thus easier to remove. Part of the reason they are more often pedunculated is that they are commonly located on the left of the large intestine, where the more forceful peristalsis draws out a stalk. The location of polyps is important for other reasons. Right-sided polyps are potentially more complicated because the colon wall is thinner and there is a greater chance of haemorrhage and perforation after resection. Most right-sided polyps are sessile or flat, increasing concerns about complications. Right-sided polyps may be more difficult to reach, and if there are loops distally, the colonoscope may be more difficult to manipulate. Rectal polyps are often sessile but their endoscopic removal is much less concerning because of the thickness of the rectal wall, the ease of insertion, the lack of loops, and the extra-peritoneal
location of the lower half of the rectum. Table 5.3 shows that studies of large polyps are mostly populated by left-sided, rectosigmoid lesions. These are the “low-hanging fruit” of colonoscopic polypectomy, in that they tend to be pedunculated and have lower rates of complications. They are also predominantly adenomas. The complication and persistent polyp rates for adenomas in this study must be taken in the context of being mostly for right-sided lesions, while the data for serrated lesions are almost exclusively from right-sided polyps, the majority of which are in the caecum and ascending colon.

Although difficult to encapsulate with data, snare polypectomy of large serrated lesions is often easier than polypectomy for large adenomas. The looseness of the serrated mucosa seems to allow the polyp to be drawn up into the snare and stay there. Specialised techniques such as endoscopic mucosal resection and endoscopic submucosal dissection were rarely used in this study, explaining the relatively high rate of piecemeal polypectomy (78% serrated polyps, 79% adenomas). However, the residual polyp rate was relatively low and there were no perforations.

This current study lacks lengthy and complete follow-up. This is the missing piece in any discussion of effective management of serrated colorectal lesions. Some studies report an aggressive natural history (148, 149). If serrated
polyposis is an amplified, accelerated version of the natural history of serrated polyps in the same way as familial adenomatous polyposis is used as an amplified, accelerated version of the natural history of adenomas, the importance of removing solitary, large serrated polyps is reinforced. Teriaky et al. (150) report a 5-year follow-up of 22 patients of 34 with SSA/Ps. The mean size of the index lesions was 11 mm. They have found a high rate of synchronous adenomas initially (45%) and on follow-up (45%), and found 21 new serrated polyps in the 22 patients followed. A high incidence of metachronous serrated polyps in this cohort of patients is found, much higher than for adenomas. Of course, this begs the question of the incidence of missed lesions and the impact of a conscious decision not to remove synchronous lesions. It is our policy to leave smaller lesions behind when a large index lesion is removed, so that the source of a post-polypectomy complication is not in doubt. The synchronous polyps are removed at the inevitable follow-up examination. This policy distorts the findings on follow-up. On the other hand, sessile serrated lesions are sometimes very subtle and easy to miss. Therefore, the true incidence of serrated lesions at presentation and their development on follow-up is hard to extrapolate from the data and needs longer follow-up, so that all synchronous lesions can be dealt with and the true incidences of missed and metachronous lesions can be determined.

Another potential limitation of this study is that only one endoscopist was
involved. Therefore, the applicability of these data to other endoscopists can be questioned, but the data for adenomas are in line with other studies available in the literature. Furthermore, a single-endoscopist study removes any inter-endoscopist variance in polyp recognition and size estimation. However, further studies of polypectomy for serrated lesions are needed to establish their natural history and optimal treatment.

5.5 Summary

Removal of large serrated colorectal polyps is no more complicated than polypectomy of similarly sized adenomas. However, large serrated polyps have a higher rate of metachronous polyps than similarly sized adenomas, and surveillance should be adapted to reflect these findings. In the next section, a literature review is presented to clarify further the cancer risk of serrated polyps if the lesions are left untreated.
6.1 Historical evidence

In the previous chapters, the natural history, detection rates, and endoscopic management of serrated polyps have been explored. The evidence suggests these precursor lesions may develop into serrated CRCs if they are not removed (93, 151, 152). Colorectal cancer develops from an accumulation of genetic abnormalities in a line of colonocytes that are usually the result of many years of exposure to environmental carcinogens. These genetic abnormalities produce cells that are capable of escaping controls on cell growth, death, and differentiation, and are able to travel from their site of origin and establish viable colonies in distant locations. For cancer to develop, the neoplastic cells must also overcome the defense mechanisms of the host, which often recognize them as foreign and try to destroy them. Therefore, the timing of the appearance of CRC is the result of a balance of tumour factors and patient factors.

Colorectal cancer does not arise de novo from normal epithelium. There is a sequential change through polyps to cancer. The polyp histology depends on the underlying genetic changes in epithelial cells. The most common sequence
in which a colonic cancer develops follows the adenoma-carcinoma pathway, but widespread methylation of mucosal DNA leads to development of cancer via the serrated polyp-cancer pathway (see Chapter 7). Certain polyp characteristics predict the risk of metachronous, advanced neoplasia; these are invasive carcinomas, adenomas 10 mm or larger, or adenomas with any villous histology or high-grade dysplasia (11-14). For sporadic cancers, the time between appearance of an adenoma and the change to carcinoma is approximately 10 years (153). The behaviour of serrated adenomas appears to be different.

The natural history and risk of progression from serrated polyps to malignancy are not well understood. Eighty cases of colorectal adenocarcinoma arising from SSA/Ps or large hyperplastic polyps had been reported since their initial discovery. Serrated pathway colorectal cancer is a distinct variant of CRC and accounts for approximately 7.5% of all CRCs (154), 9.3% of all CRCs in females, and 5.8% of CRCs in men (154, 155). Prior to 2004 the distinction between SSA/P and TSA was not recognised, and these terms were used interchangeably. During this time, Longacre and Fenoglio-Preiser (7) reported that 37% of serrated adenomas contained foci of significant dysplasia and 11% contained intramucosal carcinoma. Others have suggested a prevalence rate of 4%–16% for TSA with high-grade dysplasia or intramucosal carcinoma (78-80, 86). Iwabuchi et al. reported that 1.5% of TSAs smaller than 10 mm
had concomitant carcinoma compared with 10% of those 10 mm or larger (86). In addition, patients with TSA were found to have a higher propensity for metachronous TSA (26%) or synchronous cancer (19.6%–24%) than those with serrated adenoma (118, 119). Furthermore, in a few case reports, high-grade TSA has been associated with rapid recurrence and, ultimately, the development of serrated cancer pathway adenocarcinoma (120, 121). In addition, 5.3% of patients with TSA will have metachronous CRC in contrast with 2.2% of patients with classical adenoma (98).

6.2 Recent evidence

In recent years, after establishment of consistent histological diagnosis of serrated polyps, it is noteworthy that people who harbor serrated neoplasms are at high risk of also having synchronous serrated polyps and advanced adenomatous neoplasia. A recent cohort study analysed 290,810 colonoscopic specimens from 179,111 patients to estimate the progression rate of SSA/Ps. SSA/Ps with or without dysplasia/carcinoma were identified in 2416 specimens from 2139 patients, of whom 54% were women. In this group of patients, 1815 (85%) had no evidence of dysplasia, 257 (12%) had low-grade dysplasia, 45 (2%) had high-grade dysplasia, and 21 (1%) had adenocarcinoma. It was also important to note that the age distribution of the patients indicated a significant increase in progression of polyp to cancer with increasing age. The median age for SSA/P without dysplasia was 61 years,
that for SSA/P with low-grade dysplasia was 66 years, that for SSA/P with high-grade dysplasia was 72 years, and that for SSA/P with cancer was 76 years (101). In addition, Pai et al. (156) has found that patients with one sessile serrated polyp are four times more likely to have other serrated polyps at the same time compared with an unselected population. Schreiner et al. (157) has suggested that patients with either a proximal or a large serrated polyp are at higher risk of synchronous advanced neoplasia compared with patients who do not have these lesions. Li et al. (158) has reported that large serrated polyps (>10 mm) are associated with a three-fold increase in risk of synchronous advanced neoplasia. It is also noted that patients who had both SSA/Ps and conventional adenomas have significantly larger and more numerous lesions of both types (159). In addition, these lesions are more pathologically advanced when compared with people having only one or the other type.

On follow-up colonoscopy, patients with advanced neoplasia and proximal serrated polyps at baseline examination are twice as likely to have advanced neoplasia during subsequent surveillance than those with only advanced neoplasia at baseline examination (157). Therefore, it appears that the presence of large or proximal serrated polyps or serrated neoplasms predict the presence of synchronous and likely metachronous advanced neoplasms.
6.3 Unpublished data from the Cleveland Clinic laboratory

Jarrar et al. had previously reported (unpublished data) on a series of cases that provide further evidence of progression from the initial polyp stage to cancer. Between 2004 and 2009, the institutional review board-approved pathology database was queried to identify colorectal SSA/Ps with and without high-grade dysplasia or adenocarcinoma. Eleven patients with SSA/P and advanced neoplasia were identified. Six of eleven patients had SSA/P with a focus of adenocarcinoma and five patients had SSA/P with high-grade dysplasia. This represented 2% of all SSA/Ps in the database during the study period. Ten of these eleven polyps were located in the right colon and one was located in the sigmoid colon. The mean size of the SSA/Ps with high-grade dysplasia was 24±23 mm, and 17±8 mm for SSA/Ps with adenocarcinoma. Five of the adenocarcinomas were moderately differentiated and one was poorly differentiated. Five patients had stage I cancer and one patient had stage III cancer. Of the SSA/Ps with high-grade dysplasia, four of five polyps were felt to be completely removed by snare polypectomy, while the other was deemed too large to be resectable (63 mm). This patient subsequently underwent right hemicolectomy. This case series clearly demonstrated the malignant potential of sessile serrated polyps and the need for close surveillance. These data were reported in the hope of increasing awareness of such lesions and to provide an insight into the management strategies.
6.4 Guidelines for post-polypectomy surveillance

A guideline for post-polypectomy surveillance in individuals with serrated lesions of the colon has recently been published and is shown in Table 6.1 (153). The guideline recommends that patients with a small sessile serrated polyp (<10 mm) and no dysplasia should have a repeat colonoscopy in five years. Patients with a sessile serrated polyp ≥10 mm, a sessile serrated polyp with dysplasia (any size), or a TSA (any size) should undergo repeat colonoscopy in three years. Serrated polyposis syndrome should be followed up at one year, although subsequent examinations that identify a decrease in polyp prevalence can be followed at expanded intervals. If a patient with a sessile serrated polyp and also an adenoma, the surveillance interval should be the shortest interval recommended for either lesion (159). The importance of detection and removal of SSA/Ps and TSA (75) have recently been emphasised, given that up to 50% are microsatellite unstable and that these CIMP-positive tumours are overrepresented in interval cancers, particularly in the proximal colon.
<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Surveillance interval (years)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1–2 tubular adenomas &lt;10 mm</td>
<td>5–10</td>
<td>Depending on other risk factors, e.g., previous colonoscopic findings, family history, smoking, obesity</td>
</tr>
<tr>
<td>3–10 adenomas, any adenoma &gt;10 mm, with villous pathology or HGD</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>More than 10 adenomas in one examination</td>
<td>&lt;3</td>
<td>Consider a genetic syndrome</td>
</tr>
<tr>
<td>Hyperplastic rectosigmoid polyps &lt;10 mm</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Sessile serrated polyps &lt;10 mm without dysplasia</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Sessile serrated polyps &gt;10 mm with dysplasia or a traditional serrated adenoma</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.1 Colonoscopy surveillance. Adapted with permission from Lieberman et al (153). Abbreviation: HGD, high-grade dysplasia.

6.5 Summary

The clinical relevance of detection and removal of SSA/P and TSA is becoming abundantly clear as our understanding of the various types of serrated polyps improves. Further longitudinal and follow-up studies will be required to clarify how the subtype, number and location of serrated lesions influence this risk. Given that average-risk individuals account for almost 75% of patients with CRC, the detection and removal of precursor lesions in this population provides the best opportunity for improving cancer prevention. Hence, further knowledge about the prevalence of SSA/P and TSA in the screening population will allow us to estimate the true rate of malignant progression of these lesions. The following chapters of this thesis delve deeper
into the molecular aspects of various types of CRCs and uncover a novel association with a specific miRNA that potentially has a profound effect on the development of serrated adenocarcinoma in the colon and rectum.
7.1 Key molecular pathways in colorectal cancer

Over the last 20 years, our understanding of the pathogenesis of CRC has evolved from the concept of a single disease progressing through a sequence of morphological and genetic alterations (160) to the concept of molecular heterogeneity and tumour uniqueness (161). Three major pathways have been proposed to result in the development of sporadic CRCs.

7.1.1 Classical "suppressor" pathway

This is the pathway by which classical adenomas progress to microsatellite stable (MSS) cancers (160). It is characterised by a "gatekeeper" Adenomatous polyposis coli (APC) mutation and progression is secondary to chromosomal deletions (loss of heterogeneity). The suppressor pathway is the most common type of genomic instability (162), encompassing 50%-85% of CRCs (163, 164). This pathway is characterised by karyotypic variability resulting from gains and/or losses of whole/portions of chromosomes (165). Various mechanisms have been identified, and include sequence changes, chromosome number alterations, chromosome rearrangements, and gene amplification (166). Additional alterations identified include chromosomal
segregation defects or microtubule dysfunction, abnormal centrosome number, telomere dysfunction or telomerase overexpression, DNA damage, and loss of heterozygosity (165-167). The resulting CRC has multiple structural chromosomal abnormalities and aneuploidy.

7.1.2 Microsatellite instability-high (MSI-H) pathway
Microsatellite instability is exemplified by multiple mutations in areas of repeating nucleotide sequences (microsatellites) that occur throughout the genome, and include key growth control genes and oncogenes. The underlying defect is inactivation of DNA repair genes, most notably hMLH1. This pathway culminates in a CRC described as MSI-H because the conventional assay for microsatellite status typically shows positivity in two or more of five microsatellites. These neoplasms are found in the proximal colon, and are often poorly differentiated, with lymphocyte infiltration and a Crohn’s-like lymphocyte response.

7.1.3 "Mild mutator" Microsatellite instability-low (MSI-L) pathway
This is associated with inactivation of mutator genes other than hMLH1, such as MGMT. The MSI assay shows methylation in only one of the five microsatellites probed, hence the designation of MSI-L. Loss of heterogenicity of suppressor genes is also suggested in this pathway. The endpoint is a carcinoma that shows features of MSS as well as some MSI cancer (mucin
production) and this usually locates at the left side of the colon.

### 7.2 Molecular alterations in serrated pathway lesions

Several articles have provided data highlighting the lack of genetic alterations in SSA/Ps that are seen in adenomas. (17, 22, 168, 169). Mutations of APC, KRAS and p53 and loss of heterozygosity, which are key features in the APC pathway, are uncommon in serrated polyps. In addition, immunostaining for the WNT pathway transcriptional activator beta-catenin shows a normal membranous distribution consistent with the presence of wild-type APC and CTNNB1 genes in serrated polyps (17, 168-170).

Eventually, studies began to demonstrate a set of changes in serrated polyps that are also present in hyperplastic polyps and a subset of CRCs. These changes include mutually exclusive mutations of BRAF or KRAS (22, 59, 126), DNA MSI (50, 112), mutation of TGFβRII (112), abnormal DNA methylation (59, 112), and loss of expression of the DNA repair gene MGMT (17, 171) and hMLH1 (172).

#### 7.2.1 MAPK-ERK pathway

The earliest genetic alterations in serrated lesions are BRAF and KRAS mutations, which are observed in aberrant crypt foci (24). These genes transcribe proteins that have crucial roles in the cell signaling pathway known
as RAS/RAF/MERK/ERK. This pathway controls fundamental cellular processes such as proliferation, differentiation, survival and apoptosis (173).

The KRAS gene is known to be one of the most commonly mutated oncogenes in the classic adenoma-carcinoma sequence (APC pathway). The BRAF gene is immediately downstream from KRAS, so conceptually, mutational activation of both KRAS and BRAF would be redundant because they would both activate the same signaling system. In fact, mutations in these two genes appear to be mutually exclusive in human CRCs (174). This indicates that both mutations occur early in development of a neoplasm and also supports the notion that there are two parallel pathways that rarely cross over.

KRAS mutations are found in up to 82% of aberrant crypt foci-hyperplastic (175, 176) but are seen less frequently in hyperplastic polyps (4%–37%) (22, 59, 126, 177, 178). In addition, KRAS mutations are rare in SSA/Ps (0%–10%) (59, 60, 126, 177, 179). The most common BRAF mutation (V600E, formerly V599E) results in a constitutively activated enzyme (174). These mutations are rare in sporadic aberrant crypt foci-hyperplastic and in hyperplastic polyps (24) but very frequent in SSA/Ps (75%–82%), in mixed polyps (40%–89%) (50, 177) and in lesions associated with hyperplastic polyposis (24).

The high frequency of BRAF mutations in SSA/Ps is surprisingly similar to the
76% incidence reported for MSI-H CRCs (126, 180) and in sharp contrast with the 0% incidence of *BRAF* mutations in hereditary non-polyposis CRCs (180). This also lends support to the concept that most sporadic MSI-H CRCs originate from SSA/Ps, and this is discussed in the following sections (180-182).

### 7.2.2 Alterations in regulation of apoptosis

Human cells do not proliferate indefinitely but instead they are “mortal.” Their proliferative capacity displays three phases which can be identified on cell culture. Phase I, corresponding to a period of little proliferation before the first passage. Phase II, characterized by rapid cell proliferation. Phase III, during which proliferation gradually grinds to a complete halt (183). In this process telomeres are progressively shortened, ultimately causing cells to reach their “Hayflick limit.” This barrier has been termed replicative or cellular senescence. It denotes a stable and long-term loss of proliferative capacity, despite continued viability and metabolic activity.

Early SSA/Ps represent a "safe haven", where genetic alterations are allowed to accumulate without cells being signaled to undergo apoptosis. This self-perpetuating cycle is maintained by diminished apoptosis from methylational silencing of apoptosis-associated genes such as *p14* and *p16* (180, 184) and activation of the anti-apoptotic signal of *BRAF* (185). These two
mechanisms are frequently found in serrated polyps and serrated pathway colorectal cancers.

7.2.3 Abnormal methylation

Methylation of DNA is fundamental for many epigenetic mechanisms used in gene regulation, such as DNA imprinting, X chromosome inactivation, and controlling tissue-specific gene expression (186, 187). In carcinogenesis, disruption of DNA by methylation is seen in two forms, i.e., hypermethylation of DNA in the promoter regions of certain genes and global hypomethylation (188, 189).

CpG islands are especially common in promoter sequences, being found in over half of these sequences (190). In normal tissue, methylation of cytosine is common outside of the exons (162). The likelihood of methylation naturally increases with age, injury, and in patients with chronic inflammation (191). Binding of methyl groups to CpG islands (stretches of recurrent cytosine-guanine dinucleotide sequences) is frequently present in the promoter regions of the genes affected in SSA/Ps (186, 192). This mechanism often results in epigenetic inactivation of genes and is functionally comparable with the mutational inactivation of tumour suppressor genes seen in the classical APC pathway or the mutational inactivation of DNA repair genes in Lynch syndrome. It is a stable alteration, although potentially reversible, and is
transmitted into cell progeny (192). When this alteration confers a growth advantage by inactivating both alleles of a tumour suppressor gene in an affected cell, it gives rise to an expanding clone and progression of neoplasia (23). This concept is explored further in Section 7.3. If CpG island methylation involves a DNA repair gene such as *hMLH1*, inactivation of both alleles results in MSI and the potential to progress to an MSI-H CRC.

Colorectal cancers with concordance of DNA methylation across multiple genes or loci are designated as being CIMP (193). The CIMP status of tumours is measured by comparing the methylation status of a set of these cancer-associated genes in the tumour with that found in normal tissue. Several studies have used sets of five markers, such as MINT 1, 2, 12, and 31, and p16, and definition of CIMP-positive status is based on the presence of methylation of at least two of these markers (193, 194). More recently, Neurog1, CACNA1G, IGF2, RUNX3, and SOCS1 have been used in the laboratory setting to characterize CIMP status, and these markers were utilised in our own experiments to classify tumours and cancer cell lines (195).

Excess methylation has been observed in about 30%–50% of CRCs and can be divided into low-level (CIMP-L) and high-level (CIMP-H) types. CIMP-H CRC is distinguished from its low counterpart by having a predilection for females of advanced age, tending to locate in the proximal colon, and
frequently presenting as mucinous and/or poorly differentiated cancer. In addition, it frequently harbours MSI and the \textit{BRAF} gene is often mutated. (126, 171, 193, 194). Interestingly, CIMP is found not only in cancer but also in the normal-appearing mucosa of some subjects with CIMP-positive CRC (196), suggesting the possibility of using CIMP status as a biomarker for risk stratification.

\textbf{7.2.4 Microsatellite instability}

A microsatellite is a stretch of DNA containing a pattern of 1–5 nucleotides with tandem repeats (197, 198). Microsatellites are found abundantly throughout the genome and are unique in uniform and length within the tissue of each individual (191). A minimum of 500,000 microsatellites is estimated to be present within the genome, occurring in the intron, untranslated terminal regions, and the coding exon itself (199). Microsatellites may be classified as monomorphic (the same number of repeats in all individuals) or polymorphic (variable number of repeats between individuals) (199). Elongation or shortening of the microsatellite is primarily due to inactivation of DNA mismatch repair genes, which are responsible for correcting base-base DNA replication errors. At regions of short repeats within the genome, DNA polymerase is particularly susceptible to making mistakes; therefore, when DNA mismatch repair is inactivated, these errors will not be corrected, resulting in MSI. This inactivation may be genetic or acquired. These tumours
are not usually associated with mutations of KRAS or p53; however, genes such as TGFβ RII, EGFR, and BAX, which contain simple repeats, are often mutated in these tumours (163). Additional genes affected by MSI include regulators of proliferation (GRB1, TCF-4, WISP3, ACVR2, IGF2R, axin-2, CDX), the cell cycle or apoptosis (caspase-5, RIZ, BCL-10, PTEN, hG4-1, FAS), and DNA repair (MBD-4, BLM, CHK1, MLH3, RAD50, MSH3, MSH6) (191).

Inactivation of DNA mismatch repair may be due to either an inherited germline mutation in one allele with somatic inactivation of the other or somatic inactivation of both alleles (199). The most common mechanism of inactivation of DNA mismatch repair is through an acquired methylation of the hMLH1 gene promoter (164). The DNA mismatch repair system comprises seven proteins (MLH1, MLH3, MSH2, MSH3, MSH6, PMS1, PMS2) which combine and form functional heterodimers (200, 201). These mutations allow repeats within the microsatellite to accumulate or to be lost through clonal propagation (202).

In Lynch syndrome, the affected individual develops CRC because of an inactivating germline mutation in one of several genes required for DNA mismatch repair, whereas in sporadic cancers with MSI, loss of DNA mismatch repair occurs not as a result of mutations but from hypermethylation. It has
now been shown that these two classes of MSI-H CRCs can be distinguished by the presence of activating mutations in the BRAF oncogene in sporadic MSI cancers but not in Lynch syndrome cancers (203).

Not only do sporadic MSI-H CRCs and serrated polyps share BRAF mutation and CIMP, but more advanced serrated polyps also often show an increase in methylation and loss of expression of MLH1 (112, 172). In contrast, methylation of other microsatellite DNA mismatch repair genes such as p14, p16 and EpHB2 is very rare (60, 171, 180, 184, 204-210). In recent times, methylation of the DNA repair gene (MGMT) has been reported to be involved in the serrated pathway. It has been postulated that a deficiency in this gene overrides the microsatellite mismatch repair system and gives rise to the MSI-L status (97, 171, 184). Methylation of MGMT is rare (6%) in hereditary nonpolyposis CRC, (180), but has been detected in 22% of hyperplastic polyps (60), 24.6% of SSA/Ps (60), 16%–22% of TSAs with a variable degree of dysplasia (184), and 50% of serrated cancers (184). It may also occur with hMLH1 methylation in 20% of sporadic MSI-H cancers (180). However, the significance of MSI-L itself in the serrated pathway has not yet been determined.

4.2.5 Key molecular changes in colorectal polyps

Key molecular changes in the various CRC pathways are summarised in Table
7.1, with the serrated polyp pathway again being characterised by \(BRAF\) mutation, CpG island methylation, and microsatellite instability, while the classical pathway is characterized by \(KRAS/APC\) mutation and loss of heterogeneity. This is supported by the similarity in percentages of \(BRAF\) mutation, CIMP and MSI status in colon cancers with a high MSI level when compared with their precursor lesions, i.e., SSA/Ps and serrated polyps with dysplasia (Table 7.2).

<table>
<thead>
<tr>
<th></th>
<th>Serrated polyp pathway</th>
<th>Classical APC pathway</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instigating mutation</td>
<td>(BRAF)</td>
<td>(APC/KRAS)</td>
</tr>
<tr>
<td>Precursor lesion</td>
<td>HPs, SSA/Ps with or without dysplasia, TSAs</td>
<td>Conventional adenomas</td>
</tr>
<tr>
<td>Main mechanism</td>
<td>CpG island methylation</td>
<td>Loss of heterogeneity</td>
</tr>
<tr>
<td>Pathway endpoints</td>
<td>MSI-H CRC</td>
<td>MSS cancer</td>
</tr>
<tr>
<td></td>
<td>MSI-L/MSS CRC</td>
<td></td>
</tr>
</tbody>
</table>

Table 7.1 Summary of key molecular changes in colorectal polyps. **Abbreviations:** CRC, colorectal cancer; HPs, hyperplastic polyps; SSA/Ps, sessile serrated adenomas/polyps; TSAs, traditional serrated adenomas; MSI-H, methylator phenotype-high, MSI-L, methylator phenotype-low; MSS, microsatellite stable.
<table>
<thead>
<tr>
<th>Type</th>
<th>BRAF mutation</th>
<th>KRAS mutation</th>
<th>Strong CIMP positivity</th>
<th>High MSI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goblet cell serrated polyp</td>
<td>21%</td>
<td>43%</td>
<td>14%</td>
<td>0%</td>
</tr>
<tr>
<td>Microvesicular serrated polyp</td>
<td>76%</td>
<td>13%</td>
<td>47%</td>
<td>0%</td>
</tr>
<tr>
<td>Sessile serrated polyp/adenoma</td>
<td>83%</td>
<td>7%</td>
<td>76%</td>
<td>0%</td>
</tr>
<tr>
<td>Serrated polyp with dysplasia</td>
<td>62%</td>
<td>24%</td>
<td>79%</td>
<td>50%</td>
</tr>
<tr>
<td>Colon cancer with high MSI</td>
<td>76%</td>
<td>2%</td>
<td>61%</td>
<td>100%</td>
</tr>
<tr>
<td>Large tubular adenoma</td>
<td>0%</td>
<td>26%</td>
<td>44%</td>
<td>0%</td>
</tr>
<tr>
<td>Colon cancers without MSI-H</td>
<td>9%</td>
<td>44%</td>
<td>12%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 7.2 Comparison of the molecular profiles of serrated polyps, traditional colonic adenomas and colorectal cancers. Data from O’Brien et al\textsuperscript{51} and Kambara et al\textsuperscript{117}. **Abbreviations**: CIMP, CpG island methylator phenotype; MSI, microsatellite instability.

7.3 miRNAs and colorectal cancer

miRNAs are highly conserved sequences of RNA in both eukaryotes and prokaryotes, and were first reported by Lee et al. when they found that the developmental time controlling gene, *lin-4*, of *Caenorhabditis elegans* produced a pair of unknown RNAs instead of proteins (211). Despite this early discovery, miRNAs did not attract much attention until 2001 when different laboratories began to detect and clone large numbers of these small antisense molecules (212).

miRNAs are a class of small (19–22 bp) non-coding, single-stranded RNAs that negatively regulate gene expression at the post-transcriptional level. They
are located in intergenic regions and also within introns of either protein-coding or non-coding genes; only a few are located in exons of non-coding RNAs or the untranslated region (UTR) of protein-coding genes. They can also cluster in the same chromosomal region, sharing the same expression patterns and being transcribed as polycistronic transcripts (213). These single-stranded RNAs are considered to play crucial roles in many normal cellular processes, such as proliferation, development, differentiation and apoptosis, by regulating target gene expression through imperfect pairing with target mRNAs of protein-coding genes, inducing direct mRNA degradation or translational inhibition (214, 215).

The biogenesis of miRNA is shown in Figure 7.1. The primary transcripts (Pri-miRNA), which can be up to 1 kb in length, are synthesised in the nucleus by the RNA polymerase II enzyme. In the cropping step, the pri-miRNA, forms a hairpin loop and is cleaved by Drosha, an RNAIII enzyme, on the non-loop end, producing pre-miRNAs of 70 nucleotides. These pre-miRNAs are then exported to the cytoplasm by an Exportin-5/RanGTP-dependent mechanism (216). They are further processed in the cytoplasm by Dicer, another RNAIII enzyme, to produce miRNA-miRNA duplexes that are unwound by a helicase to release mature miRNAs (22 bp), one or both of which can be active (217-219). Although miRNAs gain their mature shape and function through a series of actions, some steps of which are shared by small interfering RNAs,
they are still distinguishable from small interfering RNAs in terms of several key differences, including their origin, evolutionary conservation, and the types of genes that they silence (214).

**Figure 7.1** Pathways of microRNA (miRNA) biogenesis and mechanism of action. The genes encoding miRNAs transcribe the primary transcript, the pri-miRNA, which is processed to a short 70-nucleotide stem-loop structure known as pre-miRNA by the ribonuclease “Drosha”. The pre-miRNA is transported to the cytoplasm by the special exporting protein. In the cytoplasm, the pre-miRNA is processed to mature miRNAs by its interaction with the endonuclease enzyme “Dicer”. The resulting 19–23-nucleotide mature miRNA binds with RNA-induced silencing complex (RISC). This RISC-bound miRNA pairs with complementary or near-complementary messenger RNA (mRNA) and induces cleavage or inhibits the translation of the target mRNA. miRNAs that bind to their mRNA targets with perfect complementarities induce target mRNA cleavage, but miRNAs that bind with imperfect complementarities modulate gene expression by inhibiting protein translation. The overall role of miRNAs is therefore to regulate target gene expression to control normal rates of cellular growth, proliferation, differentiation and cell death. Reproduced with the permission of Aslam (220) and the publisher.
miRNAs work in two ways. First, miRNA is incorporated into an RNA-induced silencing complex that targets and cleaves mRNA. Second, miRNAs can cause epigenetic gene silencing through imperfect alignment with the 3'-UTR of their target mRNAs, resulting in repressed translation. Depending on the biological function of the targeted mRNA, both mechanisms ultimately result in a reduction of the target protein downstream and hence translational repression of target genes (221, 222).

The “seed region” of a miRNA, which typically localizes to the 3'-UTR of the target mRNA, although may sometimes fall outside of this region (223), is of special relevance because it mediates target recognition. Complete and partial complementarity between the seed region (nucleotide positions 2–8) of miRNA and its target mRNA governs its function (224). Imperfect sequence complementarity often results in large numbers of targets for each miRNA (222). Recent bioinformatics and experimental reports suggest that over 30% of human genes are direct targets of miRNA (225-227).

miRNAs are known to regulate many known oncogenic and tumour suppressor pathways involved in the pathogenesis of CRC (228-231). Expression profiles of hundreds of different miRNAs have been shown to have a much higher potential as biomarkers than their mRNA counterparts. Thus far, 2042 mature
human miRNAs are listed in the updated database (http://www.mirbase.org) accounting for approximately 2%–3% of the human genome (225, 227). However, despite their discovery, only a few miRNA targets and pathways have been explored.

The discovery of miRNA genes strongly suggests that many critical genes involved in oncogenesis may be derived from non-coding regions that make up approximately 98% of the human genome. While it was originally reported that miRNA expression levels are globally reduced in cancers (232), most studies examining colon tumours have found the opposite. The majority of published studies that have evaluated global miRNA expression pattern in colon tumours indicate that there is an increased expression of miRNAs in colon cancers (233-235). Furthermore, recent studies have shown that deregulation of miRNA plays a critical role in human oncogenic signaling pathways including oncogenesis, progression, invasion, metastasis, and angiogenesis (236-238). Figure 7.2 proposes a model of how miRNAs are involved in the CRC network (239). A possible role of miRNA in tumorigenesis is its effect on tumour suppressor genes; this has been suggested to be linked to hypermethylation. With this in mind, the next section further explores the epigenetic mechanism of gene silencing.
Figure 7.2 Model of the colorectal cancer network. The role of miRNAs and mutations in colorectal cancer pathogenesis. Selected miRNAs along with their targets are indicated. Mutations are derived from recent high-throughput studies. Abbreviations: ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; ICAM, intercellular adhesive molecules; MMP, matrix metalloproteinase. Reproduced with the permission of Schweiger et al (239) and the publisher.

7.4 Epigenetic changes in the serrated pathway

7.4.1 microRNA and methylation

Identification of miRNAs and their role adds another layer of complexity to the control of protein expression. miRNAs can be abnormally upregulated or downregulated in CRCs. Although the mechanisms for such dysregulation of miRNAs are still largely unknown, one theory proposes that hypermethylation of the promoter regions of miRNAs govern their expression.
7.4.2 DNA methylation

DNA methylation occurs by covalent addition of a methyl group at the 5’ carbon of the cytosine ring, resulting in 5-methylcytosine. These methyl groups project into the major groove of DNA, effectively inhibit transcription, and occur most often in CpG islands (240). In contrast with the bulk of genomic DNA, in which most CpG sites are heavily methylated, CpG islands in germ-line tissue and promoters of normal somatic cells remain unmethylated, allowing gene expression to occur. The aberrant promoter CpG island methylation in cancer is viewed as conversion of a transcriptionally active to a transcriptionally repressive chromatin domain.

A prime role of DNA methylation is to "lock in" the transcriptionally repressed state characterizing the majority of human DNA (heterochromatin). Normally, this acts to suppress expression of any potentially harmful viral sequences or transposons that may have integrated into sites containing highly repetitive sequences. A minority of the genome is not maintained in the repressive chromatin (euchromatin) state, and is actively transcribed. The essence of this concept is that heterochromatic, transcriptionally repressive chromatin has the potential to spread into adjacent regions of euchromatin unless boundary elements maintain and protect active domains from this process.
7.4.3 Epigenetics and regulation of methylation

Epigenetics is defined as inheritance of changes in gene activity that are independent of DNA sequence. The two main epigenetic events involved in gene regulation, development and carcinogenesis are DNA methylation and histone modification. One of the mechanisms by which a cell can completely silence gene expression is by modification of its promoter region and preventing certain transcription factors from interacting with its DNA. This is mediated by DNA methyltransferases (DNMTs) that catalyse the covalent addition of a methyl group to the 5' carbon of cytosine, creating 5-methylcytosine, which some have called the “fifth base” of DNA.

CpG islands are defined as sequences of at least 200 bases in length (usually >500) with >50% cytosine-guanine content and “a ratio of observed to expected CpGs that is >60% (241). Most CpG sites are maintained in a methylated state, especially those that are not in the promoter region of a gene, but are not methylated in actively expressed genes. Methylation starts at one end of the CpG island and extends through the promoter and start site of the gene; this alters the three-dimensional configuration of the DNA, inhibiting its interaction with transcription factors and silencing gene expression (242). Importantly, this epigenetic modification is stably passed to the progeny of the parent cell, maintained by “histone marking” at the sites of methylation. Permanent silencing of genes is a prominent part of normal development,
because certain genes are no longer used after the embryonic stage; moreover, maladaptive cellular behaviors may occur if certain developmental genes are inappropriately expressed in differentiated tissues. Figure 7.3 demonstrates that aberrant promoter methylation serves to turn off critical genes that could lead to tumour progression. Therefore, promoter hypermethylation can account for the initial hit, with subsequent mutations or deletions eliminating the second gene copy. Again, DNA methylation is regulated by DNMTs, i.e., DNMT1, DNMT2, DNMT3a and DNMT3b. In the mouse, DNMT1 appears to be responsible for maintenance of the established pattern of DNA methylation while DNMT3 seems to mediate formation of de novo methylation patterns. The effects of these DNMTs are less well established in humans.

![Diagram](image)

**Figure 7.3** Regulation of gene transcription by DNA methylation. Promoter region of a certain tumour suppressor (rectangular boxes) is hypomethylated (open circle) in normal tissues. However, in cancer tissue, some co-factors recruit DNMT and hypermethylate the promoter region (close circle), resulting in suppression of gene transcription.
Treatment with a demethylating agent can cause global DNA changes that can increase levels of suppressed genes. In 1998, azacitidine (AZA) was approved by the US Food and Drug Administration for the treatment of hematopoietic cancers; however, it has also been widely used in the laboratory setting. Figure 7.4 shows that the 5'-Aza moiety is incorporated into DNA in place of cytidine residues. It acts by preventing resolution of a covalent reaction intermediate that traps and inactivates DNMT in the form of a covalent protein-DNA adduct, resulting in rapid depletion of DNMT and concomitant demethylation with continued DNA replication (243). The newly replicated DNA will have unmethylated cytosine residues, hypomethylation of DNA, and subsequent expression of previously repressed genes.

**Figure 7.4** Suppression of DNMT by 5'-Aza. 5'-Aza has high affinity for DNMT thus interfering with binding of DNMT to cytosine. **Abbreviation**: DNMT, DNA methyltransferase.
7.5 Rationale for laboratory research

CRCs are a group of heterogeneous tumours, and questions remain regarding the effect of miRNAs in CRC of various subtypes. In the following chapters a series of studies have been conducted looking specifically at the significance of miRNAs in the serrated neoplasia pathway. The hypothesis centers around that hypermethylation of CpG islands in the promoter region of key miRNAs would result in epigenetic silencing of miRNAs that function as tumour suppressors. This would subsequently lead to overexpression of their regulatory genes which will confer cell growth and tumour formation. The next chapter explains the rationale for choosing the specific two miRNAs and following from that the subsequent chapter contains the results of a series of experiments carried out to explore how one of these miRNAs is involved in the serrated pathway.
miRNAs regulate gene expression and play pivotal roles in normal cellular processes, including proliferation, differentiation, and apoptosis (214). Both aberrant expression and silencing of miRNAs have been observed in human cancers, suggesting potential oncogenic and tumour suppressor functions for these miRNAs (236). Of particular interest with regard to this research is miRNA transcriptional regulation by DNA methylation. Generally, gene promoter DNA methylation is negatively correlated with gene expression and can account for abnormal tumour suppressor gene silencing in a variety of human cancers (244). Epigenetically silenced miRNAs have been discovered in cancers based on differential expression between normal tissues and tumours and between baseline and DNA-demethylated cancer cells. Some of these miRNAs are emerging as significant contributors to carcinogenesis in the colon. Examples of these include miR-143 and miR-145, two of the first miRNAs identified as having reduced expression in colon tumours (245). These findings have been validated in multiple studies, and miR-143 and miR-145 behave with tumour suppressor activity in colon cancer (231, 246, 247). In addition, one of the target of let-7 is the RAS oncogene (248, 249) and this microRNA has important tumour suppressor activity in multiple cancer
types (250). An oncogenic miRNA, miR-21, has been found to be overexpressed in colon (233, 251) and other solid cancers that have been studied (252). This miRNA appears to be an important factor in the initiation, progression and metastasis of colon and other cancers. Other important miRNAs that appear to contribute to colon cancer are the miR-17-92 cluster, miR-135a/b and miR-200c.

Thus far, the hypermethylated sites used to characterise CIMP have not been standardised, leading to inconsistencies as to whether a colon cancer is appropriately described as CIMP (253-256). Consequently, clinical studies attempting to correlate CIMP with prognosis and responsiveness to 5-fluorouracil chemotherapy have produced conflicting results (257-262). While genome-scale, array-based platforms may be sufficient for visualizing subgroups of colon cancers with a distinct pattern for DNA methylation, this type of approach cannot assess if:

1. DNA methylation patterns in all genomic contexts are significantly different between the subgroups and are similar within each subgroup
2. CpG islands are truly the preferential methylation targets in CIMP
3. Additional molecular features, other than CpG island DNA methylation, can distinguish CIMP

On the other hand, sequencing-based approaches can objectively survey the
genome to allow construction of individual DNA methylome profiles to answer the above questions. Currently, the most widely used marker panel for defining CIMP consists of CpG islands at CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1 (263).

Xu et al (195) has used parallel sequencing technology to test the whole genome directly, with minimum ascertainment bias, to see whether these markers truly differentiate between subgroups of colon cancers with different propensities for CpG island DNA hypermethylation as seen in Figure 8.1. To do so, the DNA methylomes of three sets of specimens from normal colon tissues, non-CIMP colon cancers, and CIMP colon cancers. Briefly, they incubated randomly sheared genomic DNA from each sample with recombinant methyl CpG-binding domain proteins to capture methylated DNA fragments for sequencing on the Genome Analyzer II (Illumina Inc, San Diego, CA, USA). The CIMP tumours were selected based on DNA methylation at the five markers described by Weisenberger et al. and the presence of MSI and BRAF mutations (263). The non-CIMP tumours had no methylation at the five markers and harbored KRAS mutations, which were mutually exclusive of BRAF mutations in colon cancer. At a threshold of a <5% false discovery rate, 6135 differentially methylated sites were found between the normal, non-CIMP, and CIMP sets. The samples clustered to form three distinct groups consisting of normal, non-CIMP and CIMP specimens shown in Figure 8.1.
This result suggests that the sample selection criteria convincingly identified two populations of colon cancers that differ in their genome-wide DNA methylation profiles.

**Figure 8.1** Mapping and comparisons of DNA methylomes in normal colon, non-CIMP and CIMP colon cancer specimens. (A) Multidimensional scaling plot of the nine samples using their individual genome-wide DNA methylation profiles: red dots are normal colon samples, green dots are non-CIMP colon cancer samples, blue dots are CIMP colon cancer samples. (B) Heatmap and unsupervised hierarchical clustering of the nine samples based on their individual DNA methylation status at 6135 sharp yes/no differentially methylated sites. N1, N2, N3 are normal colon specimens, T1, T2, T3 are non-CIMP cancer specimens, T4, T5, T6 are CIMP cancer specimens. (C) Spatial distribution of sharp yes/no differentially methylated sites in the genome. Each sharp yes/no differentially methylated site is represented by a vertical tick below each chromosome. Each differential methylation pattern is represented by one colour according to the colour key. The blue horizontal line under each chromosome represents regions of the chromosome where DNA methylation was detected in any of the nine samples. Reproduced with the permission of Xu et al. (195) and the publisher.

Xu et al. concluded that CIMP tumours had significantly more CpG island DNA
methylation on a genomic scale (195). Consistent with previous reports, CIMP and non-CIMP colon cancers shared a large number of hypermethylated sites (3780 sites) when compared with normal colon tissue (263, 264). In addition to shared hypermethylation, CIMP tumours have an additional 2026 unique hypermethylated sites compared with only 49 sites that are unique to non-CIMP tumours. CIMP-specific hypermethylation is not restricted to any one genomic context, but CpG islands account for 80% of these sites, and this increase is largely attributable to CpG island hypermethylation in the promoter sites. These data from Xu et al. demonstrate on a genome-wide scale that CIMP cancers truly have a higher propensity for CpG island hypermethylation (195).

In support of this, Lanza et al. also compared the miRNA expression patterns of MSS versus MSI-H tumours (265). The expression of eight miRNAs could distinguish between MSS from MSI tumours, including expression of the miR-17-92 cluster. Valeri et al. also demonstrated that miR-155 could target the mismatch repair genes MLH1, MSH2 and MSH6 and contribute to the MSI-H phenotype in colon cancer (266). Sarver et al. investigated the miRNA expression patterns of DNA mismatch repair-proficient and DNA mismatch repair-deficient tumours and identified six miRNAs that were differentially expressed between these subtypes of colon cancer (267). Although most work with p53 has been performed in vitro with little support from human tissues, the
relevant studies have identified miRNAs regulated by p53 in colon cancer cell lines (268-270).

Xu et al. (195) identified a total of 17 miRNAs to be differentially hypermethylated and one to be differentially hypomethylated in six CRC specimens when compared with three normal specimens. Of the 17 miRNAs, only miR-1247 and miR-1258 were differentially hypermethylated in CIMP tumours when compared with non-CIMP tumours. A Medline search was performed to determine whether miR-1247 or miR-1258 had been reported. In the literature, miR-1247 had only been explored by Yan et al. (271); in their research, they used genome sequencing (MiGS) to evaluate genome-wide DNA methylation patterns and microarray analysis to determine miRNA expression levels in CRC cell lines. A total of 64 miRNAs were located within 500 bp of robust DNA methylation in HCT116 or DKO cells. Of those, five miRNAs were located in the gene imprinting regions, 13 miRNAs had been previously reported to be regulated by DNA methylation, and 46 miRNAs had not been reported to be regulated by methylation. The DNA methylation status of 18 unreported miRNAs was consistent with their expression levels in HCT116 and DKO cells. Finally, eight miRNAs were upregulated by treatment with 5'-Aza and identified to be novel miRNAs regulated by DNA methylation. In addition, they demonstrated that miR-941, miR-1247 and miR-1237 had functional relevance with regard to inhibition of growth and migration of cancer
cells.

The only published study on miR-1258 considered its relationship with brain metastatic breast cancer (BMBC). Heparanase is a potent protumorigenic, proangiogenic and prometastatic enzyme that is overexpressed in BMBC. Using miRanda and RNAhybrid, Zhang et al. (272) identified heparanase to be a candidate target for miR-1258, and he hypothesised that it suppresses BMBC. In a series of experiments, an inverse correlation was noted between miR-1258 level and heparanase expression, enzymatic activity, and the metastatic propensity of cancer cells, being lowest in highly aggressive BMBC cell variants compared with either non-tumorigenic or non-metastatic human mammary epithelial cells. These findings were validated by analysis of miR-1258 and heparanase content in paired clinical specimens of normal mammary gland tissue versus invasive ductal carcinoma. In functional experiments, stable expression of miR-1258 in BMBC cells inhibited cell invasion. The same authors concluded that miR-1258 was linked to BMBC through heparanase control, hence the rationale to develop heparanase-based therapeutics.

Next, by using the Ingenuity software and its "Pathway Analysis", top pathways associated with miR-1247 were identified as shown in Figure 8.2. The Wnt pathway was noticeably the second most common pathway after the axonal
guidance signaling pathway.

Figure 8.2 Top predicted pathways for miR-1247.

The canonical Wnt pathway has been well studied and is closely associated with the development of CRC. When Wnt proteins bind to and activate the cell surface receptors, these Frizzled proteins activate Dishevelled family proteins, which in turn inhibit the “destruction complex” that includes Axin, glycogen synthase kinase-3β, and APC. This allows β-catenin within the cytoplasm to translocate into the nucleus, where it acts as a cofactor for T-cell factor/lymphoid enhancing factor transcription factors and regulates a wide variety of specific cells. When Wnt ligand binding is absent, phosphorylation of
β-catenin will result in ubiquitination and proteolytic degradation. Mutations of the APC gene result in a stable protein that is resistant to degradation of the “destruction complex”. Correspondingly, there will be an increase in the cytoplasmic β-catenin level and its migration into the nucleus. Therefore, APC mutations indirectly induce genes targeted by TCF/LEF transcription factors, including the proto-oncogenes, c-myc and cyclin D1, and genes encoding membrane factors (matrix metalloproteinase-7, CD44), growth factors, and Wnt pathway feedback regulators (273-287). Furthermore, with Ingenuity software, the predicted gene targets of miR-1247 in the Wnt pathway are identified, this is demonstrated by the shaded boxes in Figure 8.3.
**Figure 8.3** Predicted gene targets for miR-1247 in the Wnt pathway. The grey areas are genes targeted by miR-1247.

Again, Ingenuity software was used to determine the predicted pathways for miR-1258 as demonstrated in Figure 8.4. Unlike miR-1247, the Wnt pathway was not within the top ten predicted pathways for miR-1258, but interestingly the molecular mechanism of cancer was within this group.

**Figure 8.4** Top predicted pathways for miR-1258.
Given the close association between the Wnt pathway and CRC, potential gene targets of miR-1258 in this pathway were again identified with “Pathway Analysis”, even though Wnt was not one of the top ten predicted pathways. The genes targeted by miR-1258 are demonstrated in the shaded boxes in Figure 8.5.

Figure 8.5 Predicted gene targets for miR-1258 in the Wnt pathway. The grey areas are genes targeted by miR-1258.
In summary, with the identification of 2 novel miRNAs by Xu et al. (195) a series of experiments was planned to look into the potential tumour suppressor effects of miR-1247 and miR-1258 in vitro and in vivo. The next chapter covers miR-1247 exclusively, first looking at its expression levels in various subtypes of CRCs, then a series of in vitro experiments focusing on cell proliferation, apoptosis and motility. These will be followed by a series of functional experiments to determine the effect of methylation in both serrated and conventional CRC cell lines. To further validate the tumour suppressive effects of miR-1247, experiments will be replicated in nude mice. Lastly, to allow us to determine the potential targets of the miRNAs, the 3'-UTR assay will be used to detect direct binding of miRNA to its target mRNA. By identifying its target and its associated pathways, this will broaden our understanding of the importance of miRNAs and their intricate relationship with the serrated colorectal neoplasia pathway.
CHAPTER IX
miR-1247 FUNCTIONS AS A TUMOUR SUPPRESSOR AND IS EPIGENETICALLY REGULATED BY DNA METHYLATION IN SERRATED COLORECTAL CANCERS

9.1 Introduction

Unlike the well studied classical adenomatous polyps, SSA/Ps evolve through a distinct molecular pathway. Several papers have provided data highlighting a lack of the genetic alterations in serrated polyps that are seen in adenoma. Further, studies have started to demonstrate a set of changes that differentiate serrated polyps from traditional adenomas, including \textit{BRAF} mutation (22, 59, 126), CpG island methylation (59, 112), microsatellite instability (50, 112) and loss of \textit{hMLH1} (172). Hence, progression of methylated (BRAF/CIMP+/MSI-H) tumours cannot be explained by the well established Vogelstein’s APC theory (160), and an alternative mechanism such as epigenetic regulation of tumour suppressors may be responsible.

As mentioned in Chapter 7, miRNAs are a class of small (19–22 bp) non-coding, single-stranded RNAs that negatively regulate gene expression at the post-transcriptional level. These single-stranded RNAs are considered to play crucial roles in many normal cellular processes, such as proliferation,
development, differentiation and apoptosis, by regulating expression of their target genes via complete or incomplete pairing with their target mRNAs. This induces either direct mRNA degradation or translational inhibition (224, 288, 289). Recent work has shown aberrant miRNA expression patterns in a range of human diseases, including many cancers (290). Although the mechanisms for dysregulation of miRNAs are still largely unidentified, it has been proposed that hypermethylation of the promoter region of miRNAs may govern their expression (291). Here the promising role of miR-1247 as a tumour-suppressive miRNA in methylated CRCs is explored.

9.2 Methods and materials (see Appendix)

9.2.1 Patient and sample collection

The research protocol was approved by the regional institutional review board at Cleveland Clinic, Ohio. Twenty methylated (BRAF/CIMP+/MSI-H) and 20 non-methylated (KRAS/CIMP-/MSS) CRC samples, as well as 10 normal colonic mucosa samples, were retrieved from the tumour bank. A histological diagnosis of CRC was confirmed by pathologists from surgical specimens. Characterisation of BRAF, CIMP and MSI status has been described previously (134). Demographic and tumour characteristics were abstracted from the Colorectal Cancer Registry at Cleveland Clinic.
9.2.2 Antibodies and reagents

Antibodies against cleaved caspase-3, i.e., 9661S (Cell Signaling Technology Inc, Danvers, MA, USA), MYCBP2 (ab86078, Abcam, Cambridge, UK), c-Myc (sc-40 Santa Cruz Biotechnology), and tubulin (T6074, Sigma-Aldrich) were used in immune staining or Western blot at a concentration recommended by their respective manufacturers. Biotinylated goat-anti-rabbit secondary antibody for immunohistochemistry was purchased from Vector Laboratories. Alexa-conjugated secondary antibodies used for immunofluorescence were purchased from BD Biosciences (Franklin Lakes, NJ, USA).

miR-1247 mimic and inhibitor (anti-miR-1247) were purchased from Qiagen. miRNA mimic is a double-stranded synthetic RNA synthesised and chemically modified using proprietary procedures, whereas the miRNA inhibitor is engineered by proprietary synthetic hairpins to generate an antisense RNA to the mature miR-1247. Both the mimic and inhibitor were designed based on microRNA sequences available in miRBase databases (microrna.org).

Demethylation of DNA was achieved using 5-aza-2’-deoxycytidine (A3656, Sigma-Aldrich) dissolved in dimethyl sulfoxide. Cells were treated at a
concentration of 15 µM for the desired time points.

9.2.3 Cell lines and miR transfection

RKO, SW480 and SW620 were kind gifts from Dr Janet Houghton (Cancer Biology, Lerner's Research Institute, Cleveland Clinic) and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. LS411N was purchased from the American Type Culture Collection and cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum. For transient transfection, 20 µM of miR mimic or 100 µM inhibitor were suspended in 6 µL of RNAiMAX to transfect 250,000 cells in a 35 mm dish according to the manufacturer's instructions. Scrambled control AllStars Negative was purchased from Qiagen. Forty-eight hours post transfection, the cells were harvested or replated for other experiments.

9.2.4 Taqman quantitative PCR

Quantitative real-time PCR was performed to assess miR-1247 levels using TaqMan Universal PCR Master Mix (ABI 4324020). Specific probes for hsa-miR-1247 (ABI 4427975), and endogenous control U6 snRNA (ABI 4427975) were used for PCR-based detection of mature miR-1247. In brief, miRNAs were isolated using the mirVana miRNA kit (Ambion AM1560) followed by reverse transcription with a TaqMan miRNA reverse transcription kit (ABI 4366596). TaqMan PCRs were carried out with miR-1247-specific
primers (forward 5'-CTGCGAGTCCCTGGAGTTAG-3'; reverse 5'-CGGTCCCCTA-ACTACCCATT-3') along with Taq-Man probes for miR-1247 or MAM6 control. PCR assays were performed using a 7900 thermal cycler (Applied Biosystems). PCR results were recorded as Ct numbers, normalised against MAM6, and expressed as the log-fold change.

9.2.5 Genomic DNA isolation, bisulphite conversion, and methylation-specific PCR (MSP)

Genomic DNA was isolated using a QIAmp DNA Mini Kit as per protocol (Qiagen 51304). For study of methylation, 1 μg of genomic DNA was bisulphite-converted using an EZ DNA methylation kit (Zymo D5001) according to the manufacturer's instructions. Methylation-specific PCR was done with methylation-specific primers:

- forward 5'- GACAACGAAAAAACGTATCGAA-3'
- reverse 5'-TTTAGGTAGTGTACGTTACCGG-3'

or non methylations specific primers:

- forward 5'-TTCAACCAACAAAAAAACATCATAACTAAA-3'
- reverse 5'-TTTAGGTTAGTGTAGTTATGGG-3'

Universally methylated and nonmethylated controls were purchased from Zymo Research (D5014).
9.2.6 Cell proliferation, migration and anoikis assays

For the cell proliferation assay, performed 48 hours post miRNA transfection, 500 cells were seeded in each well of a 96-well plate. Cell viability was determined at indicated time points with the CellTiter Glo luminescent assay.

Cell migration was examined in Transwell cell culture chambers (354578, BD Biosciences). Forty-eight hours post miRNA transfection, 50,000 cells were added into the upper well of each chamber and migration assay was performed in Dulbecco's modified Eagle's medium or Roswell Park Memorial Institute 1640 medium supplemented with 15% fetal bovine serum for 48 hours. Migrated cells were stained with 0.5% crystal violet and photographed with a 20× microscope (Leica). Cell numbers were calculated with ImageJ version 1.47 software.

To assess cell resistance to anoikis, 50,000 cells were seeded in one well of a 24-well plate precoated with 10% poly-HEMA (P3932, Sigma-Aldrich) and cultured for 24 hours. Cells were suspended in 0.4% Trypan blue (15250-061, Invitrogen) and cell viability was determined using a TC-10 automated cell counter (Bio-Rad).

9.2.7 3'-UTR luciferase reporter assays

The plasmid containing the full-length human MYCBP2 3'-UTR inserted
downstream of the luciferase reporter gene was purchased from Abmgood (MT-h15016). The luciferase reporter construct, along with β-galactosidase control plasmid, was transfected into 293T cells. Twenty-four hours post transfection, the cells were split into 24-well plates followed by transfection of miR-1247 mimics or AllStars Negative control mimics at 50 nM. At 72–96 hours post miRNA transfection, luciferase activity was determined using a luciferase assay system (E4030, Promega) and normalised to β-galactosidase activity measured with a β-galactosidase enzyme assay system (E2000, Promega). The results represented three independent experiments, and each was performed in triplicate.

9.2.8 Immunohistochemistry

Expression of MYCBP2 protein in human colon cancer specimens was examined by immunohistochemistry. Anti-MYCBP2 polyclonal rabbit antibody was used at a 1:200 dilution in phosphate-buffered saline with 10% goat immunoglobulin G. Cryosections were incubated with the first antibody overnight at 4°C, washed with phosphate-buffered saline three times, followed by incubation with biotinylated goat-anti-rabbit secondary antibody in phosphate-buffered saline with 1% bovine serum albumin for 30 minutes at room temperature. Staining of different sections was performed in parallel using a Vectastain ABC kit per the manufacturer’s instructions.
9.2.9 Immunofluorescence

Cells grown on cover slips were fixed in 4% paraformaldehyde at room temperature for 15 minutes followed by permeabilisation in 0.3% Triton X-100 for 30 minutes. After blocking with 1% bovine serum albumin for one hour, cells were stained with the first antibody at 4°C overnight. The next day, the cover slips were washed three times with phosphate-buffered saline and the cells were incubated with Alexa-conjugated secondary antibody along with Hoechst at room temperature for one hour. Cells were mounted using mounting medium (Sigma) and the preparations were visualised with a microscope (Leica).

9.2.10 Lentivirus packaging and infection

Lentiviral constructs containing miR-1247 (ABM Mh10072) was purchased from ABMgood. For virus package, 293Ta cells at 70%–80% confluence were transfected with lentiviral construct along with psPAX2 and VSVG. At 48–72 hours post transfection, the viral supernatant were collected and transduced into colon cancer cells at a multiplicity of infection of 1, per the manufacturer's protocol. Transfection efficiency was monitored by GFP imaging. To obtain stable cells, infected cells were selected with 1 µg/mL puromycin for 4 weeks until >95% cells turned to being GFP-positive. The miR expression level in the stable cells were determined by quantitative PCR.
9.2.11 Xenografts and treatment with 5'-Aza

LS411N cells infected with control or miR-1247-overexpressing lentivirus were selected for 4 weeks. These cells were further infected with luciferase, and $1 \times 10^6$ cells were then subcutaneously injected into athymic BALB/c nude mice (Charles River Laboratory, Wilmington, MA, USA). The control cells were injected into the left flank, while the miR-1247-overexpressing cells were injected into the right flank of the same mouse.

LS411N cells infected with luciferase were subcutaneously injected into nude mice, which were started the next day on daily intraperitoneal injections with 5'-Aza 1 µg/5 g in dimethyl sulfoxide. Growth of the xenografts was monitored using IVIS at indicated time points on days 0, 7, 14, 21 and 28.

9.3 Statistical analysis

The results for cell viability, anoikis, and migration experiments were expressed as the mean ± standard deviation of at least three different experiments performed in triplicate. The data were analyzed with GraphPad Prism (Intuitive Software for Science; GraphPad Software Inc, San Diego, CA, USA). Statistical significance was determined by the Student’s t-test (two-tailed), and differences were considered to be statistically significant at a $p$-value of $<0.05$. 
9.4 Results

9.4.1 miR-1247 expression is attenuated in methylated colon cancer cells and human colon cancers

Our previous genome-wide methylation screening study suggested a distinct methylation pattern in BRAF/CIMP+/MSI-H CRCs (271). One of the two miRNAs that were differentially hypermethylated in this specific subtype of CRC was miR-1247, suggesting that DNA methylation could potentially influence its expression. To address this issue, 20 patients with methylated (BRAF MUT/CIMP+/MSI-H) CRC and 20 patients with non-methylated (KRAS MUT/CIMP-/MSS) CRC were included in this study. The mean age of the patients with methylated CRC was 75.50±10.47 years and 68.25±12.35 years for those with non-methylated CRC. All of the methylated tumours were right-sided, while only five of 20 (25%) non-methylated tumours were right-sided ($p<0.05$).
Figure 9.1 miR-1247 expression in methylated vs non methylated colorectal patients and colorectal cancer cell lines (A, B). TaqMan quantitative PCR of the miR expression levels in the methylator (n=14) versus non-methylator (n=16) colon cancer patients. Relative miR-1247 expression levels were calculated as fold changes. Results were normalised to average miR-1247 expression level in ten normal colonic mucosa. (C) Genetic and methylated status of different colon cancer cell lines. (D) TaqMan quantitative PCR of the miR-1247 expression levels in methylator versus non-methylator colon cancer cell lines. Relative miR-1247 expression levels were calculated as fold changes. (**p<0.001; *p<0.05).

Abbreviation: PCR, polymerase chain reaction.
Total RNA was extracted from the frozen tissue samples followed by reverse transcription for miR-1247. Thereafter, TaqMan quantitative PCR was performed to determine the miR-1247 level in the different tumour subtypes. The results were normalised to ten normal colonic mucosa. A significant decrease in the miR-1247 level was observed in methylated CRC patients compared with non-methylated CRC patients (Figure 9.1A and Figure 9.1B).

To study the effect of miR-1247 in CRC, two BRAF MUT/CIMP+/MSI-H colon cancer cell lines (RKO and LS411N) and two KRAS MUT/CIMP-/MSS colon cancer cell lines (SW480 and SW620) were chosen; the miR-1247 level was decreased in methylated cell lines (Figure 9.1C and Figure 9.1D). Thus, miR-1247 expression is reduced in the methylated subtype of CRC and representative cell lines.

9.4.2 miR-1247 inhibits colon cancer cell proliferation via cell apoptosis

Colorectal cancer cell lines were forced to overexpress miR-1247 by transient transfection of synthesised miRNA. TaqMan quantitative PCR confirmed the success of overexpression (Figure 9.2A). The cells were replated in 96-well dishes 48 hours post transfection and cell viability was determined at various time points. Overexpression of miR-1247 in either RKO (BRAF MUT/CIMP+/MSI-H) or SW480 (KRAS MUT/CIMP-/MSS) resulted in attenuated cell growth. A more noticeable decline of cell viability was observed in methylated CRC cells but not in non-methylated CRC cells after day 5
(Figure 9.2B and 9.2C, Figure 9.3A and 3C), one of the possible causes of such an observation is apoptosis. The miR-1247-transfected and mock-transfected cells were stained with Annexin V and PI. The majority of RKO cells transfected with miR-1247 were PI-positive, indicating that the methylated CRC cells were in the late stage of apoptosis (Figure 9.2D). Meanwhile, miR-1247 overexpression only marginally increased Annexin V staining in SW480, suggesting a mild early apoptotic effect in KRAS Mut/CIMP-/MSS cells (Figure 9.2E). Similar results were also observed in the LS411N (BRAF Mut/CIMP+/MSI-H) cells and the SW620 cells (KRAS Mut/CIMP-/MSS cells) (Figure 9.3B and 3D). To further confirm miR-1247-induced cell apoptosis, overexpression of miR-1247 in the LS411N cells with lentiviral infection and apoptotic cells were detected by immunofluorescence staining for cleaved caspase-3. Again, an increase in cleaved caspase-3-positive cells in miR-1247-infected cells were noted when compared with mock-infected cells (Figure 9.3E and 3F).
Figure 9.2 miR-1247 regulates methylated CRC cells’ proliferation via promoting cell apoptosis. (A) TaqMan quantitative polymerase chain reaction of miR-1247 level 48 hours after transfection with mimic miR-1247. Relative miR-1247 expression levels were calculated as LogRQ by normalizing to miR-1247 expression before transfection in each type of cells, respectively. (B) Cell viability curve of methylator RKO cells with miR-1247 or mock RNA overexpression. (C) Cell viability curve of non-methylator SW480 cells with miR-1247 or mock RNA overexpression. (D, E) Annexin V (early apoptosis marker) plus propidium iodide (late apoptosis marker) staining of CIMP-positive RKO cells or CIMP-negative SW480 cells transfected with miR-1247 or mock RNA. (*p<0.05; **p<0.01; ***p<0.001)
**Figure 9.3** miR-1247 regulates CIMP+ colorectal tumour cell proliferation via promoting cell apoptosis. (A) Cell viability curve of methylator LS411N cells with miR-1247 or mock RNA overexpression. (B) Annexin V (early apoptosis marker) plus propidium iodide (late apoptosis marker) staining of CIMP-positive LS411N cells transfected with miR-1247 or mock RNA. (C) Cell viability curve of non-methylator SW620 cells with miR-1247 or mock RNA overexpression. (D) Annexin V (early apoptosis marker) plus propidium iodide (late apoptosis marker) staining of CIMP-negative SW620 cells transfected with miR-1247 or mock RNA. (E, F) Confirmation miR-1247-induced cell apoptosis by an increase in cleaved caspase-3-positive cells in miR-1247-infected cells compared with mock-infected cells, RT-PCR and immunofluorescence. (*p<0.05; **p<0.01; ***p<0.001).

### 9.4.3 miR-1247 regulates colon cancer cell motility

Next, the ability of miR-1247 to regulate the motility of CRC cells was investigated. Cells transfected with miR-1247 or scrambled RNA were subjected to Transwell assays. Briefly, for each Transwell chamber, 50,000 cells were seeded on the upper well and allowed to migrate for 48 hours. Overexpression of miR-1247 inhibited both RKO and SW480 cells (Figure 9.4A and 9.4C, Figure 9.5A and 5B). However, the inhibitory effect was more significant in the BRAF Mut/CIMP+/MSI-H RKO cells (~70%) than in the KRAS Mut/CIMP-/MSS SW480 cells (~30%, Figure 9.4B and 9.4D). To determine the role of endogenous miR-1247 in cell motility, cells were treated with miR-1247 inhibitory anti-sense RNA or scrambled RNA, and cell migration ability was then tested under the conditions described above. As expected, inhibition of endogenous miR-1247 dramatically increased the migration of BRAF Mut/CIMP+/MSI-H RKO cells (~400%) but had only a mild effect on KRAS Mut/CIMP-/MSS SW480 cells (~30%) (Figure 9.4E–H,
Figure 9.5C and 9.5D). Overexpression of miR-1247 or its inhibitor in BRAF Mut/CIMP+/MSI-H LS411N cells produced the same results as those seen with RKO cells.

The ability of cancer cells to metastasize is often linked to resistance to anoikis, a form of programmed cell death induced by anchorage-dependent cells detaching from the surrounding extracellular matrix. The ability of miR-1247 and scrambled RNA transfected cells to survive under this condition was examined. Again, 50,000 cells were seeded in 24-well plates precoated with 10% poly-HEMA and allowed to grow for 24 hours. The percentage of live cells was then determined by Trypan blue staining and cell counting. Transfection of miR-1247 significantly lessened the resistance of RKO, LS411N and SW480 cells to anoikis (Figure 9.5E). Taken together, the above observations strongly suggest that miR-1247 has an inhibitory effect on the motility of CRC cells and their potential to metastasise.
Figure 9.4 miR-1247 regulates colorectal cancer cell motility. (A) Representative Transwell images of CIMP-positive RKO cells transfected with miR-1247 or mock RNA. (B) Quantification of (A). (C) Representative Transwell images of CIMP-negative SW480 cells transfected with miR-1247 or mock RNA. (D) Quantification of (C). (E) Representative Transwell images of CIMP-positive RKO cells treated with anti-sense miR-1247 inhibitor or control RNA. (F) Quantification of (E). (G) Representative Transwell images of CIMP-negative SW480 cells treated with anti-sense miR-1247 inhibitor or control RNA. (H) Quantification of (G). (*p<0.05; **p<0.01; ***p<0.001).
Figure 9.5 Colorectal cancer cell motility is regulated by miR-1247. (A) Representative Transwell images of CIMP-positive LS411 cells transfected with miR-1247 or mock RNA. (B) Quantification of (A). (C) Representative Transwell images of CIMP-positive LS411 cells treated with anti-sense miR-1247 inhibitor or control RNA. (F) Quantification of (E) Anoikis assay demonstrating an increase in programmed cell death induced by anchorage-dependent cells detaching from the surrounding extracellular matrix in miR-1247 transfected cells (grey) compared with mock (white) (*p<0.05; **p<0.01; ***p<0.001).
9.4.4 DNA methylation regulates miR-1247 expression which has a reciprocal effect on cell proliferation and migration in colorectal cancer cells

To further clarify the relationship between DNA hypermethylation and miR-1247 expression, the four CRC cell lines were treated with demethylation drug, 5'-Aza, and the expression level of miR-1247 was determined by TaqMan quantitative PCR. Methylation-specific PCR showed that the naive BRAF Mut/CIMP+/MSI-H RKO cells and the LS411N cells, but not the KRAS Mut/CIMP-/MSS SW480 and SW620 cells, are hypermethylated in the miR-1247 5' region (Figure 9.6A). Treatment with 5'-Aza resulted in a substantial decrease in methylation of the two BRAF Mut/CIMP+/MSI-H cell lines (Figure 9.6A), and as expected, had no effect on the two KRAS Mut/CIMP-/MSS cell lines. After treatment with 5'-Aza, there was a consistent simultaneous increase in the miR-1247 expression level in RKO cells (>10-fold) and in LS411N cells (>5-fold), with minimal effects observed in KRAS Mut/CIMP-/MSS cells (Figure 9.6B). These data suggest that DNA hypermethylation negatively regulates miR-1247 expression in BRAF Mut/CIMP+/MSI-H CRC cells.
Figure 9.6 miR-1247 expression in colon cancer cells is regulated by DNA methylation. (A) Methylation-specific PCR of the miR-1247 5' region in methylator and non-methylator colon cancer cells before and after treatment with 5'-Aza. M: methylation-specific primer. U: unmethylation-specific primers. (B) TaqMan PCR of miR-1247 expression levels in methylator and non-methylator colon cancer cells after treatment with 5'-Aza. Results were calculated as fold changes by normalizing to miR-1247 levels in each cells treated with dimethyl sulfoxide. Abbreviation: PCR, polymerase chain reaction.

Next, the upsurge in miR-1247 level post demethylation and its functional effect on cell proliferation and migration were explored. Cells were treated with 5'-Aza for five days, and the cells were then trypsinised and seeded in 96-well plates. Cell viability was determined at indicated time points. Treatment with 5'-Aza was associated with an increase in miR-1247 expression levels and a parallel inhibition of cell growth in both RKO cells
(Figure 9.7A) and LS411N cells (Figure 9.7B). Consistent with this finding, Transwell assays of cells treated with 5'-Aza indicated a significant reduction in cell migration (Figure 9.7C and 9.7D). In summary, miR-1247 expression was repressed by DNA hypermethylation in BRAF Mut/CIMP+/MSI-H cells, and release from such inhibition on treatment with 5'-Aza did result in a rise in miR-1247 levels. This subsequently impeded cell growth and motility.

**Figure 9.7** DNA methylation regulates viability and motility of methylator colon cancer cells. (A) Viability curve of methylated RKO cells treated with 5'-Aza or the dimethyl sulfoxide control. (B) Viability curve of methylated LS411N cells treated with 5'-Aza or the dimethyl sulfoxide control. (C) Quantification of Transwell assays of methylated RKO cells treated with 5'-Aza or dimethyl sulfoxide. (D) Quantification of Transwell assays of methylated LS411N cells treated with 5'-Aza or the dimethyl sulfoxide control. (*p<0.05; **p<0.01; ***p<0.001).
9.4.5 miR-1247 suppresses colon cancer xenograft progression *in vivo*

Nude mouse xenografts were established to study the influence of miR-1247 on colon cancer *in vivo*. miR-1247 or control virus, along with luciferase, was introduced into LS411N cells by lentiviral infection. Infected cells were selected with puromycin for 4 weeks to obtain stable cells, and the miR-1247 expression level was determined using the enhanced GFP reporter assay (Figure 9.9A). Cells overexpressing miR-1247 were subcutaneously injected into the right flank and cells infected with control virus were injected into the left flank of the same mouse. Xenograft progression was monitored using IVIS at indicated time points. Xenograft growth recapitulated that of colon cancer cells *in vitro*, i.e., overexpression of miR-1247 greatly delayed tumour progression *in vivo* throughout the whole period of tumour growth. The inhibition of tumour growth could be observed from an early stage (Figure 9.8A). Furthermore, miR-1247 overexpression resulted in a significant reduction in tumour mass and size (Figure 9.8B and 9.8C). Specifically, injection of control cells yielded an average tumour weight of 424.5 mg, whereas injection of cells overexpressing miR-1247 yielded an average tumour weight of 73.3 mg, i.e., an 82% reduction in tumour mass (Figure 9.8D). The inhibitory effect of miR-1247 on xenograft growth was evident using IVIS, on palpation, and with objective measurement of size and weight. Interestingly, on further analysis of the tumour formed in the miR-1247-overexpressed flank, the majority of cells had low levels of GFP signals, implying that miR-1247 expression had
somehow been lost (Figure 9.9A and 9.9B).

Figure 9.8 Overexpression of miR-1247 suppresses growth of the colorectal cancer xenograft. (A) Representative in vivo images of xenografts derived from cells infected with miR-1247 or mock lentivirus. The images showed tumour growth by ten days post injection. (B) Image of mice bearing control (left) or miR-1247-overexpressing (right) LS411N cell-derived xenografts in the terminating point. (C) Image of explants from control (upper) or miR-1247-overexpressing (lower) LS411N cells. (D) Scatter plotting of the weight of explants.
**Figure 9.9** miR-1247 or control virus, along with luciferase, was introduced into LS411N cells by lentiviral infection. miR-1247 expression level was determined using the enhanced green fluorescent protein (eGFP) reporter assay. (A) Tumour formed in the miR-1247-overexpressed flank demonstrated low levels of GFP signals, implying that miR-1247 expression had somehow been lost. (B) Mock-infected LS411N cells. (C) Methylation-specific polymerase chain reaction did not detect demethylation of the miR-1247 promoter in the tumours that developed in the mice treated with 5'-Aza, suggesting that these tumours had arisen from cells that had somehow evaded treatment with 5'-Aza.

As previously demonstrated, treatment with 5'-Aza upregulated miR-1247 expression and inhibited proliferation of colon cancer cells *in vitro*; this effect on tumour progression was further studied *in vivo*. Nude mice were subcutaneously injected with LS411N cells and then treated or not treated with 5'-Aza. Again, the effect on tumour progression was monitored by IVIS. Treatment with 5'-Aza resulted in a significantly delayed rate of tumour growth and diminished tumour sizes (Figure 9.10A and 9.10B). Large palpable tumours were observed in 5/5 mice in the control group versus 1/5 in the treated group. The remaining two mice in the treatment group developed very small tumours and two had no tumour growth (Figure 9.10B and 9.10C). Methylation-specific PCR did not detect demethylation of the miR-1247 promoter in the tumours that developed in the mice treated with 5'-Aza (Figure 9.9C), suggesting that these tumours had arisen from cells that had somehow evaded treatment with 5'-Aza. These data, combined with the fact that most cells in miR-1247-overexpressing explants actually lost miR-1247 overexpression, strongly indicate a suppressive effect of miR-1247 on colon cancer growth *in vivo*. These sets of experiments support that increased
miR-1247 expression, either by genetic manipulation for overexpression or pharmacological restoration via demethylation agents, inhibits tumour progression of colon cancer xenografts \textit{in vivo}.

\textbf{Figure 9.10} 5’-Aza treatment suppresses growth of colorectal cancer xenografts. (A) Representative image of mice bearing LS411N-derived tumours treated with control treatment (upper) or 5’-Aza (lower). (B) Representative image of explants from control-treated (upper) or 5’-Aza-treated (lower) mice. (C) Scatter plot of the weight of explants with or without 5’-Aza treatment.
9.4.6 miR-1247 targets MYCBP2 in colon cancer

TargetScan, microRNA.org, and miRBase were utilised to predict the putative coding gene target of miR-1247 (Figure 9.11).

**Figure 9.11** TargetScan was used to predict the putative coding gene target of miR-1247

The resulting database search converged on Myc-binding protein 2 (MYCBP2), a protein reported to bind to myc, a well known tumour-associated transcriptional factor. Western blots was performed to validate the effect of miR-1247 on MYCBP2. RKO or LS411N cells were transfected with miR-1247 or scrambled RNA and lysed with RIPA buffer 48 hours post transfection. As expected, MYCBP2 protein levels were significantly decreased in miR-1247-transfected samples compared with controls (Figure 9.12A). The luciferase reporter assay was then used to
determine if this decline in protein could be secondary to a direct or indirect effect of miR-1247 on MYCBP2. Briefly, miR-1247 and luciferase constructs containing MYCBP2 3′-UTR or an empty control were cotransfected into 293T cells. Luciferase activity was decreased by approximately 60% in miR-1247-transfected cells when compared with the control (Figure 9.12C). At the same time, overexpression of miR-1247 had no significant effect on luciferase activity in the control construct (Figure 9.12C). Taking this further by investigating the potential target of MYCBP2, a decrease of the tumour-associated transcriptional factor c-myc protein level in cells overexpressing miR-1247 was observed (Figure 9.12A). In contrast, overexpression of the miR-1247 inhibitor dramatically increased the MYCBP2 protein level in RKO and LS411N cells when compared with mock-transfected cells (Figure 9.12B). Again, this translated directly into an increase in c-myc protein (Figure 9.12B). Although the molecular mechanism of the positive regulation of c-myc by MYCBP2 remains unknown, these data demonstrate that miR-1247 directly targets and degrades MYCBP2, and this in turn suppresses the c-myc protein level. This may partly explain the tumour suppressive function of miR-1247. By analyzing the public mRNA microarray dataset, a high MYCBP2 mRNA level correlates with poorer survival in patients with CRC (Figure 9.12D).
Figure 9.12 MYCBP2 is the direct target of miR-1247 in colorectal cancer. (A) Western blot of MYCBP2 and Myc protein levels before and after miR-1247 transfection. (B) Western blot of MYCBP2 and Myc protein levels before and after miR-1247 inhibitor transfection. (C) Luciferase assays demonstrating miR-1247 targeting the 3'-UTR region of MYCBP2. (D) Survival curve of colorectal cancer patients with different levels of MYCBP2 mRNA. (E) Representative immunohistochemistry images of MYCBP2 staining in two CIMP-positive colorectal cancers. (F) Representative immunohistochemistry images of MYCBP2 staining in two CIMP-negative colorectal cancers. (***p<0.001; ns, p>0.05).
In view of the observed inverse correlation between MYCBP2 and miR-1247 *in vitro*, the relationship was further evaluated in human specimens. Cryosections of seven BRAF MUT/CIMP+/MSI-H patient samples along with seven KRAS MUT/CIMP-/MSS patient samples were stained for MYCBP2 expression by immunohistochemistry. miR-1247 levels were previously determined with Taqman quantitative PCR. All of the BRAF MUT/CIMP+/MSI-H samples showed medium to high expression of MYCBP2, whereas the KRAS MUT/CIMP-/MSS samples showed minimal to low levels of MYCBP2 (Figure 9.12E and 9.12F). These findings point to an inverse relationship between miR-1247 and the MYCBP2/c-myc axis.

### 9.5 Discussion

Serrated pathway colorectal cancer is a recently described distinct variant of CRC. The earliest genetic alterations in serrated lesions are *BRAF* mutations, and studies have shown that *KRAS* and *BRAF* mutations are mutually exclusive in most colorectal polyps, indicating that both mutations occur early in development of neoplasia. This supports the idea that these are two parallel pathways that rarely cross over. The most common *BRAF* mutation (V600E) results in a constitutively activated enzyme and hence augments cellular responses to many extracellular signals regulating cell growth and differentiation and apoptosis, and BRAF mutation alone has been associated with a poorer prognosis (292, 293). The intermediate step involves CpG island
methylation. The binding of a methyl group to CpG islands, which are stretches of recurrent cytosine-guanine dinucleotide sequences, causes silencing of the associated genes. This may confer a growth advantage by inactivating both alleles of a tumour suppressor gene in an affected cell, thus giving rise to an expanding clone and progression of neoplasia. Loss of the mismatch repair mechanism represents the final step in the development of serrated cancers. Microsatellites are short, repetitive DNA sequences that are scattered throughout the genome. These are often more pronounced in tumour tissue than in normal tissue. In contrast with the traditional MSS cancers, recent published data imply that standard 5-fluourouracil-based chemotherapy confers a survival advantage in patients with advanced MSI-H cancer (294).

Several mechanisms contribute to the initiation and progression of cancer. One of the most fascinating aspects of tumorigenesis is the epigenetic regulation of cell homeostasis. The previous genome-wide work has deciphered a distinct methylation pattern in the CIMP subset; two putative miRNAs that are differentially hypermethylated in CIMP when compared with non-CIMP CRCs. (195). In the present studies, I have explored the role of miR-1247. First, with quantitative PCR and 20 tumours of each subtype, there is a significant decrease in miR-1247 expression in methylated (BRAF Mut/CIMP+/MSI-H) cancers compared with non-methylated (KRAS Mut/CIMP-/MSS) cancers. Second, rescue of miR-1247 expression in
methylated CRC cells results in a significant decline in cell viability and motility. Third, methylation-specific PCR confirmed that the 5' regions of miR-1247 in methylated CRC cell lines are indeed hypermethylated and that treatment with the demethylation reagent 5'-Aza results in increased miR-1247 expression. Expression of miR-1247 is preferentially inhibited by DNA hypermethylation in the methylated subset of cancers. Finally, similar results were replicated in xenografts, thereby demonstrating the tumour-suppressive role of miR-1247 in methylated CRCs in vivo.

Furthermore, the research has identified MYCBP2 as the coding gene target of miR-1247. MYCBP2, also known as PAM (protein associated with Myc), is a large and highly conserved protein that interacts directly with the transcriptional activating domain of Myc (295). Functioning as an adenyl cyclase and an E3 ubiquitin ligase, MYCBP2 has been demonstrated to have an important role in the nervous systems (296-298). In addition, research in T-cell lymphoma has established a link between overexpression of MYCBP2 and a poorer prognosis, indicating the involvement of MYCBP2 in cancer (299). By analyzing the public mRNA microarray dataset, it is noted that high MYCBP2 mRNA level correlates with poorer survival in CRCs (Figure 9.13). Therefore, it is quite possible that miR-1247 exerts its tumour-suppressive function by targeting MYCBP2, the putative oncogene.
The molecular pathways downstream of MYCBP2 are largely unknown. Previous research has suggested that the negative regulator TSC2 is a substrate of the E3 ubiquitin ligase activity of MYCBP2 in the mTOR pathway. Thus, MYCBP2 may promote tumorigenesis by inhibiting the tumour suppressor TSC2 via ubiquitination-mediated degradation (300, 301). Using Western blotting, a decrease in MYCBP2 protein levels in cells transfected with miR-1247 was observed; however, this had no effect on downstream TSC2 or mTOR protein levels (data not shown). Therefore, the myc proto-oncogene, the other probable downstream target of MYCBP2 in CRC cell lines, was investigated.

A notable discovery was the appreciable decline of c-myc protein levels in cells overexpressing miR-1247. By regulating approximately 15% of all genes, myc proto-oncogene has a global effect on cellular proliferation (upregulating
cyclins, downregulating p21), cell growth (upregulating ribosomal RNA and proteins), apoptosis (downregulating Bcl-2), differentiation and stem cell renewal, and hence is considered as a key oncogenic transcription factor (302-304). Although MYCBP2 was initially identified as a myc-associated protein, no report has interpreted its direct interaction with Myc. It is possible that MYCBP2 may stabilize Myc by regulating its localisation or it may function as an E3 ligase to negatively regulate some of its suppressors, resulting in upregulation of Myc expression. On the other hand, MYCBP2 has been suggested as a putative transcriptional factor and may affect Myc directly or indirectly through transcriptional regulation. Given all these assumptions, further studies are warranted to elucidate the mechanisms by which MYCBP2 works on the MYCBP2/Myc axis in tumorigenesis.

The discovery of miRNAs has substantially changed the view of gene regulation, and new findings over the past few years have catapulted miRNAs to the center stage of cancer molecular biology. It is now evident that dysregulation of miRNAs is an important step in the development of many cancers, including CRCs. miRNAs are a set of small non-coding regulatory genes that are highly conserved in sequence between distantly related organisms, indicating their participation in essential biological processes. They regulate a broad spectrum of physiological and pathological processes (288). Dysregulation of miRNA expression can influence carcinogenesis if target
mRNAs are encoded by tumour suppressor genes or oncogenes. Relatively minor variations could have important consequences for the cell because of the large number of targets for each miRNA (305, 306). An accumulating body of evidence suggests that miRNA may be controlled by epigenetic modifications, especially under pathological conditions such as DNA methylation of the 5'-promoter region of miRNA genes, resulting in initiation and progression of several cancers, including lung adenocarcinoma, epithelial ovarian cancer, hepatocellular carcinoma, breast cancer, and endometrial cancer (307-312). The functional effect of miRNAs depends on their complementarity with the 3'-UTR of their target mRNA (313).

Recent studies have suggested that miRNA dysregulation may contribute to the development of CRCs (314-317). Bandres et al were able to demonstrate restoration of miR-9, miR-129 and miR-137 expression after simultaneous treatment of CRC cell lines with both a methyltransferase inhibitor and a histone deacetylase inhibitor (230). In addition, miR-148a and miR-34b/c were found to be re-expressed in metastatic colorectal cell lines following treatment with 5'-Aza. This re-expression of epigenetically silenced miR-148a and miR-34b/c led to inhibition of cell motility in cell lines and a reduction in metastatic potential in xenografts. So far, studies of DNA methylation-regulated miRNA have focussed on the difference between normal tissue and cancer tissue, and to the best of our knowledge, this is the
first time this concept has been further explore in different subtypes of CRCs. Although the initial endogenous miR-1247 level is lower in BRAF Mut/CIMP+/MSI-H CRC cells, the effect on cell mobility is more obvious in these cells when miR-1247 is inhibited by synthesised anti-sense RNA when compared with KRAS Mut/CIMP-/MSS cells. Thus, miR-1247 may play a pivotal role in tumorigenesis in the BRAF Mut/CIMP+/MSI-H subset of colorectal tumours.

Finally, unlike genomic changes, which are essentially "locked in" forever, epigenetic modifications are intrinsically reversible, and altered gene expression can be turned on and off by the cell via a mechanism such as methylation. This makes miRNAs attractive candidates for therapeutic intervention. The results of this current research suggest a novel approach in the treatment of methylated CRCs. Some DNA-demethylating drugs, e.g., 5-azacitidine and 5-aza-2’ deoxycytidine (decitabine), are already approved by the US Food and Drug Administration for clinical use in a various human malignancies, including myelodysplastic syndrome (318). These drugs act through their ability to be incorporated into DNA and by preventing resolution of a covalent reaction intermediate that traps and inactivates DNMT in the form of a covalent protein-DNA adduct, resulting in rapid depletion of DNMT and concomitant demethylation with continued DNA replication (243). Arnold et al have previously shown that simultaneous use of a demethylating agent
together with 5-fluorouracil in a CIMP CRC cell line (SW48) overcomes resistance to cell death, suggesting its use as an alternative therapy (319), but no association has been made between use of 5’-Aza and miRNAs. To explore this therapeutic option further, clinical trials are needed to establish the safety and efficacy profiles of 5’-Aza in this group of patients.

The key challenge for future epigenetic therapies will be the development of miR mimics or more region-specific demethylating agents. Although still in its infancy overexpression of miRNAs can be induced using either synthetic miRNA mimics or chemically modified oligonucleotides (320). Conversely, miRNAs can be silenced by antisense oligonucleotides and antagonirs (synthetic analogues of miRNAs) (321). Although organ-specific delivery of miRNA remains a major challenge, luminal delivery of small RNA for the prevention of CRC in mice through use of bioengineered probiotic bacteria has been reported, and a similar strategy can be devised for miRNA (322). In the future, the findings of these studies suggest great potential for miRNAs as a novel class of therapeutic targets and as a powerful intervention tool in the treatment of CRC.

In summary, in this chapter I have demonstrated the epigenetic regulation of miR-1247 in the pathogenesis of serrated CRC. DNA hypermethylation plays a crucial role in this subtype of CRC, by sequentially suppressing
miR-1247 expression which results in an increase in MYCBP2 and its downstream c-myc protein, thereby leading to development of a tumour. In light of the new findings, novel therapeutic approaches to the treatment of serrated CRC, i.e., use of demethylating agents and miR-1247 mimics can be further explored.
10.1 Summary of clinical research

This PHD project provides new information regarding the natural history of various types of serrated polyps. In addition to detailing the characteristics of the more widely described SSA/Ps with and without dysplasia, TSAs were also examined. TSAs are a rare type of serrated polyps that share some characteristics with SSA/Ps without dysplasia and others with SSA/P with dysplasia. However, they are smaller than either SSA/Ps with or without dysplasia, are more evenly distributed between the left and right colon, and are usually sessile or pedunculated. TSAs comprise almost 16% of advanced serrated lesions and are found in 2.6% of colonoscopies. These incidence rates are higher than those previously reported, and emphasize the need for colonoscopists to be aware of these potentially premalignant lesions as well as SSA/Ps.

Detection and removal of serrated polyps requires keen colonoscopic skills that differ from those needed for recognition of classical adenomas. For screening and all examinations, the strong relationship between scope withdrawal time and serrated polyp detection rate is an indication that careful
examination of the colorectal mucosa is key to identifying serrated lesions. On colonoscopies performed for polyp or cancer surveillance, the ability to find adenomas does not necessarily translate into ability to find serrated polyps. In the last clinical section, the results have demonstrated that removal of large serrated polyps is no more problematic than polypectomy for adenomas of similar size. However, one needs to realize that large serrated polyps are associated with a higher rate of metachronous polyps than are adenomas of similar size, and surveillance should be adapted to reflect these findings.

Over the past ten years, there has been an explosion of literature designed to increase our awareness and understanding of the pathways leading to serrated neoplasia in the colorectum. In the realm of management of CRCs, a large disparity in five-year survival exists between stage I and stage IV CRCs, despite improvements in early diagnosis, surgical techniques, and general patient care. In recent times, the focus of colorectal research has expanded from a clinical perspective towards developing a detailed knowledge of the molecular basis of this malignancy, including individual risk stratification, and exploring the initiation, progression, response and resistance to anti-tumour treatment and metastatic spread. This highlights a need for better understanding of tumour biology in order to identify novel molecular targets for early disease detection and individualisation of therapy.
10.2 Overview of experimental findings

In serrated adenocarcinoma of the colorectum, the fundamental event is methylation of the DNA repair mechanism, leading to downregulation of associated genes and development of cancer. This concept provides us with a valuable framework within which to further delineate other molecular aberrations, such as those occurring with miRNAs, that can be epigenetically altered to promote tumorigenesis. miRNAs have been identified in a variety of neoplastic diseases, such as prostate cancer (miR-141 and miR-375) (323, 324), breast cancer (miR-195) (325), gastric cancer (miR-17-5p, miR-21, miR-106a and miR-106b) (326), pancreatic cancer (miR-210) (327), oral cavity cancer (miR-31) (328), and lung cancer (miR-486, miR-30d, miR-1 and miR-499) (329). A number of miRNA genes are thought to be located in cancer-associated regions or at fragile sites of chromosomes that are prone to deletion, amplification and mutations in cancer cells (290). Given that miRNAs function as negative regulators of gene expression, overexpression of oncogenic miRNAs can contribute to tumorigenesis by promoting cellular proliferation and evasion of apoptosis. Reduced expression of tumour-suppressive miRNAs may have similar effects (330). Elucidation of the cause and consequence of miRNA dysregulation will help us to better understand the pathogenesis of CRC and discover novel molecular targets for development of anticancer therapeutics. In this thesis, I have focused
specifically on miR-1247 as a potential tumour suppressor in serrated neoplasia of the colorectum. Instead of focusing on in vitro work initially, I had examined miR-1247 expression levels in human serrated CRC specimens and compared them with classical CRCs. It was reassuring to find that miR-1247 was indeed suppressed in the serrated cancers. This observation allowed further exploration of miR-1247 as a tumour suppressor in serrated neoplasia pathway in the laboratory.

The in vitro experiments demonstrated that transfection with miR-1247 inhibits proliferation of CRC cells by increasing cell apoptosis, and furthermore miR-1247 negatively regulates motility of CRC cells and their metastatic potential. With the use of 5-Aza, the previously repressed expression of miR-1247 in serrated cancer cell lines were reversed. In turn, this exerted a reciprocal effect on cell proliferation and migration in CRC cells.

Taking this further, tumours with miR-1247-overexpressing serrated cells and mock-infected serrated cells were established in xenografts. By far the most impressive findings so far are that miR-1247-transfected and 5’-Aza-treated serrated CRC cells produce significantly smaller tumours when compared with their non-transfected and non-treated counterparts.
Further analysis of these tumours from miR-1247-treated and 5'-Aza-treated mice by immunofluorescence and methylation-specific PCR confirmed minimal miR-1247 expression or persistent methylation of the miR-1247 locus. The diminished level of miR-1247 in the explants suggests that only cells that were unsuccessfully infected (80%–90% confluence) or still methylated could survive and contribute to tumour growth.

To complete the story, MYCBP2 was shown to be a target of miR-1247 in serrated CRC by Western blot and luciferase reporter assay. Even more fascinating was the observed decrease in the tumour-associated transcriptional factor c-myc protein level in cells overexpressing miR-1247. Myc protein is a transcription factor that activates expression of many genes through binding on consensus sequences (Enhancer Box sequences, also known as E-boxes) and recruiting histone acetyltransferases. Myc is activated in response to various mitogenic signals, such as Wnt, Shh and epidermal growth factor (via the MAPK/ERK pathway). By modifying the expression of its target genes, activation of Myc results in numerous biological events. The first to be discovered was the capability of Myc to drive cell proliferation, but it also plays a very important role in regulating cell growth, apoptosis, differentiation and stem cell self-renewal. Myc overexpression stimulates gene amplification presumably through overreplication of DNA and hence is a prominent proto-oncogene. The results demonstrate that miR-1247 directly targets and
degrades MYCBP2, and this in turn suppresses the c-myc protein level, which may explain the marked anti-tumour effect of this miRNA in the development of serrated CRC.

10.3 Clinical implications

In recent years, there has been increasing interest in epigenetic regulation and development of cancer, as shown by a PubMed literature search. For the period from 2002 to 2014, a total of 2698 epigenetic research papers were retrieved, compared with a total of 40 papers from 1978 to 2002. This eruption in publication rates is an acknowledgment by the scientific community that epigenetic deregulation is a crucial player in manipulating the landscape of the normal cell, resulting in malignant transformation. A key contributor to the growth of the field is the speed at which technology has moved, allowing researchers to address the underlying complexities of epigenetic control.

From a clinical perspective, dysregulated miRNAs have emerged and hold enormous potential to revolutionize the management of CRCs. miRNAs could be used as markers for screening purposes, as diagnostic markers, as prognostic markers, and for monitoring disease recurrence (233, 331). While research on plasma and faecal-based miRNA profiling is still largely in its infancy when compared with research on tissue-based miRNA profiling, it has the potential to contribute to the development of new approaches of
non-invasive or minimally invasive CRC screening in the foreseeable future. The current American Joint Committee on Cancer staging system is based on histopathologic parameters, and one can anticipate that the additional molecular prognostic markers will allow better risk stratification of patients in the future. These markers will influence the decision whether or not to provide adjuvant chemotherapy or what types of therapy are appropriate.

Unlike genetic changes, which are essentially fixed forever, epigenetic changes are intrinsically reversible, and altered gene expression can be turned on and off by the cell. This makes miRNAs attractive candidates for therapeutic intervention. Furthermore, there is growing evidence supporting the hypothesis that epigenetic alterations may be a driving force for drug resistance in human cancer (332, 333), a phenomenon that has been reported for many solid tumours, including CRC cells (319). Figure 10.1 (239) shows some of the published miRNAs involved in polyp-cancer progression as well as their effects on p53, RAS and several epithelial-mesenchymal transcription transcription factors in colorectal carcinogenesis. Along with miR-1247, these serve as potential targets for future therapeutic interventions.
To mitigate the repressed state of miRNAs, either by DNA demethylation or by altering its miRNA tertiary structure, two classes of chemical compound, i.e., DNMT inhibitors (5-azacytidine and 5-aza-2’-deoxycytidine) and histone deacetylase inhibitors (vorinostat, suberoylanilide hydroxamic acid) have undergone major preclinical investigations and are currently being explored for efficacy in the treatment of various human cancers in several clinical trials. It is known that serrated CRCs have a diminished response to adjuvant chemotherapy, so trials of epigenetic modifiers seem to be warranted (294). These drugs have potent activity *in vitro*, and some responses are achieved clinically, but the demethylation activity is nonspecific and the toxicities need to

**Figure 10.1** Changes in expression of miRNA in colorectal adenomas, colorectal carcinomas, and their correlation with Union for International Cancer Control stage or survival. Reproduced with the permission of Schweiger et al. (239) and the publisher.
carefully weighed (334). Arnold et al. had previously shown *in vitro* that the simultaneous use of a demethylating agent together with 5-fluorouracil in a CIMP CRC cell line (SW48) overcame resistance to cell death, suggesting an alternative approach to adjuvant therapy (319), but this study did not identify the molecular targets on which the treatment acts. Given the close collaboration between DNA methylation and histone modifications in inhibiting transcription of tumour suppressor genes, another strategy is to combine DNMT inhibitors with histone deacetylase inhibitors, which may have a more synergistic effect in epigenetic silencing of genes (335). Indeed, combined treatment with 5-azacitidine and valproic acid in a Phase I clinical trial in patients with refractory solid tumours (including CRC) resulted in a significant decrease in global DNA methylation and induced histone deacetylation, with stable disease lasting up to 12 months in a subset of patients (336). Currently, despite some discouraging results, DNMT inhibitors and histone deacetylase inhibitors are being tested in patients with metastatic CRC to improve their quality of life and survival (337). Our recommendation is to further stratify these patients on the basis of the molecular profile of their tumours and only offer DNMT inhibitors and histone deacetylase inhibitors to those with serrated CRC.

It is anticipated that miRNA-based therapeutics will be upcoming additions to the armamentarium in the fight against human cancer in the near future.
Therapeutically, underexpressed miRNAs may be replenished by administration of miRNA mimetics, whereas overexpressed miRNAs can be targeted by a novel class of synthetic oligonucleotides known as antagomirs (320). miRNA replacement involves reintroducing synthetic miRNA mimics or expression vectors that will produce the microRNA of interest. This strategy has shown promise in preclinical murine models, where reintroduction of miR-145 and miR-33a had an antitumour effect in a mouse model of colon cancer (338). Direct inhibition of miRNAs can be achieved using antisense oligonucleotides or miRNA sponges to bind and sequester the target miRNA. Antagomirs are modified antisense oligonucleotides that have been used to inhibit miRNAs in vivo (320) in mouse studies. Similar strategies have been used to inhibit miR-122 function effectively in primates (321), and anti-miR-122 treatment in chimpanzees chronically infected with hepatitis C virus improved the related liver pathology (339). This anti-miR-122 drug has progressed to Phase II clinical trials to treat hepatitis C infection in humans, suggesting that antisense-based miRNA therapeutics may soon be available.

The critical issue to be addressed with miRNA-based therapies is development of a delivery system that allows prolonged stability of the therapy and provides sufficient uptake in target cells while minimizing off-target side effects. These strategies include optimizing the chemistry and size of the synthetic oligonucleotides to achieve better binding efficiency and increased stability.
Delivery mechanisms range from adenoviral vector-based delivery, to cationic liposomes, to polymer-based nanoparticles. Although organ-specific delivery of miRNA remains a major challenge, luminal delivery of small interference RNA for prevention of CRC in mice using bioengineered probiotic bacteria has been reported, and a similar strategy can be devised for miRNA (322). All in all, there are great expectations that these strategies will result in effective therapies for CRC.

10.4 Future perspectives and conclusion

miR-1247 clearly has a role in the development of serrated CRCs. This is the first time the importance of epigenetic regulation of miR-1247 as a tumour suppressor in serrated colorectal cancers is demonstrated. miR-1247 acts on its target protein of MYCBP2 which, in turn, modulates the downstream expression of Myc. This suggests alternative adjuvant therapy either with a demethylating agent or miR mimics should be considered in patients with this specific molecular profile. Although the results of experimental miRNA therapy look promising, future research is required in a population-based cohort to validate these findings of an antitumour effect of miR-1247, both \textit{in vitro} and \textit{in vivo}, before they can be formally introduced into clinical practice.
Chapter XI
APPENDIX: LABORATORY METHODS AND MATERIALS

11.1 Human tissue sample

A panel of colorectal carcinomas and corresponding normal colon mucosa tissue samples were collected from patients treated in the Colorectal Surgery Unit, Cleveland Clinic, Ohio. The study concept and tissue collection were approved by the institutional review board in Ohio. The samples were snapfrozen and stored at −80°C. The cancers had been previously typed for BRAF, KRAS, and CpG island methylation status (134). Normal colon mucosal biopsies from individuals who were disease-free on colonoscopy were also collected and used as control samples.

11.2 Colorectal cancer cell lines

11.2.1 Selection

The BRAF, KRAS, CpG methylation status, and MSI of the different CRC cell lines are profiled in Table 11.1 (340). RKO and LS411N cells were selected to represent serrated adenocarcinoma (BRAF MUT/CIMP+/MSI-H) while SW480 and SW620 cells were chosen as representative of traditional CRC (KRAS MUT/CIMP-/MSS).
<table>
<thead>
<tr>
<th>Cell lines</th>
<th>CACNA1G</th>
<th>IGF2</th>
<th>NEUROG1</th>
<th>RUNX3</th>
<th>SOCS1</th>
<th># of loci Methylated</th>
<th>MSI status</th>
<th>BRAF exon 15</th>
<th>KRAS exon 2</th>
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<td>Caco2</td>
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<td></td>
<td></td>
<td></td>
<td>MSS WT</td>
<td>MSS WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>COLO 320DM</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>MSS WT</td>
<td>MSS WT</td>
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<td>WT</td>
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<td></td>
<td></td>
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<td>MSS WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>MSS WT</td>
<td>MSS WT</td>
<td>G12S</td>
<td></td>
</tr>
<tr>
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<td></td>
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<td>MSS WT</td>
<td>G12V</td>
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<tr>
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<td>MSS WT</td>
<td>G12V</td>
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<td>MSS WT</td>
<td>WT</td>
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</tr>
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<td></td>
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</tr>
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<td>MSI WT</td>
<td>G13D</td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>MSI V600E</td>
<td>MSI V600E</td>
<td>WT</td>
<td>WT</td>
</tr>
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<td>MSI V600E</td>
<td>MSI V600E</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
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<td></td>
<td>MSI WT</td>
<td>MSI WT</td>
<td>G13D</td>
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<tr>
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<td></td>
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<td>MSI WT</td>
<td>MSI WT</td>
<td>G13D</td>
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<tr>
<td>LS411N</td>
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<td>MSI V600E</td>
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</table>

Table 11.1 Profiling of colorectal cancer cell lines. The BRAF, KRAS, and CpG methylation status of different colorectal cancer cell lines were profiled. RKO and LS411N cells were selected as representatives for CIMP+ while SW480 and SW620 cells were chosen as representatives for CIMP- cells. Courtesy of Hinoue et al (340).

11.2.2 Origin

The RKO, SW480 and SW620 cell lines were generously provided by Dr Janet Houghton (Cancer Biology, Lerner’s Research Institute, Cleveland Clinic, Ohio)
and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The LS411N cell line was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum.

11.2.3 Maintenance

The following method was used to grow and maintain all cell lines for DNA, RNA and protein extraction. This method was also used to provide cells for transfection, proliferation, apoptosis, migration and anoikis assays. Cell culture was carried out under sterile conditions in a biosafety level 1 tissue culture hood.

The cell were defrosted from liquid nitrogen rapidly by thawing in a 37°C water bath, and then placed into the appropriate medium immediately and centrifuged at 1000 rpm for three minutes. Supernatants containing dimethyl sulfoxide were suctioned and the cells were resuspended in fresh medium, supplemented with 10% fetal bovine serum and penicillin/streptomycin, and seeded into tissue culture dishes.

For subculture, the cells were washed twice with phosphate-buffered saline (0.1 M NaH₂PO₄·H₂O, 0.1 M Na₂HPO₄·7H₂O, pH 7.4) and then trypsinised for
three minutes at 37°C. Detached cells were collected by centrifuge at 1000 rpm for three minutes and replated into a new dish at a ratio of 1:10.

To maintain a renewable stock of the cell lines, at least $3 \times 10^6$ cells were split from the main culture, centrifuged at 1000 rpm, and resuspended in 1 mL of freezing medium (fetal bovine serum supplemented with 10% dimethyl sulfoxide). The cells were then frozen at −80°C before being transferred to liquid nitrogen (−160°C).

11.3 Purification of DNA and RNA

11.3.1 Purification of DNA from cell lines and human tissues

All DNA purifications were carried out using a Tissues QIAamp DNA mini kit (Qiagen NV, Venlo, The Netherlands). The details of the protocols used are as follows:

**Step 1** Section the frozen tissue samples and determine the weight of the tissue; 1 mg of tissue will yield approximately 0.2–1.2 µg of DNA

**Step 2** Cut up 25 mg of tissue into small pieces; place the tissue in a 1.5 mL microcentrifuge tube, and add 180 µL of Buffer ATL (Qiagen); proceed to step 3 (it is important to cut the tissue into small pieces to decrease lysis time).
**Step 3** Add 20 µL of proteinase K, mix by vortexing, and incubate at 56°C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample. Proteinase K must be used because Qiagen protease has reduced activity in the presence of Buffer ATL. Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 hours. Lysis overnight is possible and does not influence the preparation.

**Step 4** Add 200 µL of Buffer AL (Qiagen) to the sample, mix by pulse-vortexing for 15 seconds, and incubate at 70°C for 10 minutes. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from inside the lid. It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL, which in most cases will dissolve during incubation at 70°C.

**Step 5** Add 200 µL of ethanol (96%–100%) to the sample, and mix by pulse-vortexing for 15 seconds. It is essential that the sample, Buffer AL, and ethanol are mixed thoroughly to yield a homogeneous solution. A white precipitate may form on addition of ethanol. It is essential to apply all of the precipitate to the QIAamp Mini spin column.

**Step 6** Carefully apply the mixture from step 6 (including the precipitate) to the QIAamp Mini spin column (in a 2 mL collection tube) without wetting the rim.
Close the cap, and centrifuge at 6000x g (8000 rpm) for one minute. Place the QIAamp Mini spin column in a clean 2 mL collection tube, and discard the tube containing the filtrate.

**Step 7** Carefully open the QIAamp Mini spin column and add 500 µL of Buffer AW1 (Qiagen) without wetting the rim. Close the cap, and centrifuge at 6000x g (8000 rpm) for one minute. Place the QIAamp Mini spin column in a clean 2 mL collection tube, and discard the collection tube containing the filtrate.

**Step 8** Carefully open the QIAamp Mini spin column and add 500 µL of Buffer AW2 (Qiagen) without wetting the rim. Close the cap and centrifuge at full speed (20,000x g; 14,000 rpm) for three minutes. Place the QIAamp Mini spin column in a new 2 mL collection tube and centrifuge at full speed for one minute.

**Step 9** Place the QIAamp Mini spin column in a clean 1.5 mL microcentrifuge tube, and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 50 µL of Buffer AE (Qiagen) or distilled water. Incubate at room temperature for one minute, and then centrifuge at 6000x g (8000 rpm) for one minute.

**Step 10** Repeat step 9. A five-minute incubation of the QIAamp Mini spin
column loaded with Buffer AE or water before centrifugation generally increases the DNA yield.

**Step 11** For long-term storage of DNA, eluting in Buffer AE and placing at –20°C is recommended, given that DNA stored in water is subject to acid hydrolysis. Yields of DNA will depend both on the amount and type of tissue processed; 25 mg of tissue will yield approximately 10–30 µg of DNA in 400 µL of water (25–75 ng/µL), with an A260/A280 ratio of 1.7:1.9.

11.3.2 Extraction of RNA

All RNA extractions were carried out using mirVana (Ambion®, Life Technologies, Carlsbad, CA, USA). The details of the protocols are as follows:

**Step 1** Collect $10^2$–$10^7$ cells or 0.5–250 mg of tissue. Aspirate and discard the culture medium, and rinse with phosphate-buffered saline. Trypsinize cells to detach them from the tissue culture plate. Next, inactivate the trypsin, pellet the cells, and discard the supernatant. Wash the cells by gently resuspending in about 1 mL of phosphate-buffered saline or RNA later and pelleting at 1000 rpm for three minutes. Place the cells on ice.

**Step 2** Remove the phosphate-buffered saline and add 600 µL of
Lysis/Binding Solution (Life Technologies). Vortex or pipette vigorously to lyse the cells completely and obtain a homogenous lysate.

**Step 3** Add a 1/10 volume (60 µL) of miRNA homogenate additive and incubate for 10 minutes on ice.

**Step 4** Extract with a 600 µL volume of acid-phenol:chloroform equal to the initial lysate volume. Be sure to withdraw from the bottom phase in the bottle of acid-phenol:chloroform because the upper phase consists of an aqueous buffer. Vortex for 30–60 seconds to mix.

**Step 5** Centrifuge for five minutes at maximum speed (14,000 rpm) and room temperature to separate the aqueous and organic phases. After centrifugation, the interphase should be compact; if not, repeat the centrifugation.

**Step 6** Carefully remove the aqueous (upper) phase without disturbing the lower phase, and transfer it to a fresh tube. Note down the volume removed.

**Step 7** Preheat Elution Solution or nuclease-free water to 95°C; this is used to elute the RNA from the filter at the end of the procedure. Warm 100% ethanol to room temperature.

**Step 8** Add 1.25x volumes of room temperature 100% ethanol to the aqueous
phase. For each sample, place a filter cartridge into one of the collection tubes supplied. Pipette the lysate/ethanol mixture (from the previous step) onto the filter cartridge. Up to 700 µL can be applied to a filter cartridge at a time. For samples larger than this, apply the mixture in successive applications to the same filter.

**Step 9** Centrifuge for approximately 15 seconds to pass the mixture through the filter. Centrifuge at a relative centrifugal force of 10,000 g. Spinning harder than this may damage the filters.

**Step 10** Discard the flow-through, and repeat until all of the lysate/ethanol mixture is through the filter. Reuse the collection tube for the washing steps.

**Step 11** Apply 500 µL of wash solution 2/3 (working solution mixed with ethanol) and draw it through the filter cartridge as in the previous step.

**Step 12** Repeat with a second 500 µL aliquot of wash solution 2/3.

**Step 13** After discarding the flow-through from the last wash, replace the filter cartridge in the same collection tube and spin the assembly for one minute to remove residual fluid from the filter.
**Step 14** Transfer the filter cartridge into a fresh collection tube (provided with the kit). Apply 50 µL of preheated (95°C) elution solution or nuclease-free water to the center of the filter, and close the cap. Spin for about 20–30 seconds at maximum speed to recover the RNA.

**Step 15** Repeat step 14. Collect the eluate (containing the RNA) and store it at −20°C or below.

11.3.3 Estimation of DNA and RNA concentration

NanoDrop (Thermo Scientific, Wilmington, DE, USA) was used to estimate the concentration of DNA and RNA purified from the protocols listed above. A concentration in ng/µL was given and the quality was checked against the absorbance range of 280–260 nm.

**Step 1** Open the NanoDrop software on the desktop.

**Step 2** Choose "Nucleic Acids".

**Step 3** Initialize the instrument and ensure upper and lower pedestal surfaces are clean by wiping with a KimWipe®. Place 2 µL of NanoPure water on the lower pedestal. Lower the sampling arm and press "OK", when this is done, wipe upper and lower pedestals with a KimWipe.
Step 4 Calibrate the instrument by placing 2 µL of elution buffer on the pedestal. Click "Blank", and when it is done, wipe upper and lower pedestals with a KimWipe.

Step 5 Measure sample by placing 2 µL of sample on the pedestal. Enter sample ID and click "Measure", then wipe upper and lower pedestals with a KimWipe after each sample.

Step 6 Save data by clicking "Show Report" followed by clicking "Reports", "Save Report", and "Export Report Table Only"

11.4. Techniques for studying DNA methylation

Two methods were used for detecting DNA methylation, i.e., MethyLite and methylation-specific polymerase chain reaction (PCR) (341).

11.4.1 Treatment with 5’-Aza-2’-deoxycytidine

The cell lines were treated with 15 µM of 5-Aza-2’-deoxycytidine (5’-Aza; Sigma-Aldrich, St Louis, MO, USA) dissolved in 50% dimethyl sulfoxide for the desired time points (5 days). Fresh 5’-Aza was added at 24-hourly intervals following phosphate-buffered saline wash and addition of fresh medium. Control cells were treated with 15 µM of dimethyl sulfoxide for five days.
11.4.2 Bisulphite conversion

All bisulphite conversions were carried out using an EZ DNA methylation kit (Cat# 5001, Zymo Research Corporation, Irvine, CA, USA). The details of the protocols are as follows:

**Step 1** Add 5 μL of M-Dilution Buffer to 1 μg of the DNA sample and adjust the total volume to 50 μL with water. Mix the sample by flicking or pipetting up and down.

**Step 2** Incubate the sample at 37°C for 15 minutes. Meanwhile, prepare CT Conversion Reagent by adding 750 μL of water and 210 μL of M-Dilution Buffer to a tube of CT Conversion Reagent, and mix at room temperature for 10 minutes.

**Step 3** Add 100 μL of the prepared CT Conversion Reagent to each sample and mix. Incubate the samples in a thermocycler at 95°C for 30 seconds, 50°C for 60 minutes × 16 cycles, then “hold” at 4°C.

**Step 4** Add 400 μL of M-Binding Buffer to a Zymo-Spin™ IC column and place the column into the collection tube provided.
Step 5 Load the sample (from Step 4) into the Zymo-Spin IC column containing the M-Binding Buffer. Close the cap and mix by inverting the column several times.

Step 6 Centrifuge at full speed (>10,000x g) for 30 seconds. Discard the flow-through.

Step 7 Add 100 µL of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds.

Step 8 Add 200 µL of M-Desulphonation Buffer to the column and allow to stand at room temperature (20°C–30°C) for 15–20 minutes. After incubation, centrifuge at full speed for 30 seconds.

Step 9 Add 200 µL of M-Wash Buffer to the column. Centrifuge at full speed for 30 seconds. Add another 200 µL of M-Wash Buffer and centrifuge for a further 30 seconds.

Step 10 Place the column into a 1.5 mL microcentrifuge tube. Add 25 µL of M-Elution Buffer directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA. The DNA is ready for immediate analysis or can be stored at or below –20°C for later use.
Step 11 For long-term storage, store at or below –70°C. 1–4 µL of eluted DNA is used for each PCR; however, up to 10 µL can be used if necessary.

11.4.3 MethyLite primer design and sequence

Primers were designed according to the following strategy:

Step 1 UCSC Genome Browser was used to determine the base sequence of the associated CpG island of miR-1247. In Figure 10, the red represents the base sequence of miR-1247 and the dark green represents the CpG island for which miR-1247 was located.

Figure 11.1 CpG island in the promoter region of the has-mir-1247 gene.
Step 2 Insert the CpG island sequence into Beacon Design (version 5.10) to design MethyLite primers. Because the CpG island for miR-1247 was >1000 bp, I have used the site of the differentially hypermethylated CpG Island region specified by Dr Angela Ting, i.e., chr14: 101095650 to 101096350.

Step 3 "Design" sense and anti-sense primers and probes to the sequence after bisulphite conversion.

Step 4 "Export" result to Microsoft® Excel files.

Step 5 Select the most complementary primers and probe (Table 11.2).

<table>
<thead>
<tr>
<th>Probe dequence miR-1247</th>
<th>ACTACAAATACCCTCGCTACGCTAACCCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sense primer miR-1247</td>
<td>ATATTATTTTATAGTATCGGAGTTTGGAGG</td>
</tr>
<tr>
<td>Anti-sense primer miR-1247</td>
<td>GAAAACCAAAACGCAACCGAATAC</td>
</tr>
</tbody>
</table>

Table 11.2 MethyLite primers for miR-1247.

We repeated the above steps to obtain the appropriate primers and probe for miR-1258 but, unfortunately, no complementary primers or probe were found for miR-1258.

11.4.4 Protocol: MethyLite
**Step 1** Bisulphite-converted DNA preparation

a. A preliminary MethyLite control PCR reaction with a small amount of bisulphite-converted sample using the ALU-C4 (Alu repeats) bisulphite control reaction is performed. The bisulphite-converted DNA recovered using the Zymo protocol is in a final volume of approximately 25 µL. The sample is then diluted to 1:22 with molecular grade water (Sigma-Aldrich). The threshold cycle (Ct) value generated from this 1:22 dilution (1 µL of bisulphite-DNA sample + 21 µL of water) gives an indication of the approximate bisulphite-converted DNA sample amounts, and the degree to which the bisulphite-DNA sample can be further diluted when performing MethyLite assays.

b. Given that the ALU-C4 reaction is highly sensitive and will generate low Ct values, a sample that gives a Ct of 22 is considered maximally diluted. However, it should be noted that lower Ct values are always preferred to achieve the best possible data.

c. The bisulphite-converted M.SssI-DNA sample was also diluted to 1:22; 10 µL of this is removed for use in each MethyLite reaction.

**Step 2** Preparation of MethyLite primer/probe

a. MethyLite primers and probes need to be dissolved in sterile water because
they are lyophilised after synthesis. The forward and reverse primers are prepared at a concentration of 300 µM and the probe at a concentration of 100 µM. Small aliquots of the primers at these concentrations should be made to prevent repeated freeze/thaw events.

b. Prepare an Oligo Mix containing both primers and the probe.

   Forward primer: 300 µM stock; use 4 µL; 2 µM concentration in Oligo Mix
   Reverse primer: 300 µM stock; use 4 µL; 2 µM concentration in Oligo Mix
   Probe: 100 µM stock; use 4 µL; 0.67 µM concentration in Oligo Mix
   Water: add 588 µL
   Total volume: 600 µL

**Step 3** Use 4.5 µL of this Oligo Mix per 30 µL MethylLite reaction. After addition to the PCR reaction mixture, the forward/reverse primers are at a concentration of 0.3 µM and the probe at 0.1 µM.

**Step 4** PCR reaction

a. Prepare the PCR component (final concentration)

   4.2 µL of 25 mM MgCl₂ (3.5 mM)
   3.0 µL 10× buffer (1×)
   3.0 µL 10× stabilizer (1×)
   0.6 µL 10 mM dNTPs (200 µM)
4.5 µL of this Oligo Mix

4.6 µL water

0.1 µL Taq Gold polymerase

10.0 µL DNA sample

b. PCR conditions

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial PCR activation</td>
<td>5 minutes</td>
<td>95°C</td>
</tr>
<tr>
<td>Two-step cycling:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>15 seconds</td>
<td>95°C</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>60 seconds</td>
<td>60°C</td>
</tr>
<tr>
<td>Cycles (n)</td>
<td>40–45</td>
<td></td>
</tr>
</tbody>
</table>

**Table 11.3** Polymerase chain reaction conditions.

**Step 5** Data analysis using SDS version 2.1 program (Applied Biosystems, Foster City, CA, USA)

a. Threshold cycle values were used to create relative values for each sample by generating standard curves. These were created by plotting the Ct against the logged quantity of a calibrator, which was serially diluted bisulphite-treated, fully methylated CpGenome™ Universal Methylated DNA (Zymo)

b. The coefficient of linear regression ($r$) was calculated for each standard curve, and curves with values close to 1 were used further.
c. Samples were normalised for initial bisulphate-treated DNA quantity variations by dividing relative values by β-actin values.

d. Percentage methylation ratio values were calculated by dividing normalised values with fully methylated CpGenome DNA relative values and multiplying by 100. Less than 10% is considered to be demethylated.

11.4.5 Methylation-specific PCR primer design and sequence

Methylation-specific PCR primers for both miR1247 and miR-1258 were designed according to the following protocol, but only the steps for miR-1258 are shown here.

**Step 1** The UCSC Genome Browser was used to determine the base sequence of the associated CpG island of miR-1258. The red in Figure 11 represents the base sequence of miR-1258 and the dark green represents the CpG island from which miR-1258 was located.
Figure 11.2 CpG island in the promoter region of the has-mir-1258 gene.

**Step 2** Identifies the differentially hypermethylated CpG Island region specified by Dr Angela Ting, i.e., chr2: 180433600 to 180434700

**Step 3** MSP uses primers which are designed to distinguish unconverted methylated cytosines from converted unmethylated cytosines using bisulphate-treated DNA as a template. Hence, two PCR reactions are performed, and methylated sequences or unmethylated sequences are amplified depending on the methylation status of the sample. Bisulphate sequencing primers is designed using the MethPrimer program (342) at http://www.urogene.org/methprimer/index.html, I have chosen the most complementary primers for each microRNA (Table 11.4).
miR-1247 right unmethylated 5'-TTCAACAACAAAAAACATATCAA A-3'
miR-1247 left unmethylated 5'-TTTAGGTTTAGGAGGAGTTTATGG-3'
miR-1247 right methylated 5'-GACAACGAAAAACGTATCGAA-3'
miR-1247 left methylated 5'-TTTAGGTTTAGGAGGAGTTTACGG-3'
miR-1258 right unmethylated 5'-AAATACCCACTATATTAACCACA-3'
miR-1258 left unmethylated 5'-GTTTTGTTGTAGAATTTTTATGA-3'
miR-1258 left methylated 5'-TGGGTTTTGTAGAATTTTTATTAC-3'
miR-1258 right methylated 5'-AAATACCCCGCTATATTAACC-3'

**Table 11.4** MSP primers of miR-1247 and miR-1258 promoter regions.

### 11.4.6 Methylation-specific PCR protocol

The methylation-specific PCR protocol was carried out using an EpiTect MSP Kit (Cat# 59305, Qiagen):

**Step 1** Thaw the EpiTect Master Mix, nucleic acid template, primer solutions, and RNase-free water. Mix well before use.

**Step 2** Mix the EpiTect Master Mix by vortexing briefly and dispense 25 µL into each PCR tube according to Table 16. It is important to mix the EpiTect Master Mix before use in order to avoid localised concentrations of salt. It is not necessary to keep reaction vessels on ice because HotStarTaq d-Tect polymerase is inactive at room temperature.
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>EpiTect Master Mix (2×)</td>
<td>25 µL</td>
<td>1×</td>
</tr>
<tr>
<td><strong>Diluted primer mix</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer A</td>
<td>Variable</td>
<td>0.3–0.4 µM</td>
</tr>
<tr>
<td>Primer B</td>
<td>Variable</td>
<td>0.3–0.4 µM</td>
</tr>
<tr>
<td><strong>Template DNA</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Template DNA added at step 4</td>
<td>Variable</td>
<td>&lt;200 ng</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>Variable</td>
<td></td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td>50 µL</td>
<td></td>
</tr>
</tbody>
</table>

**Table 11.5** Components of methylation-specific polymerase chain reaction system.

**Step 3** Add the appropriate volume of the diluted primer mix into the PCR tubes containing the Master Mix.

**Step 4** Add template DNA (<200 ng/50 µL reaction) to the individual PCR tubes.

**Step 5** Program the thermal cycler according to the manufacturer’s instructions. Each PCR program must start with an initial heat activation step at 95°C for 10 minutes.

**Step 6** Place the PCR tubes in the thermal cycler and start the cycling program...
Additional comments

<table>
<thead>
<tr>
<th>Initial activation step</th>
<th>10 minutes</th>
<th>95°C</th>
<th>HotStarTaq d-Tect polymerase is activated by this heating step</th>
</tr>
</thead>
</table>

### 3-step cycling

<table>
<thead>
<tr>
<th>Denaturing</th>
<th>15 seconds</th>
<th>94°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annealing</td>
<td>30 seconds</td>
<td>50–55°C</td>
</tr>
<tr>
<td>Extension</td>
<td>30 seconds</td>
<td>72°C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Number of cycles</th>
<th>30–40</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Final extension</th>
<th>10 minutes</th>
<th>72°C</th>
</tr>
</thead>
</table>

Table 11.6 Methylation-specific polymerase chain reaction cycle settings.

### 11.5 DNA polyacrylamide gel electrophoresis

#### 11.5.1 Buffers and solutions

- Acrylamide:bisacrylamide (29:1) (30% w/v)

- Ammonium persulphate (10% w/v): ammonium persulphate is used as a catalyst for the copolymerisation of acrylamide and bisacrylamide gels. The polymerisation reaction is driven by free radicals that are generated by an oxido-reduction reaction in which tetramethylethylenediamine is used as the adjunct catalyst

- 5× TBE electrophoresis buffer: polyacrylamide gels are poured and run in 0.5× or 1× TBE at low voltage (1–8 V/cm) to prevent denaturation of small fragments of DNA by heating.
11.5.2 DNA polyacrylamide gel electrophoresis protocol

**Step 1** Clean the glass plates and spacers thoroughly. Hold the plates by the edges or wear gloves, so that oils from the hands do not become deposited on the working surfaces of the plates. Rinse the plates with deionised water and ethanol and set them aside to dry. The glass plates must be free of grease spots to prevent air bubbles from forming in the gel.

**Step 2** Assemble the glass plates with spacers in Gel Caster® (Bio-Rad, Hercules, CA, USA).

**Step 3** Prepare the gel solution with the desired polyacrylamide percentage according to the table below, which gives the amount of each component required to make 12 mL of gel.

<table>
<thead>
<tr>
<th>Volume of reagents used to cast polyacrylamide gels</th>
<th>6%</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gel concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30% acrylamide (29:1)</td>
<td>3.2 mL</td>
<td>4.0 mL</td>
<td>4.8 mL</td>
<td>5.6 mL</td>
</tr>
<tr>
<td>5x TBE</td>
<td>2.4 mL</td>
<td>2.4 mL</td>
<td>2.4 mL</td>
<td>2.4 mL</td>
</tr>
<tr>
<td>10% APS</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µL</td>
<td>10 µL</td>
<td>10 µL</td>
<td>10 µL</td>
</tr>
<tr>
<td>Double-distilled H₂O</td>
<td>6.4 mL</td>
<td>5.6 mL</td>
<td>4.8 mL</td>
<td>4.0 mL</td>
</tr>
</tbody>
</table>

**Table 11.7** Recipe for sodium dodecyl sulphate polyacrylamide gel electrophoresis gel. **Abbreviation:** TEMED, tetramethylethylenediamine

**Step 4** Wear gloves. Work quickly after addition of tetramethylethylenediamine to complete the gel before the acrylamide polymerises.
Step 5 Immediately insert the appropriate comb into the gel, being careful not to allow air bubbles to become trapped under the teeth. Clamp the comb in place with bulldog paper clips. Make sure that no acrylamide solution is leaking from the gel mold.

Step 6 Allow the acrylamide to polymerise for 30–60 minutes at room temperature.

Step 7 When ready to proceed with electrophoresis, remove gels from gel caster, carefully clean spilled gel from back of white plates, and insert gels into Hoefer gelbox. Add running buffer and carefully pull the combs from the polymerised gel. Use a Pasteur pipette or a syringe to flush out the wells once more with 1× TBE.

Step 8 Mix the DNA samples with the appropriate amount of gel-loading buffer. Load the mixture into the wells using a micropipette equipped with a drawn out plastic tip. Do not attempt to expel all of the samples from the loading device, because this almost always produces air bubbles that blow the sample out of the well. It is important not to take too long to complete loading the gel; otherwise, the samples will diffuse from the wells.
**Step 9** Connect the electrodes to a 100 V power pack, turn on the power, and begin the electrophoresis run.

**Step 10** Run the gel until the marker dyes have migrated the desired distance. Turn off the electric power, disconnect the leads, and discard the electrophoresis buffer from the reservoirs.

**Step 11** Detach the glass plates. Lay the glass plates on the bench. Use a spacer or plastic wedge to lift a corner of the upper glass plate. Pull the upper plate smoothly away. Remove the spacers.

**Step 12** Stain gels with ethidium bromide exposed to Phospholmager screen.

### 11.6 Real-time quantitative PCR

11.6.1 Instrument

**Figure 11.3** Real-time quantitative polymerase chain reaction instruments and workflow
Relative quantitation using comparative Ct determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample. The calibrator sample can be an untreated control or a sample at time zero in a time-course study (343).

Relative quantitation provides an accurate comparison between the initial amount of template in each sample, without requiring the exact copy number of the template. Also, the relative levels of templates in samples can be determined without the use of standard curves.

Relative quantitation assays are performed using real-time PCR where the progress of the PCR is monitored as it occurs (344). Data are collected throughout PCR rather than at the end of the process (end-point PCR). In real-time PCR, reactions are characterised by the point in time during cycling when amplification of a target is first detected rather than by the amount of target accumulated at the end of PCR (345). There are two types of quantitative real-time PCR, i.e., TaqMan and SYBR Green, absolute and relative (Figure 11.4).
Figure 1
1.4 Representative TaqMan relative quantitation amplification plot.

<table>
<thead>
<tr>
<th>Chemistry</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan® reagents or kits</td>
<td>TaqMan reagent-based chemistry uses a fluorescent probe to enable detection of a specific PCR product as it accumulates during PCR cycles.</td>
</tr>
<tr>
<td>Description</td>
<td><strong>Advantages</strong></td>
</tr>
</tbody>
</table>
| TaqMan reagent-based chemistry uses a fluorescent probe to enable detection of a specific PCR product as it accumulates during PCR cycles. | • Increases specificity with a probe. Specific hybridization between probe and target generates fluorescence signal.  
• Provides multiplex capability.  
• Optimized assays available.  
• Alters 5′-nuclease assay to be carried out during PCR.  
• Improves detection of low-copy target genes in relation to SYBR Green I dye. |
| SYBR® Green I reagents | SYBR Green I dye, a double-stranded DNA binding dye, is used to detect PCR products as they accumulate during PCR cycles. |
| Description | **Advantages** |
| SYBR Green I dye, a double-stranded DNA binding dye, is used to detect PCR products as they accumulate during PCR cycles. | • Economical (no probe needed)  
• Amplifies all double-stranded DNA  
• Yields a melting profile of distinct PCR runs  
• Increases signal fluorescence as amplification product length increases.  
• Improves detection of low-copy target genes. |
| Limitations | Brings nonspecifically to all double-stranded DNA sequences. To avoid false positive signals, check for nonspecific product formation using dissociation curve or gel analysis. |

**RQ Studies with the 7900HT Fast System**

The data-collection part of an RQ assay is in single plate document called the RQ plate. Amplification data from PCR runs are stored with sample setup information on the RQ plate.

**Notes**

---

4 Relative Quantitation U
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>Initial cycles of PCR in which there is little change in fluorescence signal.</td>
</tr>
<tr>
<td>Threshold</td>
<td>A level of $\Delta R_0$, automatically determined (or manually set) by the SDS software, used for $C_T$ determination in real-time assays. The level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the amplification plot defines the $C_T$.</td>
</tr>
<tr>
<td>Threshold cycle ($C_T$)</td>
<td>The fractional cycle number at which the fluorescence passes the threshold.</td>
</tr>
<tr>
<td>Passive reference</td>
<td>A dye that provides an internal fluorescence reference to which the reporter fluorescence signal can be normalised during data analysis. Normalisation is necessary to correct for fluorescence fluctuations caused by changes in concentration or in volume.</td>
</tr>
<tr>
<td>Reporter dye</td>
<td>The dye attached to the 5’ end of a TaqMan probe. The dye provides a fluorescent signal that indicates specific amplification.</td>
</tr>
<tr>
<td>Normalisation reporter ($R_n$)</td>
<td>The ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.</td>
</tr>
<tr>
<td>Delta $R_n$ ($\Delta R_n$)</td>
<td>The magnitude of the signal generated by the specific set of PCR conditions ($\Delta R_n = R_n - \text{baseline}$)</td>
</tr>
</tbody>
</table>

Table 11.8 Terminology of TaqMan quantitative PCR.

For every sample in a relative quantitation experiment, we specified:

A target – the nucleic acid sequence that we are studying.

A calibrator – the sample used as the basis for comparative results.
An endogenous control – a gene present at a consistent expression level in all experimental sample sets. By using an endogenous control as an active reference, we can normalize quantitation of a cDNA target for differences in the amount of cDNA added to each reaction.

Replicate wells – for comparative Ct studies, Applied Biosystems recommends using three or more.

Typically, housekeeping genes such as β-actin, glyceraldehyde-3-phosphate, and ribosomal RNA, are used as endogenous controls because their expression levels tend to be relatively stable (346).

11.6.2 Reverse transcription of microRNA

**Step 1** Remeasure RNA concentration with NanoDrop.

**Step 2** Prepare RNA volume of 10 μL with 40 ng total RNA for three miRNAs (2.5 μL RNA /miRNA reverse transcription step necessary) for every sample.

**Step 3** Prepare RT Master Mix:

<table>
<thead>
<tr>
<th></th>
<th>n=1</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTPs</td>
<td>0.075 μL</td>
</tr>
<tr>
<td>10× RT buffer</td>
<td>0.75 μL</td>
</tr>
<tr>
<td>Multiscribe</td>
<td>0.5 μL</td>
</tr>
<tr>
<td>RNAse inhibitor</td>
<td>0.094 μL</td>
</tr>
<tr>
<td>RNAse-free H₂O</td>
<td>2.081 μL</td>
</tr>
<tr>
<td>Total volume</td>
<td>3.5 μL</td>
</tr>
</tbody>
</table>

*Table 11.9 Recipe for microRNA reverse transcription Master Mix.*
Step 4 Prepare reverse transcription reaction for every microRNA and every time point.

<table>
<thead>
<tr>
<th>Reverse transcription Master Mix</th>
<th>3.5 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan primers 5×</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>Total RNA</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>7.5 µL</td>
</tr>
</tbody>
</table>

Table 11.10 Components of microRNA reverse transcription.

Step 5 Cycling conditions for reverse transcription on a thermocycler:

- 30 minutes at 16°C
- 30 minutes at 42°C
- 5 minutes at 85°C
- Hold at 4°C

11.6.3 Quantitative RT-PCR: Taqman® analysis

a. qRT-PCR was performed to quantify relative levels of miRNA expression using a Taqman Gene Expression Assay (Applied Biosystems) on an ABI PRISM® HT7900 Sequence Detection System thermal cycler (Applied Biosystems).

b. The miRNA probe was labeled with the reporter dye MAM and the beta actin probe labeled with the reporter dye VIC at the 5' end of the oligonucleotide. A
quencher dye (TAMRA) was used to label the 3' ends of both probes.

c. PCR reaction (total reaction volume 10 µL), for every miRNA and time point. For each cDNA sample, triplicate amplifications were carried out in a 96-well plate:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>TaqMan Fast Advanced PCR Master Mix</td>
<td>5 µL</td>
</tr>
<tr>
<td>RNAse free H2O</td>
<td>3.835 µL</td>
</tr>
<tr>
<td>TaqMan microRNA assay 20×</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>RT product</td>
<td>0.665 µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>10 µL (well)</td>
</tr>
</tbody>
</table>

**Table 11.11** Components of the TaqMan relative quantitation of microRNAs.

d. PCR plates (Applied Biosystems) were sealed using optically clear adhesive sheets (Applied Biosystems). Negative reverse transcriptase reactions and non-template controls were run on each plate.

e. Cycling conditions for PCR reaction:

10 minutes at 95°C

40 cycles: 0.15 minutes (15 seconds) at 95°C

One minute at 60°C

11.6.4 Data analysis (ΔΔCT)

**Step 1** For the SDS software to analyze relative quantitation plate data after
the run, click or select Analysis > Analyze. The SDS software mathematically transforms the raw fluorescence data to establish a comparative relationship between the spectral changes in the passive reference dye and those of the reporter dyes.

**Step 2** Once the plate is analysed, the SDS software displays the flags associated with each well in the plate grid. It also displays the resulting plate data in a table underneath the plate grid. The Results tab displays the results data of each well, including an amplification plot with the calculated Rn value.

**Step 3** Experimental error may cause some wells to be amplified insufficiently or not at all. These wells typically produce Ct values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outlying data (outliers) can result in erroneous measurements.

**Step 4** To ensure precision, carefully view replicate groups for outliers. I have removed outliers manually using the Ct versus well position amplification plot.

**Step 5** Export raw or analysed data from plate documents into tab-delimited text files (*.txt). The text file was then imported into spreadsheet software, such as Microsoft Excel software. Select File > Export
**Step 6** The comparative Ct method ($2^{- \Delta \Delta Ct}$) of relative quantitation was performed.

**11.7 Transient transfection of cell lines**

Cell lines were transfected with miRNA mimic, miRNA inhibitor and AllStars Negative (Qiagen) using RNAiMAX in six-well plates.

**Step 1** Twenty-four hours before transfection, seed 250,000 cells per well of a six-well plate in 2000 µL of an appropriate culture medium containing serum and antibiotics and incubate the cells under normal growth conditions (37°C and 5% CO$_2$).

**Step 2** On the day of transfection, dilute miRNAs (mimics, antagomirs and negative controls) to a 30 µM solution.

**Step 3** Add 6 µL of mimics, 60 mL of antagomirs, and 6 µL of negative controls to 200 µL of Opti-MEM I medium and allow it to stand at room temperature for five minutes.

**Step 4** Mix RNAiMAX before use. Add 6 µL of of RNAiMAX to 200 µL of Opti-MEM I medium and allow to stand at room temperature for five minutes.
Step 5 Combine diluted RNA with diluted RNAiMAX, and incubate at room temperature (15–25°C) for 20 minutes to allow the formation of transfection complexes.

Step 6 Add 400 µL of RNAiMAX /RNA to each six-well plate. Add the complexes dropwise onto the cells. Gently swirl the plate to ensure uniform distribution of the transfection complexes.

Step 7 Incubate for 48 hours at 37°C in 5% CO₂. Cells are harvested after 24 hours, and replated for viability, migration and invasion tests.

Step 8 Transfection efficiency was determined by monitoring miR expression with quantitative PCR after 48 hours.

11.8 Cell proliferation assays

Cell proliferation assays were performed over a period of seven days of growth in 96-well plates, in triplicate, using the CellTiter® Glo Cell Proliferation Assay (Promega, Madison, WI, USA).

Step 1 500 cells were seeded in an opaque 96-well plate 48 hours after transfection with 100 µL of medium.
Step 2 Equilibrate the plate and its contents at room temperature for approximately 30 minutes.

Step 3 Add a volume of CellTiter Glo Reagent equal to the volume of cell culture medium present in each well (add 100 µL of reagent to 100 µL of medium containing cells for a 96-well plate).

Step 4 Mix contents for two minutes on an orbital shaker to induce cell lysis.

Step 5 Allow the plate to incubate at room temperature for 10 minutes to stabilize luminescent signal.

Step 6 Record luminescence.

11.9 Annexin V apoptosis assay

11.9.1 Principles

Fluorescein isothiocyanate (FITC) Annexin V staining precedes the loss of membrane integrity that marks the late stage of cell death resulting in either apoptosis or necrosis. Viable cells with intact membranes excrete propidium iodide (PI), whereas the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are FITC
Annexin V-negative and propidium iodide-negative, cells that are in early apoptosis are FITC Annexin V-positive and PI-negative, and cells that are in late apoptosis or already dead are both FITC Annexin V-positive and PI-positive.

This assay does not distinguish between cells that have undergone apoptosis and those that have died as a result of necrosis, because in either case the dead cells will stain with both Annexin V-FITC and PI. However, when apoptosis is measured over time, cells can be often tracked from FITC Annexin V-negative and PI-negative (viable, or no measurable apoptosis) to FITC Annexin V-positive and PI-negative (early apoptosis, membrane integrity is present), and finally to FITC Annexin V-positive and PI-positive (end stage of apoptosis and death). The movement of cells through these three stages suggests apoptosis. In contrast, a single observation indicating that cells are both FITC Annexin V-positive and PI-positive reveals less information about the process by which the cells die.

11.9.2 Apoptosis assay protocols

Briefly, triplicate wells of cell lines (each well containing 50,000 cells) were transfected with 1.2 µL of miRNA mimic, 12 µL of miRNA inhibitor, and 1.2 µL of AllStars Negative (Qiagen) using 1.2 µL of RNAiMAX in 24-well plates with cover slip in a 24-well plate.
Step 1 Apoptosis levels were determined 24 hours following transfection.

Step 2 The cells were washed twice with cold phosphate-buffered saline and kept in phosphate-buffered saline.

Step 3 A mixture of 5 µL FITC Annexin V, 5 µL PI, and 1 µL Hoechst in 100 µL of phosphate-buffered saline was added to the cells.

Step 4 The plate was placed on a shaker and incubated for 15 minutes at room temperature (25°C) in the dark.

Step 5. The cover slip was then removed and mounted on Superfrost slides.

Step 6 Apoptotic cells were imaged using fluorescent microscopy.
11.10 Cell migration assay (Transwell®)

| Available Pore Sizes (µm) | 0.4, 1.0, 3.0, 8.0 | 0.4, 5.0 |

*Membrane manufacturing processes are inherently variable; thus, actual pore sizes may vary depending on membrane type and measurement techniques.*

2

**Figure 11.5** Instruments and principles for Transwell migration assays.

**Step 1** First, 50,000 transfected cells (mimic, inhibitor, and AllStars Negative) were placed in the upper chamber of the 8 µm insert along with 500 µL of serum-free medium for 24 hours.

**Step 2** In a new 24-well plate, 700 µL of medium containing 15% fetal bovine serum (potential migration factor) were placed in the bottom wells in triplicate.
**Step 3** The insert was placed into the 24-well plate, making sure there were no bubbles underneath the membrane.

**Step 4** Incubated for 48–72 hours at 37°C and 5% CO₂.

**Step 5** The Transwell was moved to an empty space in the 24-well plate.

**Step 6** Fixing was performed with 0.5% crystal violet dissolved in 50% methanol for five minutes.

**Step 7** The medium was removed from the top of membrane and the top of membrane was wiped with a cotton Q-tip (using four Q-tips per membrane).

**Step 8** Images were taken of five random fields with a 20× objective microscope (Leica Microsystems, Wetzlar, Germany) and cells are counted with ImageJ.

### 11.11 Western blot

**Step 1** The cells were lysed in RIPA buffer (TrisHCl pH 7.4 50 mM, NaCl 150 mM, ethylenediaminetetraacetic acid pH 8.0 2 mM, NP-40 1%, sodium dodecyl sulphate 0.1%, Proteinase inhibitor concoction [Roche, Basel, Switzerland], Phosphatase inhibitor concoction [Roche], NaF 10 mM, beta-GPA 20 mM,
Step 2 The protein supernatant was transferred to a new tube and the protein concentration was then determined by Bradford reagent (protein assay Cat#500-0006, Bio-Rad) with a BioPhotometer Plus (Eppendorf, Hamburg, Germany).

Step 3 Protein samples were prepared with 4× sodium dodecyl sulphate loading buffer (TrisHCl pH 6.8 200 mM, sodium dodecyl sulphate 8%, glycerol 40%, mercapitoethanol 4%, Bromophenol blue 0.4%), boiled on a hot block for 10 minutes and then placed on ice.

Step 4 Samples were run by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis gel on NuPAGE Novex 4%–12% Bis-Tris gels 1.0 mm, 12-wells (Cat# NP0322BOX, Invitrogen, Carlsbad, CA, USA) in 1 x NuPAGE MOPS sodium dodecyl sulphate running buffer (Invitrogen, Cat#NP0001) at 100 volts with an Owl P8DS System (Thermo Scientific) for approximately 2 hours. Pre-stained molecular weight markers, i.e., Cat#26612 (Thermo Scientific) or Cat# LC5699 (Invitrogen), were run on each gel.

Step 5 Proteins were transferred to a polyvinylidene fluoride membrane.
(Immobilon-P Transfer Membrane, Cat# IPVH00010, EMD Millipore, Billerica, MA) in transfer buffer (Tris base 25 mM, glycine 192 mM, methanol 20%) using a VEP-2 Mini Tank electroblotting system (Thermo Scientific) for 180 minutes at 250 mA in a cold room.

**Step 6** Membranes were blocked with 5% milk in TBST (NaCl 150 mM, Tris 10 mM, Tween-20 0.1%) for 30 minutes at room temperature. The membranes were incubated with primary antibodies in 1× NET-gelatin (2.5% gelatin in NaCl 150 mM, ethylenediaminetetraacetic acid 5 mM, Tris pH 7.5 50 mM, Triton™ X-100 0.05%) overnight at 4°C.

**Step 7** The membrane was washed in TBST for 3× 10 minutes, and then incubated for 2 hours with horseradish peroxidase-conjugated secondary antibody (Cat# sc-2031, sc-2033, sc-2004, Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and washed as described earlier.

**Step 8** An enhanced chemiluminescence Western blotting protocol (Cat# 34076, Thermo Scientific) was used to detect protein bands and carried out according to the manufacturer’s instructions. Densitometric analysis of protein bands was performed using ChemiDoc XRS+ system, QuantityOne version 4.4.1 software (Bio-Rad).
11.12 Immunohistochemistry

**Step 1** Human tissues are sectioned using cryosection (7 µm thickness).

**Step 2** For antigen retrieval, place slides into antigen unmasking solution 15 mL of stock solution diluted into 1,600 mL of double-distilled H₂O (Cat#H-3300, Vector Laboratories Inc, Burlingame, CA, USA). Incubate in boiling water bath for 5 minutes.

**Step 3** Wash the slides by incubating in phosphate-buffered saline for 5 minutes.

**Step 4** Circle samples with a PAP pen.

**Step 5** For blocking, incubate with 10% goat serum in phosphate-buffered saline for 30 minutes at room temperature.

**Step 6** Incubate slides overnight at 4°C with primary antibody dissolved in 10% goat serum in phosphate-buffered saline (MYCBP2 1:200).

**Step 7** Wash three times with phosphate-buffered saline for five minutes each.

**Step 8** Incubate the slides with 1:2000 biotinylated secondary antibody in 1%
bovine serum albumin in phosphate-buffered saline for 30 minutes at room temperature.

**Step 9** Prepare Vectastain® Elite ABC reagent (Vector): 2.5 mL of phosphate-buffered saline with one drop of A-reagent then one drop of B-reagent. Allow ABC reagent to stand for 30 minutes at room temperature.

**Step 10** Wash three times with phosphate-buffered saline for five minutes each.

**Step 11** Incubate sections with Vectastain Elite ABC Reagent for 30 minutes at room temperature.

**Step 12** Wash three times with phosphate-buffered saline for five minutes each.

**Step 13** Prepare DAB reagent: 2.5 mL of double-distilled H₂O with one drop of buffer provided in the kit, mix well, and then add two drops of DAB and one drop of peroxide.

**Step 14** Place the slide under the microscope, and add DAB onto sections. When optimal staining has occurred, terminate DAB staining by placing slides
into running tap water for 5 minutes.

**Step 15** Counterstain with haematoxylin

**Step 16** Dehydrate with 75%, 80%, 95%, and 100% alcohol, and xylene three times

**Step 17** Apply two drops of mounting solution (xylene-compatible) and place a cover slip

11.13 Immunofluorescence

**Step 1** Fix the cells in a 2–3 mm depth of 4% paraformaldehyde in phosphate-buffered saline for 15 minutes at room temperature.

**Step 2** Aspirate fixative, rinse twice in ice-cold phosphate-buffered saline.

**Step 3** For permeabilisation and antigen blocking, incubate the samples with 1% bovine serum albumin plus 0.3% Triton X-100 for 60 minutes in a humidified chamber at room temperature.

**Step 4** Wash cover slips with phosphate-buffered saline for 5 minutes.
Step 5 Incubate samples in primary antibody in 1% bovine serum albumin with phosphate-buffered saline-Triton X-100 0.3% in a humidified chamber at 4°C overnight.

Step 6 Wash the samples three times with phosphate-buffered saline for five minutes each.

Step 7 Incubate samples in secondary antibody (1:200) dissolved in 1% bovine serum albumin with phosphate-buffered saline plus Hoechst (1:200) in the dark for one hour at room temperature.

Step 8 Wash the samples three times for 5 minutes each with phosphate-buffered saline in the dark.

Step 9 Mount the cover slips and take photographs under a microscope at 20×.

11.14 Lentivirus package and infection

Lentiviral constructs containing miR-1247 (ABM Mh10072) were purchased from ABMgood. For virus package, 293Ta cells at 70%–80% confluence were transfected with lentiviral construct along with psPAX2 and VSVG. Forty-two to 72 hours post transfection, the viral supernatant was collected
and transduced into colon cancer cells at a multiplicity of infection of 1 as per the manufacturer's protocol. Transfection efficiency was monitored by green fluorescent protein (GFP) imaging. To obtain stable cells, infected cells were selected with 1 µg/mL puromycin for 4 weeks until >95% cells were GFP-positive. miR-1247 expression levels in these stable cells were determined by quantitative PCR.

11.15 Subcutaneous xenografts

LS411N cells infected with control or miR-1247-overexpressing lentivirus were selected for 4 weeks with puromycin. The cells were further infected with luciferase, and 1x10^6 cells were then subcutaneously injected into athymic BALB/c nude mice (Charles River Laboratories, Wilmington, MA, USA). The control cells were injected into the left flank while the miR-1247-overexpressing cells were injected into the right flank of the same mouse.

For 5'-Aza treatment, LS411N cells infected with luciferase were subcutaneously injected into nude mice. The control cells were injected into the left flank while the miR-1247-overexpressing cells were injected into the right flank of the same mouse. The next day, the mice were treated with 5'-Aza in dimethyl sulfoxide at 1 µg/5 g every day by intraperitoneal injection. Growth of xenografts was monitored using an in vivo imaging system (IVIS®,
PerkinElmer, Waltham, MA, USA) at indicated time points.

11.16 Direct target confirmation: 3'-UTR luciferase reporter assays

The plasmid containing the full-length human MYCBP2-3'-UTR inserted downstream of the luciferase reporter gene was purchased from Abmgood (MT-h15016). The luciferase reporter construct, along with β-galactosidase control plasmid, were transfected into 293T cells. Twenty-four hours post transfection, the cells were split into 24-well plates followed by transfection of miR-1247 mimics or All-star negative control mimics at 50 nM. Seventy-two to 96 hours post miRNA transfection, luciferase activity was determined using an E4030 luciferase assay system (Promega) and normalised to β-galactosidase activity measured using an E2000 β-galactosidase enzyme assay system (Promega).
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